COMBINING ONCOLYTIC IMMUNOTHERAPY WITH CONVENTIONAL CANCER TREATMENTS

Ilkka Liikanen

Cancer Gene Therapy Group

Department of Pathology and
Transplantation Laboratory,
Haartman Institute &
Doctoral Programme in Biomedicine

Faculty of Medicine,
University of Helsinki, Finland

ACADEMIC DISSERTATION

To be publicly discussed with the permission of the Faculty of Medicine of the University of Helsinki, in Haartman Institute, Lecture Hall 1, on 13th of February 2015, at 12 noon.

Helsinki 2015

Supervised by

Akseli Hemminki, M.D., Ph.D., Docent

Cancer Gene Therapy Group,
Department of Pathology and
Transplantation Laboratory,
Haartman Institute,
University of Helsinki,
Helsinki, Finland

Anniina Koski, M.D., Ph.D.

Cancer Gene Therapy Group,
Department of Pathology and
Transplantation Laboratory,
Haartman Institute,
University of Helsinki,
Helsinki, Finland

Thesis Committee

Jaana Pihkala, M.D., Ph.D., Docent

Department of Paediatric Cardiology,
Children's Hospital,
University Hospital of Helsinki,
University of Helsinki,
Helsinki, Finland

Brendan Battersby, Ph.D., Docent

Research Programs Unit, Molecular
Neurology,
Biomedicum Helsinki
University of Helsinki
Helsinki, Finland

Reviewers appointed by the Faculty

Pirkko Mattila, Ph.D., Docent

Institute for Molecular Medicine Finland
(FIMM), Technology Centre,
Biomedicum Helsinki 2
Helsinki, Finland

Kirmo Wartiovaara, M.D., Ph.D., Docent

Research Programs Unit,
Biomedicum Helsinki,
University of Helsinki,
Helsinki, Finland

Official opponent

Michal Besser, Ph.D., Assistant Professor

Department of Clinical Immunology and Microbiology,
Sackler School of Medicine,
Tel Aviv University,
Tel Aviv, Israel

Dissertationes Scholae Doctoralis Ad Sanitatem Investigandam Universitatis Helsinkiensis

No. 10/2015
ISBN 978-951-51-0576-9 (paperback)
ISBN 978-951-51-0577-6 (PDF)

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

As long as there is life, there is potential.

To my family

Table of Contents

PART A **6**

ABSTRACT 6
TIIVISTELMÄ 8
LIST OF ORIGINAL PUBLICATIONS 9
ABBREVIATIONS 10

PART B **13**

1. REVIEW OF THE LITERATURE 13
1.1 Introduction *13*
1.2 Conventional cancer therapies *16*
1.2.1 Radiotherapy 16
1.2.2 Chemotherapy 19
1.3 Novel emerging cancer therapies *21*
1.3.1 Gene therapy 21
1.3.2 Cancer gene therapy 23
1.3.3 Tumor immunology and immunotherapy 25
1.4 Adenovirus *33*
1.4.1 Adenovirus structure 34
1.4.2 Adenovirus life cycle 36
1.4.3. Transduction and biodistribution 36
1.4.4. Early phase and DNA-repair inhibition 38
1.4.5. Late phase and mechanism of cell death 40
1.4.6 Host antiviral defense mechanisms 41
1.5 Cancer-therapeutic adenovirus vectors *48*
1.5.1 Transductional targeting 49
1.5.2 Transcriptional targeting 50
1.6 Replication-deficient adenoviruses and their clinical use *52*
1.7 Oncolytic adenoviruses and their clinical use *53*
1.7.1. Oncolytic immunotherapy 55
1.7.2. Virus arming with immunostimulatory transgenes 57
2. AIMS OF THE STUDY 60
3. MATERIALS AND METHODS 61
3.1 Cell lines *61*
3.2 Adenovirus constructs *61*
3.2.1 Replication-deficient adenoviruses 62
3.2.2 Oncolytic adenoviruses 62
3.3 Preclinical in vitro experiments *63*
3.3.1 Efficacy and synergy experiments 63
3.3.2 Immunofluorescence and protein analyses 64
3.3.3 Immunogenicity of cell death experiments 65
3.4 Preclinical in vivo studies *65*
3.4.1 Animal models and efficacy experiments 65
3.4.2 Determination of functional virus 66
3.4.3 Gene expression and microarray analysis 67
3.4.4 Immunohistochemistry analyses 67
3.4.5 Electron microscopy 68

3.5 Patient series *68*
3.5.1 Advanced Therapy Access Program (ATAP) 68
3.5.2 Patient selection, treatments and follow-up 69
3.5.3 Response evaluation and survival analysis 71
3.5.4 Quantification of viral DNA in serum 71
3.5.5 Protein analyses on patient samples 72
3.5.6 Neutralizing antibody titer determination 72
3.5.7 Enzyme-Linked ImmunoSpot (ELISPOT) assay 73
3.6 Statistics and in silico analyses *73*
3.6.1 Preclinical statistics and pathway analyses 73
3.6.2 Patient series statistics and multivariate analyses 74
3.7 Ethical considerations *75*
4. RESULTS AND DISCUSSION 76
4.1 Improving efficacy of radiotherapy by recombinant adenoviruses expressing radiosensitizing proteins *76*
4.1.1 Virus characterization and *in vitro* efficacy combined with radiotherapy 76
4.1.2 Improved *in vivo* efficacy by combination therapy 77
4.1.3 Persistent DNA damage in cancer cells after combination therapy 77
4.2 Upregulation of interferon signaling mediates acquired tumor resistance to oncolytic adenovirus in vivo *79*
4.2.1 Animal model of acquired resistance and characterization of virus-resistant phenotype 79
4.2.2 Identification of potential therapeutic targets and a virus-resistance marker 80
4.2.3 Role of tumor stroma in maintaining resistance 81
4.2.4 Translational data on identified virus-resistance marker MxA in cancer patients 82
4.3 Combining oncolytic immunotherapy with low-dose temozolomide and low-dose cyclophosphamide preclinically and in cancer patients *85*
4.3.1 Preclinical efficacy, autophagy induction and immunogenicity 85
4.3.2 Safety and biological virus activity in patients 86
4.3.3 Evidence of autophagy, immunogenicity and immune responses in patients 87
4.3.4 Clinical responses and survival 88
4.4 Serum High-mobility group box 1 (HMGB1) protein is a predictive and prognostic biomarker for oncolytic immunotherapy in cancer patients *90*
4.4.1 Serum HMGB1 baseline levels, patient characteristics and treatments 91
4.4.2 Independent prognostic and predictive value of HMGB1 baseline status 92
4.4.3 Safety assessment and comparison between the patient cohorts 93
4.4.4 Correlative analyses of potential mechanistic factors 95
4.4.5 Evidence of immunological mechanisms underlying the HMGB1 status 97
4.4.6 Post-treatment changes in serum HMGB1 and outcome parameters 97
4.4.7 Potential clinical relevance of the biomarker 98
5. CONCLUSIONS AND FUTURE PROSPECTS 101
6. ACKNOWLEDGEMENTS 104
7. REFERENCES 107
PART C – ORIGINAL PUBLICATIONS 133

PART A

ABSTRACT

Cancer causes over eight million deaths per year, more than any other disease in the world, underlining the need for improved treatments. Recent years have provided the first breakthroughs of cancer immunotherapy. Cancer gene therapy with oncolytic adenoviruses is a potent form of immunotherapy, where targeted tumor-lytic viruses are used to direct patients' own immune system against their cancer. With over 1,000 trials carried out and more than 5,000 cancer patients treated, cancer gene therapy is appearing safe and promising approach for solid tumors, highlighted by the first positive phase-III trial results in the Western countries in 2013. Nevertheless, numerous other oncolytic immunotherapy trials have failed due to low efficacy. The aim of this thesis is to improve efficacy while maintaining low toxicity by combining oncolytic immunotherapy with conventional treatment modalities, and to identify resistance mechanisms and biomarkers for oncolytic immunotherapy.

Radiotherapy is widely used to treat solid tumors such as prostate and breast cancer, but large curative doses carry the risks for side-effects. Cancer cells can be sensitized to ionizing radiation by adenovirus replication *per se*, but the mechanisms remain unknown. Adenoviral proteins E1B55K, E4orf3 and E4orf6 might play a role in radiosensitation by targeting the Mre11-Rad50-NBS1 (MRN) protein complex, which is essential for DNA double-strand break repair. We showed *in vitro* that E4orf3 and E4orf6, but not E1B55K, expressing recombinant adenoviruses are effective radiosensitizers that enhance DNA damage accumulation and cell killing after radiotherapy. Moreover, the combination treatment significantly inhibited tumor growth in mice bearing prostate cancer xenografts. This intrinsic ability of adenoviruses to radiosensitize cells could be harnessed against cancer cells by selective targeting. Combination treatment with radiotherapy and oncolytic adenoviruses is therefore a promising way to increase efficacy, optimize the curative irradiation dose, and consequently reduce the harmful side-effects.

Owing to the tremendous transforming capacity, advanced tumors can develop resistance to virtually any therapeutic modality. With regard to chemotherapy and targeted therapies, many resistance mechanisms have been identified, which has allowed development of countermeasures. For oncolytic adenoviruses, however, no such data is yet available. We established two ovarian cancer mouse models of acquired resistance, where initially sensitive tumors respond to the oncolytic virus but then relapse despite the presence of functional virus. Mouse models were utilized to study the phenomenon on gene expression, protein, and tissue levels. We identified interferon signaling upregulation in the tumors of acquired resistance by microarray. Pathway analyses suggested potential therapeutic targets in adenovirus-resistant cells, and myxovirus resistance protein A (MxA) was found a useful protein level indicator correlating with resistance to virus. Furthermore, transplantation studies suggested a role for tumor stroma in maintaining resistance. Improved understanding of the antiviral phenotype causing tumor recurrence is essential for developing countermeasures. Identified resistance pathways may be targeted for improving therapeutic efficacy, while the resistance marker MxA could serve as a clinical biomarker for oncolytic adenoviruses.

Combination of standard chemotherapy with oncolytic immunotherapy has the potential to increase antitumor efficacy in a synergistic manner. There is evidence that the cytopathic effect elicited by oncolytic adenoviruses is mediated *via* autophagy and is highly immunogenic. Similarly, certain chemotherapeutics and have been shown to induce immunogenic cell death, a prerequisite for antitumor T-cell responses, which is characterized by exposure of calreticulin on the cell surface, and release of adenosine triphosphate and a nuclear protein high-mobility group box 1 (HMGB1). Meanwhile, low-dose chemotherapy with cyclophosphamide has been shown to inhibit the immunosuppressive regulatory T-cells. We demonstrated that oncolytic adenovirus together with an autophagy-inducing chemotherapeutic temozolomide and low-dose cyclophosphamide increased immunogenic cell death *in vitro*, and enhanced tumor growth inhibition that associated with increased autophagy *in vivo*. In the clinical part, combination therapy was found safe in 41 treatments given to 17 cancer patients with refractory solid tumors, who were treated in the context of an advanced therapy access program (ATAP). Treatments were well-tolerated with mostly mild grade 1-2 clinical adverse reactions. Objective signs of possible efficacy and antitumor immune activations were observed: Disease stabilization or better was achieved in 67% of evaluable treatments, and post-treatment HMGB1 release seemed to correlate with antitumor T-cell activity in blood. As an estimated effect on survival, combination-treated patients trended for increased overall survival over virus-only treated matched non-randomized control patients.

With the emergence of effective immunotherapeutic modalities, biomarkers are urgently needed for identification of cancer patients likely to benefit. HMGB1 protein is emerging as a key player in immunomodulation and has been implicated prognostic for certain conventional therapies. We addressed the biomarker value of serum HMGB1 in a clinical-epidemiological cohort of 202 cancer patients with refractory solid tumors, who were treated with oncolytic adenoviruses in the ATAP. Patients with low HMGB1-baseline level (below median concentration) showed significantly improved overall survival and disease control rate as compared to high-baseline patients, while both patient groups showed good safety. Importantly, these observations held in multivariate models adjusted for confounding factors, indicating that low HMGB1-baseline status is an independent prognostic, and the best predictive factor for disease control. HMGB1-low patients seemed to benefit from immunogenic-transgene coding adenoviruses and antitumor T-cell activity in blood, suggesting an immune-mediated mechanism. We have thus identified a novel prognostic and predictive biomarker for oncolytic immunotherapy. Our results indicate that HMGB1-baseline may distinguish between immunologically responsive and suppressed cancer patients, and could help in selecting the right patients for oncolytic immunotherapy.

Combination of oncolytic immunotherapy with conventional treatments has the potential to evoke durable responses and increase cure rates, particularly when based on basic scientific rationale. Our results provide evidence for combining oncolytic adenoviruses with radiotherapy, low-dose temozolomide and low-dose cyclophosphamide. In addition, we present novel insights into antiviral resistance mechanisms *in vivo* and biology underlying the combination treatments. Finally, we report safety, possible signs of efficacy, and immunological effects in altogether 238 patient treatments, and introduce a promising prognostic and predictive biomarker for oncolytic immunotherapy. Hence, our results may prove useful when developing oncolytic adenovirus treatments, designing clinical trials, and selecting the right patients for each therapy.

TIIVISTELMÄ

Syöpä nousi tilastoissa maailman yleisimmäksi kuolinsyyksi vuonna 2012, johtaen vuosittain 8.2 miljoonan potilaan kuolemaan, huolimatta parantuneesta ennaltaehkäisystä, diagnostiikasta ja tavanomaisista hoidoista. Uusia hoitomuotoja tarvitaan etenkin levinneiden kasvainten hoitoon, joiden ennuste on usein heikko. Lupaava syövän immunoterapia tähtää potilaan hankinnaisen immuunijärjestelmän aktivoimiseen syöpäkasvaimia vastaan. Hyvin siedetyillä onkolyyttisillä adenoviruksilla voidaan tuhota kasvainsoluja selektiivisesti ja aikaansaada terapeuttisia immuunivasteita. Geneettisen manipuloinnin keinoin onkolyyttiset adenovirukset on muokattu tuhoamaan ja lisääntymään vain syöpäsoluissa, säästäen terveet solut – niiden turvallisuus on todettu jo lukuisissa kliinisissä kokeissa ympäri maailman. Väitöskirjatutkimukseni käsittelee onkolyyttisen immunoterapian yhdistämistä tavanomaisiin syöpähoitoihin, sädehoitoon ja solunsalpaajiin, joita vastaan kehittyvä kasvainresistenssi ja haittavaikutukset rajoittavat tehoa. Ensimmäisessä osajulkaisussa raportoimme kahden virusproteiinin herkistävän tehokkaasti eturauhassyöpää sädehoidolle estämällä DNA-korjausmekanismeja, sekä hidastavan tuumorikasvua hiirimallissa. Toisessa osajulkaisussa tutkimme onkolyyttisiä adenoviruksia vastaan kehittyvää kasvainresistenssiä, ja havaitsimme interferoni-vasteen yliaktivoituvan sekä identifioimme potentiaalisesti hyödyllisen markkeriproteiinin joka korreloi virus-resistenssin kanssa. Kolmas, translationaalinen tutkimus käsitteli onkolyyttisten adenovirusten ja matala-annoksisen solunsalpaaja-hoidon, temotsolomidin ja syklofofamidin, yhteisvaikutuksia prekliinisesti eturauhassyövässä, sekä potilaiden levinneiden kasvainten kokeellisessa hoidossa. Prekliinisesti yhdistelmähoito oli synerginen teholtaan, lisäsi kuolevien syöpäsolujen autofagiaa sekä HMGB1-proteiinin vapautumista immunogeenisyyden merkkinä, ja hidasti tehokkaimmin tuumorikasvua hiirimallissa. 41 yhdistelmähoitoa annettuna 17 syöpäpotilaalle olivat hyvin siedettyjä, joskin gradus 1–2 flunssankaltaisia oireita ja pahoinvointia esiintyi yleisesti. Raportoimme viitteitä mahdollisista hoitovasteista kuvantamisessa ja tuumorimarkkereissa yhteensä 67%:lla arvioitavista potilaista. Lisäksi mittasimme HMGB1 proteiinin vapautumista vereen hoidon jälkeisesti, sekä samanaikaisia anti-tumoraalisia T-soluvasteita, sekä havaitsimme pitkittyneen elossaoloajan verrattuna ei-randomoituihin, virus-hoidettuhin kontrollipotilaisiin. Neljännessä tutkimuksessa syvennyimme havaintoomme seerumin HMGB1 proteiinin muutoksista virus-hoidetuissa potilaissa: Raportoimme onkolyyttisen adenovirushoidon terapeuttisen ja immunologisen vaikutuksen korostuvan 202 syöpäpotilaan aineistossa sillä osalla potilaista joiden veren HMGB1-taso oli alkutilanteessa matala. Raportoimme hoitojen olevan yhtä hyvin siedettyjä ja turvallisia molemmissa potilasryhmissä. Arvioimme matalan HMGB1-lähtötason ennustearvoa monimuuttuja-malleissa, ja osoitimme sen olevan itsenäinen prediktiivinen tekijä hoitovasteille kuvantamisessa, sekä prognostinen pidentyneelle elossaoloajalle verrattuna korkean lähtötason potilaisiin. HMGB1-matalat potilaat näyttivät hyötyvän erityisesti anti-tumoraalisten T-solujen aktivaatiosta, sekä hoidoista immuunijärjestelmää stimuloivilla adenoviruksista, viitaten biomarkkerin mekanismin olevan immuunivälitteinen. Tuloksemme osoittavat siten seerumin HMGB1-lähtötason olevan lupaava biomarkkeri onkolyyttiselle immunoterapialle. Väitöskirjatutkimuksemme tulokset edistävät onkolyyttisen immunoterapian tehon ja turvallisuuden parantamista erityisesti syövän yhdistelmähoidoissa, tarjoten perusteet kliinisiin jatkotutkimuksiin, ja esittelevät uusia biomarkkereita jotka voivat auttaa paremmin kohdentamaan immunoterapeuttiset hoitomuodot niistä hyötyville syöpäpotilaille.

LIST OF ORIGINAL PUBLICATIONS

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

I. **Liikanen I**, Dias JD, Nokisalmi P, Sloniecka M, Kangasniemi L, Rajecki M, Dobner T, Tenhunen M, Kanerva A, Pesonen S, Ahtiainen L, Hemminki A. Adenoviral E4orf3 and E4orf6 Proteins, but not E1B55K, Increase Killing of Cancer Cells by Radiotherapy In Vivo. *Int J Radiat Oncol Biol Phys*. 2010 78:1201-1209

II. **Liikanen I**, Monsurrò V, Ahtiainen L, Raki M, Hakkarainen T, Diaconu I, Escutenaire S, Hemminki O, Dias JD, Cerullo V, Kanerva A, Pesonen S, Marzioni D, Colombatti M, Hemminki A. Induction of Interferon Pathways Mediates In Vivo Resistance to Oncolytic Adenovirus. *Mol Ther*. 2011 19:1858-1866.

III. **Liikanen I**, Ahtiainen L, Hirvinen ML, Bramante S, Cerullo V, Nokisalmi P, Hemminki O, Diaconu I, Pesonen S, Koski A, Kangasniemi L, Pesonen SK, Oksanen M, Laasonen L, Partanen K, Joensuu T, Zhao F, Kanerva A, Akseli Hemminki A. Oncolytic Adenovirus With Temozolomide Induces Autophagy and Antitumor Immune Responses in Cancer Patients. *Mol Ther*. 2013 21:1212-1223.

IV. **Liikanen I**, Koski A, Merisalo-Soikkeli M, Hemminki O, Oksanen M, Kairemo K, Joensuu T, Kanerva A, Hemminki A. Serum HMGB1 is a Predictive and Prognostic Biomarker for Oncolytic Immunotherapy. *OncoImmunology*. In press, accepted on Nov 15th, 2014.

ABBREVIATIONS

Ad	Adenovirus
ADP	Adenovirus death protein
ALT	Alanine amino transferase
APC	Antigen-presenting cell
AR	Adverse reaction
AST	Aspartate amino transferase
ASR(W)	Weighed age-standardized rate
ATAP	Advance Therapy Access Program
ATM	Ataxia-telangiectasia mutated
ATP	Adenosine triphosphate
ATR	ATM and Rad3-related protein
BCL-2	B-cell lymphoma 2 protein
bp	Base pair
BNCT	Boron neutron capture therapy
BSA	Bovine serum albumin
CAR	Coxsackie-adenovirus receptor
CAR T-cell	Chimeric antigen receptor (CAR) T-cells
CD	Cluster of differentiation
CD40L	CD40-ligand
CEA	Carcinoembryonic antigen
CMV	Cytomegalovirus
CO^2	Carbon dioxide
Cox	Cyclooxygenase
CP	Cyclophosphamide
CPE	Cytopathic effect
CR	Complete response
CT	Computed tomography
CTCAE	Common Terminology Criteria for Adverse Events
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CXCL	C-X-C-motif ligand (chemokine)
DAI	DNA-dependent activator of IFN-regulatory factors
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
dsDNA	Double stranded DNA
DSB	Double-strand DNA break
DSG2	Desmoglein 2 protein
E1	Early region 1
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-Linked ImmunoSpot (ELISPOT) assay
EGFR	Epidermal growth factor receptor
EpCAM	Epithelial cell adhesion molecule
ER	Endoplasmic reticulum
FcgR	Fc gamma receptor
FDA	Food and Drug Administration
FDG	Fluorodeoxyglucose

FIMEA	Finnish Medicines Agency
FoxP3	Forkhead box P3 protein
GFP	Green fluorescence protein
GM	Growth media
GMCSF	Granulocyte-macrophage colony-stimulating factor
HMGB1	High-mobility group box 1 protein
HPCP	Hydroperoxycyclophosphamide
HR	Hazard ratio
HRP	Horseradish peroxidase
HSV	Herpes simplex virus
hNIS	Human sodium iodide symporter protein
hTERT	Human telomerase reverse transcriptase
ICD	Immunogenic cell death
IDO	Indolamine-2,3-dioxygenase
IFI	Interferon inducible protein
IFN	Interferon
IL	Interleukin
ILT2	Immunoglobulin-like transcript 2
i.m.	Intramuscular
i.p.	Intraperitoneal
i.pl.	Intrapleural
ir-AE	Immune-related adverse event
IRF	Interferon regulatory factor
ir-RC	Immune-related response criteria
ISG	IFN-stimulated gene
ITR	Inverted terminal repeat
i.t.	Intratumoral
i.v.	Intravenous
JAK	Janus kinase
L1	Late region 1
LC3B	Microtubule-associated protein light chain 3 isoform B
Luc	Luciferase
MAPK	Mitogen-activated protein kinase
MDa	Mega-dalton (weight)
MDSC	Myeloid-derived suppressor cell
MGMT	O6-methylguanine DNA methyltransferase
MHC	Major histocompatibility complex
MLP	Major late promoter
M phase	Mitotic phase
MR	Minor response
MRI	Magnetic resonance imaging
MRN	Mre11-Rad50-NBS1 protein complex
mRNA	Messenger RNA
MxA	Myxovirus resistance protein A
NF-κB	Nuclear factor kappa-B
NK	Natural killer
NOD	Nucleotide oligomerization domain
NR3C2	Nuclear receptor subfamily 3 group C member 2

OR	Odds ratio
orf	Open reading frame
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed death-1 protein
PERCIST	PET Response Criteria in Solid Tumors
PET	Position emission tomography
PFS	Progression-free survival
pfu	Plaque-forming units
pk7	Polylysine motif-7
PR	Partial response
PRK	protein kinase R
PSA	Prostate-specific antigen
RAGE	Receptor for Advanced Glycation End-products receptor
Rb	Retinoblastoma
RECIST	Response Evaluation Criteria in Solid Tumors
RGD	Arginine-Glycine-Aspartic acid motif
RNA	Ribonucleic acid
ROC	Receiver Operating Characteristic curve
ROS	Reactive oxygen species
RSV	Rous sarcoma virus
SAE	Serious adverse event
SCID	Severe combined immunodeficiency
SCID-X1	X-linked severe combined immunodeficiency syndrome
SD	Stable disease
SFC	Spot forming colonies
S phase	Synthesis phase
STAT	Signal transducer and activator of transcription
TCID	Tissue culture infectious dose
TCR	T-cell receptor
TGF	Transforming growth factor
Th1	T-helper type 1
TIM-3	T-cell immunoglobulin domain and mucin domain 3
TIL	Tumor-infiltrating lymphocyte
tk	Thymidine kinase
TLR	Toll-like receptor
TMZ	Temozolomide
TNF	Tumor necrosis factor
T-reg	Regulatory T-cell
VEGF	Vascular endothelial growth factor
VP	Virus particle
VSV	Vesicular stomatitis virus
WHO	World Health Organization
wt	Wild-type

PART B

1. REVIEW OF THE LITERATURE

1.1 Introduction

Cancer remains the major cause of death worldwide and the incidence is rising. According to the World Health Organization (WHO) cancer statistics 2012 in Europe, the estimated risk of getting cancer before age 75 are 21.6% for women and 29.7% for men, while rates for cancer mortality before age 75, are 9.2% and 15.6% for women and men, respectively (Ferlay et al. 2014). Corresponding incidence numbers in Finland are 23.1% for women and 29.1% for men, while mortality rates are considerably lower at 7.6% and 10.7% for women and men, respectively. Figures are estimated in the absence of other causes of death. The mortality difference is partially explained by the fact that more curable cancer types, prostate and breast cancer, are more common in Finland (**Figure 1**), but it also reflects the socioeconomical advantages in Finland: functional health care system, resources for early diagnosis, effective cancer treatments that are based on the latest medical research, and continuously growing repertoire of treatment options.
Worldwide, there were 14.1 million new cancer cases in 2012, and the incidence is expected to rise with over 20 million annual new cases expected by 2025 (Ferlay et al. 2014), all this despite the improvements in cancer prevention. For the first time in history, cancer now causes more deaths, altogether 8.2 million in 2012, than any other particular disease, bypassing even ischaemic heart disease, stroke, and infectious diseases (WHO Global Health Observatory Data Repository, 2012). Meanwhile, treatment of cancer has taken some major advances in the developed countries. Unfortunately this progress is yet largely unreachable by the low- and middle-income countries. As seen in **Figure 1**, three of the top cancer types in Finland, prostate, breast and colon cancer, are already mostly curable in majority of the cases. If comparing historically, this is very much owing to the progress in modern cancer research, since the 5-year survival rates of prostate and breast cancer in Finland in the 1960s were around 30% and 55%, respectively, after which both have increased to around 90% (Pukkala et al. 2011).

Many medical advances account for this progress. Besides earlier cancer diagnosis allowing radical treatments at a less aggressive local stage, also conventional curative therapies have improved owing to novel surgical techniques, effective combinations of chemotherapeutic drugs, and targeted optimally fractionated radiotherapy. Nevertheless, yet disappointing outcomes are seen e.g. with regards to lung, pancreatic and ovarian cancer (**Figure 1**), and similarly, with advanced metastatic disease of any type. This represents the dilemma in oncology that deals with hundreds, if not thousands, of different genetic disorders of various origins, commonly referred to as “cancer”. Therefore, it is not expected that there is a magic bullet, a miracle cure for all cancer types, but instead novel modalities together with advances in conventional therapies are gradually increasing our tool box. Combinations of different tools can be then utilized to achieve more cures. Select tumor types, subtypes, or patients first seem to respond to certain (combinatorial) therapies, which are then taken forward into clinical trial testing in order to determine whether

the treatment increases survival rates as compared to standard therapy. Eventually, a new form of standard therapy may be assigned, which is then further developed, optimized and revised to improve cure rates and reduce possible adverse reactions. Gradually, along with the progress done in both basic and clinical cancer research, the emotionally and socially challenging historical concept of cancer as a lethal, life-stopping disease could change. To achieve this, however, much work remains to be done and novel treatment options are needed.

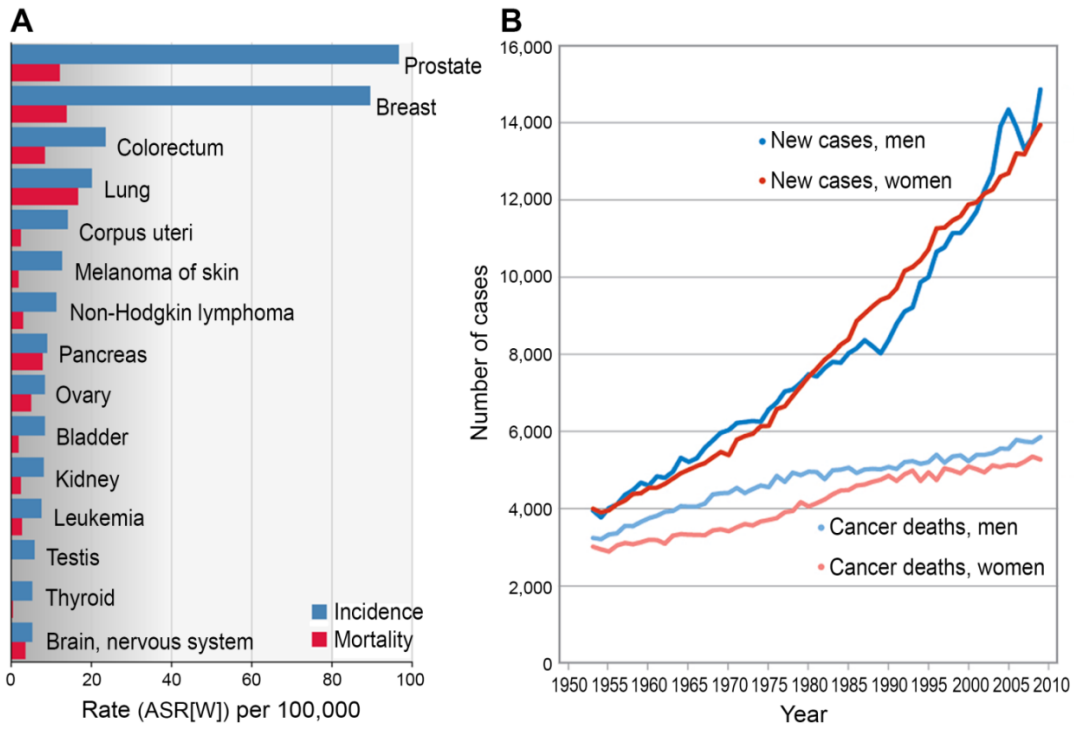

Figure 1. Cancer incidence and mortality in Finland. **A**) Estimated age-standardized rates (ASR[W]) of cancer incidence (blue) and mortality (red) in Finland in 2012, including both genders. Rates represent the number of new cases or deaths per 100,000 persons per year, which are weighted for a standard age structure. Modified from: (Ferlay et al. 2014). **B**) Development of actual cancer incidence and mortality rates in Finland during 1953–2009. Modified from: (Pukkala et al. 2011).

Biologically, cancer refers to a large group of genetic diseases, which may originate from virtually any cell type and organ in the body. All cancers arise as a result of numerous alterations occurring in the DNA sequence of cells. Consequently, some of the proteins encoded by these cells are differentially expressed or mutated, giving growth advantage and the ability to proliferate in defiance of physiological control. A localized, non-invasive tumor is called benign, whereas malignant tumor refers to a cancer which has acquired the capability to invade or disseminate from the site of primary tumor to other tissues. Metastases spawned by malignant tumors are the main cause of cancer-related deaths in humans (Mehlen and Puisieux 2006).

Oncolytic viruses are one promising treatment modality for cancer, which has recently gained attention as the first positive clinical phase III results in the Western countries were announced in 2013 (Andtbacka et al. 2013). In addition, two oncolytic adenovirus products have already been approved and are in clinical use in China since 2003 and 2005 (Guo and Xin 2006). Oncolytic adenoviruses are genetically modified to target and replicate only in cancer cells, and thus represent a form of targeted cancer gene therapy. As adenoviruses are originally human pathogens, multiple host immune mechanisms, and also counteractive circuits in the virus, have emerged during evolution. Therefore, it is not surprising that besides replicating in and lysing the infected cancer cells, oncolytic adenoviruses also induce prominent immune reactions at the tumor site. Furthermore, oncolytic viruses can be genetically modified to express immune-stimulating transgenes, which further boost immune responses, directed not only against the virus but also to the host tumor cells (Lichty et al. 2014). Hence, oncolytic virus field has naturally moved towards immunotherapy, aimed at stimulating patient's own immune system against the mutated altered self, cancer, in order to achieve long-lasting antitumor responses.

Nevertheless, as experimental virotherapy has been around since the mid-19th century, and only now the first approved cancer therapy applications are emerging, it is obvious that obstacles have been encountered. Oncolytic virotherapy is appearing safe approach with over 1,000 cancer gene therapy trials carried out and more than 5,000 cancer patients treated without treatment-related deaths or major limiting toxicity (Ginn et al. 2013). Challenges have lied in the lack of efficacy in clinical trials. This partially reflects the lack of optimal preclinical models to test efficacy, because human adenoviruses do not properly replicate in tissues of other species, forcing researchers to use xenogeneic animal models, i.e. human tumor xenografts in immunodeficient mice, which feature fundamental differences in tumor architecture and impaired immunity. Syrian hamsters have been proposed as a model to circumvent this limitation, but have been found only semi-permissive for replication of human adenovirus (Thomas et al. 2006, Bramante et al. 2014) and represent largely uncharacterized immune system. Therefore, besides developing preclinical testing and more suitable models, reporting and learning from available clinical data is of particular importance.

Both preclinical and clinical evidence suggests that efficacy can be improved, even synergistically, by combining oncolytic immunotherapy with conventional treatment modalities such as chemotherapy and radiotherapy. In preclinical part of this thesis we study combinatorial effects of oncolytic adenoviruses together with radiotherapy and certain chemotherapeutic drugs, and provide mechanistic rationale and show that improved antitumor efficacy can be achieved. In addition, we study the acquired resistance mechanisms against oncolytic adenovirus in ovarian tumors, and reveal relevant pathways, potential tumor marker and targets, which could be utilized in developing countermeasures. In the clinical part, we study altogether 238 patient treatments with oncolytic adenoviruses given in the context of an Advanced Therapy Access Program (ATAP) for patients with metastatic solid tumors progressing after conventional treatments. We demonstrate safety of the approach, and report objective signs of treatment efficacy and antitumor immune responses. In particular, we focus on patients treated, as first-in-humans, with an attractive combination of oncolytic immunotherapy and low-dose chemotherapy that was found synergistic and immunogenic preclinically. Finally, we report finding of a novel serum biomarker that is prognostic and tentatively predicts responsiveness to oncolytic immunotherapy with adenoviruses. Our findings set the stage for testing the combinations and biomarkers in clinical trials, which may ultimately have an impact on cancer therapy in practice.

1.2 Conventional cancer therapies

Conventional treatments of malignant diseases traditionally consists of surgery, chemotherapy and radiotherapy, which are all based on established scientific evidence and have been proven effective in numerous clinical situations. Surgery became the cornerstone of treatment for solid tumors with the discovery of ether anesthesia, which was first used by William T. G. Morton in 1846. Forty-six years later another William, bone surgeon William B. Coley, introduced a mixed bacterial vaccine called Coley's toxin for treatment of cancer that became the first immunotherapy preparation (Nauts et al. 1946). However, with the discovery of x-rays and radiation therapy in 1896, followed by addition of chemotherapy to the armament after the First World War, immunotherapy was long forgotten. Radiotherapy was found effective and resulted in immediate tumor reduction and pain relief, but the therapy responses remained often temporary and localized (Holsti 1995). The first cytotoxic chemotherapy agent was nitrogen mustard, adopted from its original use in chemical warfare (mustard gas), and applied for the treatment of e.g. lymphomas and leukemias in the 1940s (Goodman et al. 1984). Hormonal therapy was introduced for the treatment of prostatic cancer in 1941 (Crawford 2004). During the last twenty years, more targeted and less toxic treatments have been developed, including more tolerable chemotherapeutics, small-molecule inhibitors and monoclonal antibodies (e.g. angiogenesis and growth factor inhibitors) (Demarest et al. 2011, Hojjat-Farsangi 2014).

1.2.1 Radiotherapy

Modern radiation oncology has changed a lot from its original use of rough x-ray apparatuses in the beginning of the 20th century. Radiotherapy today can be generally divided into two main categories: teletherapy, which involves an external source delivering radiation to patient, and a newer form of brachytherapy, in which radiation is delivered in direct contact or within the patient by using an implant or mold with radioactive sources. In addition, boron neutron capture therapy (BNCT) and proton therapy are used in modern radiation oncology. Advances in targeting techniques and fractionation have improved the use of traditional teletherapy (McGovern and Mahajan 2012). Various types of solid tumors in multitude of locations can now be treated with minimal radiation exposure of normal surrounding tissues by utilizing e.g. intensity modulated techniques, arc therapies with image-guidance, and stereotactic hypofractionation.

Therapeutic effects of radiation are based on its ability to damage cellular components such as DNA or cell membranes, leading to cell death in high enough doses. An important aspect of radiotherapy is the radiosensitivity of tissues, which varies widely even within the tumor. Consequently, some tumor lesions are essentially radioresistant, while others may be curative by radiation. The molecular mechanisms underlying the differences in sensitivity and responsiveness to radiation are traditionally based on five main principles, termed as the 5Rs of radiobiology: repair, redistribution, repopulation, re-oxygenation, and radiosensitivity, which were in harmony with the original hallmarks of cancer (Harrington et al. 2007). First, sub-lethal damages in genomic DNA can be *repaired* more successfully in normal cells than in tumor cells between the treatment fractions. Second, cells are *redistributed* with regards to cell cycle, so that with fast-dividing cancer cells are more likely to be in the radiosensitive M-phase, late G1-phase or late G2-phase than normal cells. Third, *repopulation* of the irradiated necrotic tumor area seems to be slower than in healthy tissues, in part because cancer cells are susceptible to late radiation-induced death by

entering mitosis with unrepaired DNA damage (mitotic catastrophe). Fourth, tumors featuring neo-angiogenesis are effectively *re-oxygenated*, which potentiates the radiotherapy response *via* formation of oxygen radicals damaging DNA. The fifth R, the intrinsic *radiosensitivity* of cells, was later added because the previous 4Rs were insufficient in explaining some of the differences in responsiveness between certain tumors at mechanistic level.

With regards to radiotherapeutic effects, direct damage on biological material accounts for ca. 30% of the net effect, while the rest is mediated indirectly *via* formation of reactive oxygen species (ROS), such as free hydroxyl radicals, that cause lesions in cellular membranes, and macromolecules including DNA (Russi et al. 2014). Both of these are detrimental to cells: Ionizing radiation (and the generated ROS) causes hydrolysis of membrane phospholipids and fatty acids, which then act as second messengers to initiate apoptotic cascades, even in the absence of DNA-damage signaling (Haimovitz-Friedman et al. 1994). The most prominent cell death signaling, however, occurs through DNA damage. Radiotherapy leads to single- and double-strand DNA breaks, of which single-strand breaks are rarely lethal (if two breaks occur at close proximity), whereas double-strand breaks (DSB) are harmful due to potential genomic rearrangements and require immediate repair. There are several mechanisms to detect and signal, and two mechanisms to repair DSBs: non-homologous end joining and homologous recombination (Kavanagh et al. 2013). In normal cells, initiated repair cascades strive for immediate cell cycle arrest in order to prevent the transfer of damaged genomic DNA to progeny cells. The MRN complex (Mre11, Rad50, and NBS1) is a key regulator in DSB sensing, signaling and repair (Carney et al. 1998, Williams et al. 2007, Gatei et al. 2014). Followed within minutes after induction of DSBs, nuclear MRN binds to the broken ends of DNA and recruits ataxia-telangiectasia mutated (ATM) and ATM-Rad3-related (ATR) proteins to initiate repair and signal transduction pathways (**Figure 2**). These signaling cascades that include multiple DNA repair and checkpoint proteins, such as Chk1, Chk2, and H2AX, eventually lead to G_2/M or S-phase checkpoint induction and cell cycle arrest, during which DSB repair takes place (Carson et al. 2003). However, with vast enough radiation damage, DSBs cannot be repaired, cellular homeostasis is disrupted, and the cell is killed, indicating on a molecular level that cytotoxicity caused by ionizing radiation is dose-dependent after reaching a certain threshold level (Kavanagh et al. 2013). Radiotherapy induces several types of cell death mechanisms including apoptosis, autophagic cell death, mitotic catastrophe, and necrosis. In normal cells that are exposed to genomic DNA damage, p53 protein functions as the major gatekeeper in determining between growth arrest/DSB repair and cell death through classical apoptosis, whereas in cancer cells with mutated p53 (ca. 50% of all cancers), cell death usually occurs *via* other mechanisms (Golden et al. 2012). In fact, other forms of cell death may be more beneficial for overall therapeutic responses.

Recently, along with revision of the hallmarks of cancer (Hanahan and Weinberg 2011), also 5Rs of radiobiology have been revisited with a particular focus on the immune system (Good and Harrington 2013). Originally radiotherapy was regarded as a local treatment, where only tumor cells within the radiation fields are killed without much effect on the surroundings, underlined by the fact that intensive high-dose radiotherapy can be quite immunosuppressive due to radiosensitivity of lymphocytes. However, it has been long since characterized, and is now well-established, that with correct dosing and fractionating strategies, preferential elimination of suppressor T-cells over effector T-cells is attainable (North 1986). Therefore, potential for radiotherapy to induce adaptive antitumor immune responses exist.

Mechanisms of radiation-induced systemic effects are complex: As demonstrated by the adverse reactions seen after radiotherapy in e.g. head and neck tumors, tissue-specific inflammatory reactions such as dermatitis and mucositis are intertwined with systemic effects, both pathogenic and therapeutic (Russi et al. 2014). Oxidative stress first triggers a release of critical molecules from dying tumor/normal cells and surrounding stromal cells to induce innate immune responses. The production of cytokines at auto-, para-, and endocrine levels then culminates in widespread effects on immune cells and subsequently tissues throughout the body. The characteristics and magnitude of this phenomenon depends largely on the mechanism of cell death, particularly whether immunogenic or tolerogenic in nature. Autophagic and necrotic forms of cell death are, under certain circumstances, very immunogenic featuring release of danger associated molecular patterns (DAMPs) that activate dendritic cells and increase antigen-presentation to CD8+ T-cells (see below for details) (Golden et al. 2012). Finally, growing body of evidence suggests that curative effects of radiotherapy and the potential of eradicating even distant metastases depends on the activation of antitumor CD8+ T-cells (Lee et al. 2009, Takeshima et al. 2010).

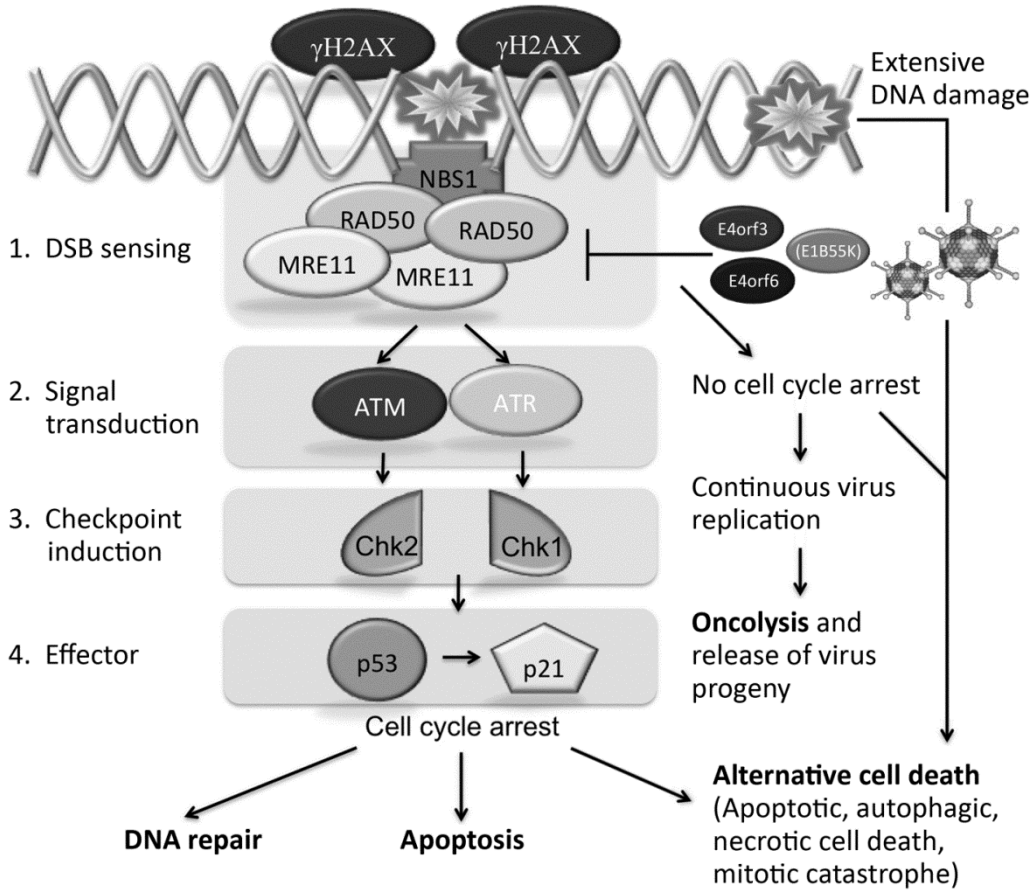

Figure 2. Repair of the double-strand DNA breaks and adenovirus-mediated inhibition of the repair. Ionizing radiation causes double-strand DNA breaks (DSBs) in the cell genome (top of the picture). Normally, the key regulator of DSB sensing and signaling, the MRN complex (Mre11, Rad50, and NBS1) binds to free DSB ends and serves as a platform for repair (left side).

Concurrently, the MRN complex upregulates ataxia-telangiectasia mutated (ATM) and ATM-Rad3-related (ATR) kinases that transduce signals to checkpoint proteins such as Chk1 and Chk2. Finally, cell faith depends on various signals transduced to major effector proteins p53 and p21 that control cell cycle progression, DNA repair, and apoptosis. The presented MRN-mediated repair and signaling pathway is the main mechanism, but also other important MRN-dependent and -independent pathways exist and thus the figure is simplified. With regards to cell faith, alternative cell death pathways, such as autophagic and necrotic death, are prominent especially in p53-mutated tumor cells, which lack the function of the main effector protein p53. Importantly, robust accumulation of extensive DNA damage (right side) can also lead to alternative types of cell death; this radiosensitivity can be enhanced by serotype 5 adenovirus infection that leads to degradation or de-targeting of the MRN complex by adenoviral proteins E4orf3, E4orf6, and to a lesser extent E1B55K, as indicated in study I. Alternatively, virus infection can lead to cell death through oncolysis (middle): adenoviral double-stranded DNA genomes entering the cell nucleus inactivate the MRN-complex to avoid being sensed as DSBs by the host cells, subsequently avoiding cell cycle arrest. Interference with the DSB sensing machinery therefore leads to promotion of S-phase, continuous virus replication, and ultimately cell lysis and release of virus progeny. The interconnected molecular mechanisms of radiotherapy and oncolytic adenoviruses provide rationale for combination approaches to improve efficacy and minimize curative radiation doses. Of note, histone protein γH2AX is phosphorylated following induction of DSBs, which serves as a mediator of repair and indicates presence of DSBs, as tested by immunohistochemistry and Western blot in study I. E4orf6 protein has been shown to inhibit dephosphorylation of γH2AX, leading to prolonged DSB signaling and atypical apoptosis. Modified from: (Mirzayans et al. 2013).

1.2.2 Chemotherapy

Traditionally, chemotherapeutic drugs were designed to mediate direct cytotoxic activity. By impairing cellular functions, e.g. *via* interfering with cell division mechanisms or damaging cellular DNA, chemotherapeutics lead to killing of essentially all cell types if given in high enough dose. However, with controlled dosage and administration (therapeutic window), the effect can be harnessed against fast-dividing cells, which is a general characteristic for malignant cells. Nevertheless, repeated monitoring of bone-marrow function is often needed during chemotherapy, since hematopoietic precursor cells that give rise to erythrocytes, platelets, and leukocytes, are also relatively fast-dividing and thus sensitive to chemotherapy. This can lead to major myelo- and immunosuppression in patients being treated with high-dose chemotherapy, which is only desirable for allogeneic bone-marrow transplantation but unfavorable in all other instances.

Given the dose-dependent cytotoxic response on different cell types, it is not surprising that certain chemotherapeutics have been found to mediate immunomodulating functions when administered in low-dosage (Ghiringhelli et al. 2004, Shevchenko et al. 2013). Furthermore, unlike the traditional direct cytotoxicity paradigm would suggest, immunogenic type of cell death and immune activation has been found essential for long-lasting antitumor effects mediated by several conventional chemotherapeutic drugs (Apetoh et al. 2008, Tesniere et al. 2010, Michaud et al. 2011). Hence, there is rationale for combining certain chemotherapeutics with oncolytic immunotherapy. In this thesis, we investigate the use of alkylating chemotherapeutics cyclophosphamide (CP) and, for the first time, temozolomide (TMZ), which were both used in low-dose as virus sensitizers preclinically and in combination treatments of advanced cancer patients.

Alkylating agents are the oldest group of chemotherapeutics derived from the aforementioned mustard gas, which act by alkylating (transferring an alkyl/methyl group to) molecules, causing cross-linking and damage of DNA that eventually leads to apoptosis (Lind 2008). Cyclophosphamide is used typically together with other chemotherapeutics to treat wide range of malignancies including lymphomas, leukemias, retinoblastoma, neuroblastoma, ovarian cancer, and breast cancer (Early Breast Cancer Trialists' Collaborative et al. 2012, Skoetz et al. 2013). CP is also widely used as a low-dose metronomic chemotherapy. A recent systematic literature analysis found that nearly half (46 studies) of the low-dose chemotherapy studies conducted, used CP either as combination or monotherapy, with promising results that are now being evaluated in phase III trials (Lien et al. 2013). The hypothesis behind most of these trials has been anti-angiogenic effects of low-dose chemotherapy, since preclinically it mainly inhibits growth of endothelial cells without much effect on fast-dividing tumor cells *per se*. Despite the apparent anti-angiogenic properties of low-dose chemotherapy, another recent literature analysis failed to identify consistent correlations between outcome and angiogenesis-related biomarkers in clinical trials, suggesting that other factors may also play a role (Cramarossa et al. 2014). Indeed, variety of other mechanisms, including alteration of the tumor microenvironment, eradication and disruption of cancer stem cells, and inhibition of immunosuppressive regulatory T-cells are likely to contribute to the antitumor activity of low-dose chemotherapy (Loven et al. 2013). In summary, given the excellent safety profile and desirable anticancer effects, a decade of research on low-dose chemotherapy, with CP in the forefront, is leading towards clinical applications, mostly likely in an adjuvant setting combined with other anticancer agents.

TMZ is also an oral alkylating agent, which is used as a standard therapy for certain gliomas, in the first line combined with radiotherapy (Stupp et al. 2005). TMZ has a favorable toxicity profile, causing relatively mild, non-cumulative myelosuppression in high dosage, which renders it attractive drug for combinations and for treatment of even metastatic disease. In fact, it has been proposed for treatment of brain metastases together with radiotherapy, regardless of the primary tumor origin (Zhu et al. 2014). In addition, TMZ has been used as an off-label treatment after standard therapies in metastatic melanoma, pituitary cancer, and lymphomas with some evidence of efficacy (Raverot et al. 2012, Velho 2012, Tatar et al. 2013). Standard dose of TMZ in adjuvant setting together with radiotherapy varies from 150 to 200 mg/m^2/day (ca. 300 – 400 mg/day) in the treatment of glioma. TMZ has also been assessed in a phase II trial of recurrent glioma, as a low-dose metronomic chemotherapy using 50 mg/m^2/day (ca. 100 mg/day) for up to 1 year or until progression (Perry et al. 2010). Interestingly, patients who had progressed during/after the conventional high-dose TMZ therapy seemed to benefit from the continuous low-dose TMZ administration, when compared to other corresponding phase II recurrent glioma trials. Resistance to high-dose alkylating TMZ has been linked to expression of the O6-methylguanine DNA methyltransferase (MGMT) protein in tumor cells, which is a repair enzyme that removes methyl and alkyl groups from guanine residues restoring the normal function of DNA (Pegg 1990). Thus, it was even more surprising, when Perry *et al.* found in their phase II study that progression-free survival was comparable in patients with and without MGMT promoter methylation (inactivation) in tumors. As speculated by the authors, efficacy of low-dose TMZ therapy may be mediated by anti-angiogenic effects, and/or inhibition of immunosuppressive regulatory T-cells, both of which have been observed to occur after low-dose TMZ treatment preclinically (Kurzen et al. 2003, Banissi et al. 2009). Thus, low-dose TMZ seems to possess some very similar immune-modulating and anti-angiogenic characteristics as low-dose CP, potentially common for many forms of low-dose chemotherapy.

Finally, an important emerging paradigm that might account for the antitumor effects mediated by chemotherapeutics involves autophagy: TMZ treatment induced autophagy can trigger autophagic cell death, especially when used in combination treatments (Kanzawa et al. 2004, Palumbo et al. 2012). This type II programmed cell death is characterized by increased turnover of cellular organelles leading to cell death, which can be useful in the treatment of apoptosis-resistant cancer cells (Lefranc et al. 2007). In addition, autophagy has recently been implicated as a prerequisite for immunogenic cancer cell death (ICD) (Michaud et al. 2011, Martins et al. 2012), which can lead to efficient antigen-presentation by dendritic cells and induction of antitumor immunity (Hannani et al. 2011). Thus, even though baseline autophagy can be viewed as a survival process (Kanzawa et al. 2004), mortal autophagic flux leading to ICD appears to be useful for anticancer therapy. With regards to TMZ, benefits of autophagic cell death have been attributed to combinatory approaches, particularly with radiotherapy and another autophagy-modulating, antiangiogenic chemotherapeutic thalidomide (Gao et al. 2009), which have been studied in phase II trials with some signs of efficacy (Hwu et al. 2003, Chang et al. 2004b, Groves et al. 2007).
Taken together, effects of radiotherapy and low-dose chemotherapy on tumor microenvironment, immune cells and alternative cell death pathways are essential considerations when designing novel combinatorial approaches. The discussed underlying biology and accumulating evidence creates strong basis for combining oncolytic adenoviruses with certain conventional treatments, such as radiotherapy, and low-dose CP and TMZ, which are discussed in more detail later. These rational multimodal treatments may lead to improved antitumor efficacy while maintaining low toxicity, and might be useful even for advanced metastatic diseases.

1.3 Novel emerging cancer therapies

1.3.1 Gene therapy

Definition of gene therapy in a broad sense is the use of nucleic acids to treat diseases. Typically this involves introduction of genetic material into body, which is then transcripted into therapeutic proteins. The concept of gene therapy in which specific known genes are introduced, dates back to early 1960s, and was originally aimed at correcting monogenic disorders, such as hemophilia or combined immunodeficiency syndromes. During the following decades, gene therapy was experimented for treatment of multitudes of diseases in laboratory. The first gene therapy, and meanwhile the first cancer gene therapy trial was conducted in 1989 by Steven Rosenberg *et al.* who used genetically modified (retroviral gene transduction) tumor-infiltrating lymphocytes to treat metastatic melanoma (Rosenberg et al. 1990). Since then, more than 1800 gene therapy clinical trials have been conducted in over 31 countries, using more than a hundred different genes or vectors (Ginn et al. 2013).

Theoretically, all cells in human body can be targeted and modified by means of gene therapy. However, ethical and legal aspects that have been developing in conjunction with the field, set important restrictions and quality standards for human gene therapy trials and treatment programs. For example, gene therapy boards monitor the quality of production, while international legislation prohibits e.g. the genetic modification of germ line (reproductive) cells, with the notable exception of the emerging mitochondrial gene transfer aiming at preventing

lethal inherited mitochondrial diseases (Vogel 2014). To date, gene therapy has been used for experimental treatment of wide range of diseases, for instance: In cardiovascular diseases, aimed at increasing angiogenesis to facilitate blood flow to ischaemic regions; In neurological diseases, to improve cholinergic transmission for Alzheimer's disease, and to protect against neurodegeneration using neurotrophic factors in Parkinson's disease; In hematological diseases, to correct many monogenic disorders including sickle-cell anemia, hemophilia and β-thalassemia; And in ocular diseases, to reverse the age-related macular degeneration, just to name a few. However, cancer is by far the biggest and increasingly popular condition for which human gene therapy has been applied, constituting around 65% of all gene therapy trials (Ginn et al. 2013).

Gene transfer to a cell can be achieved by either of the two general methods, *via* viral transduction, or non-viral transfection (e.g. plasmid or liposomes). Although the use of virus vectors comes with certain well-documented but rare pitfalls including potential for inflammatory storms and insertional mutagenesis (Ginn et al. 2013), the benefit of viral transduction lies in its superior gene transfer efficacy, and this method has been used in ca. 75% of the clinical gene therapy trials thus far. Notably, increasing amount of approaches, especially in cancer therapy, uses viral vectors *ex vivo* to transduce new genes into cells followed by adopting them the cells humans. This type of gene therapy is often referred to as cell therapy.

The first breakthrough for the field of gene therapy was achieved in 2000 with the successful treatment of a lethal monogenic disease, X-linked severe combined immunodeficiency (SCID-X1) (Cavazzana-Calvo et al. 2000). The disorder is commonly known as a form of bubble boy's disease, where infants suffer from recurrent life-threatening infections and die of an early age, as a result of impaired humoral immunity due to a single gene defect in X-chromosome. In one of the original trials, nine patients with SCID-X1 were treated with a retrovirus coding for the missing gene, and as reported in 2010, eight patients were alive after a median follow-up of 9 years, and seven had sustained immune reconstitution and lived normal lives (Hacein-Bey-Abina et al. 2010); An impressive outcome, given that allogeneic bone marrow transplantation, which is used to treat the disease if a donor exists, associates with only ca. 72% long-term survival rates (Hacein-Bey-Abina et al. 2010). The retroviral treatment was, however, later associated with leukemia in four cases, of which three patients survived. Since this was a side-effect of the therapeutic mechanism of action, insertional mutagenesis of the therapeutic gene, it has led to tightened safety precautions and development of novel retroviral vectors with safer integration profiles. Notably, retroviruses (featuring a reverse-transcriptase enzyme) and adeno-associated viruses (low degree of spontaneous integration) are the only vectors currently in clinical testing that entail potential for this side-effect. Of the retrovirus family, lentiviruses are the most used vector systems when integration into the genome of a non-dividing host cell is desired. Recent years have provided further major advances in gene therapy: In 2012, an important milestone was reached when alipogene tiparvovec (Glybera[®]) was approved in Europe, becoming the first gene therapy product in the Western countries. Alipogene tiparvovec is an adeno-associated virus encoding a variant of the human lipoprotein lipase gene for the treatment of familial lipoprotein lipase deficiency (Gaudet et al. 2013). In 2013, two clinical trials using new generation lentiviral vectors, which belong to the family of retroviruses, reported promising results in a lethal neurodegenerative lysosomal storage disease (arrested progression) and in a fatal primary immunodeficiency syndrome (immunological and hematological improvement), both mediated by successful restoration of the missing gene expression (Aiuti et al. 2013, Biffi et al. 2013).

The most used gene therapy vector is adenovirus, constituting up to 23.3% of all gene therapy trials (Ginn et al. 2013). The main advantages are good safety and large amount of clinical data supporting that, capacity to carry relatively large DNA loads as transgenes, ability to infect also non-dividing cells, and high transduction efficiency coupled with high levels of gene expression. Another *pro*, or *con* depending on the application and perspective, is that adenoviruses are common human pathogens (discussed later). Adenoviral vectors mediate only transient gene expression, which is why they are not optimal for correcting long-term genetic defects such as the aforementioned SCID-X1 disease. Thus, it is logical that for a fatal disease with no other treatment options that requires very long-term gene expression, an integrative retroviral vector with known potential risks is being tested. Analogously, the choice of vector for non-lethal, e.g. cardiovascular or ocular diseases would be a safer adenovirus or adeno-associated virus, even though therapeutic efficacy might remain lower. Thus, the field of gene therapy takes on a panel of diseases to combat, and features multiple therapeutic strategies to achieve this, with basically only the use of genetic material as the common nominator. Use of adenoviruses and some other oncolytic virus vectors in the treatment of cancer is discussed in the following sections.

1.3.2 Cancer gene therapy

In gene therapy in general, the severity of the disease has impacted the choice of vector, so that lethal (monogenic) diseases are often treated with riskier vectors. However, with regards to cancer which is the deadliest disease of all (WHO Global Health Observatory Data Repository 2012), this does not hold true due to biological reasons: Tumor cells are inherently characterized by continuous proliferation, DNA replication and cell division, which is why gene therapy vectors targeting cancer cells should entail preference for metabolically active and fast-dividing cells. In addition, high amplitude of gene expression, instead of long-term stable expression, would be desired in order to produce a maximal therapeutic bystander effect to surrounding tumor cells. In fact, common human pathogenic viruses, including adeno-, herpes simplex, and reovirus, have evolved in humans to mediate this type of rapid replication on epithelial tissues to propagate enough virus to effectively spread from host to host. This renders adenovirus, together with certain other human pathogens, an attractive vector. Moreover, owing to the common evolution and inherent innate immunity in humans, adenovirus is a safe vector, when potential for liver toxicity is taken into account (discussed later). This is underlined by the fact that over 5,000 patients have been treated and over 1,000 cancer gene therapy trials have been conducted without treatment-related fatalities (Ginn et al. 2013). To put these numbers into perspective, standard adjuvant chemotherapy in colorectal cancer using fluorouracil and oxaliplatin is associated with 1 – 3% treatment-related mortality (Sanoff et al. 2012). Further, in breast cancer, yet considered well-tolerated, the mortality rate using adjuvant anthracyclines and taxanes is still 0.2 – 0.5% (e.g. acute chemotherapy-induced leukemia, cardiotoxicity) (Early Breast Cancer Trialists' Collaborative et al. 2012).

Inherent ability of viruses to kill cancer cells has been regarded for over a century, along with observations that cancer patients who suffered from influenza infections seemed to slow down progression of their disease (Kelly and Russell 2007). However, the use of virotherapy for treatment of cancer sparked along with developments in basic virology in the 1950s. A few different viruses were tested in the first clinical trials at the time, including hepatitis B, West Nile virus, and adenovirus. Although clinical responses were observed in several different tumor types, also side-effects were notable, which is not surprising given the limited knowledge in virology and

inability to render viruses tumor-homing by means of gene therapy (Kelly and Russell 2007). These obstacles together with contemporaneous development of novel more efficient chemotherapeutic drugs, led to a few decades of decreased interest in virotherapy. In the 1990s, however, time was ready for genetic engineering of viruses, and thus virotherapy was bolstered by methods of gene therapy. Subsequently, a non-pathogenic Moloney murine leukemia virus was engineered to express thymidine kinase gene (tk) derived from a herpes simplex virus-1 (HSV), which became the first genetically-engineered virus (Ezzeddine et al. 1991). This strategy known as 'suicide gene therapy' utilizes a pro-drug conversion enzyme, here thymidine kinase, which converts a pro-drug, ganciclovir, into a toxic metabolite thus killing only the infected cells (expressing the tk protein). During the following years, similar approaches were intensively tested, mainly for treatment of malignant glioma that lacks curative treatment modalities, finally resulting in a phase III randomized trial in the late 20th century (Rainov 2000): Researchers used carrier cells that were infected *ex vivo* with a retroviral vector containing the tk gene, and tested local injections of these cells given during surgery, coupled with ganciclovir treatment, as compared to the standard treatment in previously untreated glioblastoma. Although not reaching their primary endpoints probably due to poor delivery of the HSV-tk enzyme, the study proofed the biosafety and feasibility of the approach, which encouraged other researchers from Finland to proceed to another phase III trial that started in 2005 and the final results were recently announced (Westphal et al. 2013): This large multicenter trial used the same pro-drug converting enzyme but this time mediated by a replication-deficient adenovirus vector, which rendered more promising results; Median time to death or re-intervention (interim analysis) proved longer than with current standard therapy, but the overall survival was not significantly improved.

The field of cancer gene therapy has grown from its infancy during the past decade, and the first marketing approvals have been achieved. As of 2003, China became the first country to approve a modified replication-deficient serotype 5 adenovirus coding for p53 protein, Ad-p53, which is used together with radiotherapy for the treatment of head and neck cancer (Pearson et al. 2004). Only two years later, China was again the first country in the world to approve an oncolytic, i.e. replication-competent adenovirus, H101 (Garber 2006). This serotype 5 adenovirus is rendered tumor selective by deletion of the E1B gene, which is necessary for virus replication in normal cells with intact p53 protein. H101 is also intended for the treatment of head and neck cancer. An important milestone for cancer gene therapy in the Western countries was achieved in 2013, when the first positive phase III trial results using an oncolytic herpes virus, talimogene laherparepvec (T-VEC), in metastatic melanoma were announced last year (Andtbacka et al. 2013). T-VEC codes for granulocyte-macrophage colony-stimulating factor (GMCSF) protein to improve antitumor immune responses, and is expected to receive the first marketing license for oncolytic immunotherapy agent in the Western world in the near future.

Above mentioned approaches that have reached far in clinical testing can be divided into six main categories or strategies how to combat cancer: (**1.**) HSV-tk strategy is an example of the described 'suicide gene therapy'; (**2.**) Ad-p53 therapy aims at replacing missing or altered genes in tumor cells to induce cell death (replacing p53 protein); (**3.**) H101 and T-VEC are oncolytic viruses directed to specifically replicate in, and kill cancer cells *via* oncolysis. In addition, T-VEC can be regarded as a form of gene therapy aimed at (**4.**) improving host's antitumor immune responses, which is achieved in this case by GMCSF expression as well as virus replication *per se*. Besides these approaches, researchers have investigated ways to transfer genes, usually by means of replication-deficient vectors, that would render target tumor cells (**5.**) more susceptible to radiotherapy, chemotherapy, or other treatments; or (**6.**) genes that inhibit tumor angiogenesis or

interfere with tumor metabolism, thus depriving cancer of nutrients. Nevertheless, the focus of this thesis is on oncolytic viruses, which appear to be one of the most promising cancer gene therapy approaches, as indicated by the recent success in clinical trials.

1.3.3 Tumor immunology and immunotherapy

Development of the Coley's toxin in 1892, containing killed Streptococcus pyogenes and Serratia marcescens bacteria, was the second anticancer modality after surgery to reach "clinical" testing. It was sparked from an observation by Willliam Coley that tumors of a sarcoma patient disappeared following a high fever from erysipelas infection (Nauts et al. 1946). Thereafter Coley and others, and later a pharmaceutical company reported numerous similar cases by using the toxin preparations on cancer patients, occasionally coupled with some serious adverse reactions. With the inconsistency of treatment results and contemporary emergence of radiotherapy and the first chemotherapeutic drugs, Coley's toxin, and with it the concept of activating body's own immune system against cancer, nowadays known as cancer immunotherapy, was largely forgotten for nearly half a century.

Immunosurveillance and immunoediting

Dr. William Coley and colleagues were ahead of their time, because in order to develop tumor immunotherapy, it is necessary to understand tumor immunology. This has proven evidently true when looking back to the progress and convergence of these fields. Immunotherapy began lifting its profile again in the 1970s. This happened alongside with decades long debate in the scientific community whether a concept of cancer immunosurveillance existed, which was depicted by its developer and strongest supporter, Nobel Laureate Sir Frank Macfarlane Burnet in 1970 as follows: "*In large long-lived animals, like most of the warm-blooded vertebrates, inheritable genetic changes must be common in somatic cells and a proportion of these changes will represent a step toward malignancy. It is an evolutionary necessity that there should be some mechanism for eliminating or inactivating such potentially dangerous mutant cells and it is postulated that this mechanism is of immunological character*" (Burnet 1970). The concept got support with the first experimental findings by Burnet and colleagues in their series of crucial immunological experiments (Burnet 1970, Corthay 2014). Nevertheless, debate continued as later evidence proposed a dual role for immunity in the context of cancer: it was observed that parts of the immune system also promote tumor growth, in particular by selective advantage for cancerous lesions of low immunogenicity, i.e. lesions that are not empathically recognized by the immune system. As recently as in 2002, this lead to emergence of another, supplementing concept of "immunoediting" (Dunn et al. 2002). The process constitutes of three phases based on scientific evidence, denoted as the three **E**'s of cancer immunoediting (Dunn et al. 2004): Tumor cells are ***e****liminated* by the surveying immune system, but occasionally some cancer cells with low immunogenic phenotypes are spared due to fine balance between foreign and self, which are then selected by the Darwinian law and promoted for. This can lead to an ***e****quilibrium* phase, lasting potentially for decades, where the tumor and the immune system are constantly selecting for clones and eradicating them, respectively, but maintaining a dynamic balance. Many lesions are spontaneously eradicated by the immune system during this phase, or prompted by natural infections and other external factors to be destructed. Some external factors can, however, offer additional selection advantage to the tumor lesions, such as viral oncoproteins or growth-promoting mutations *via* carcinogens. Depending on the tipping of this scale, mutational

advantage may ultimately lead to the final phase of tumor ***e**scape*, where the immune system can no longer restrain the growing tumor that effectively resists immune destruction, either *via* reduced immunogenicity (e.g. mutations leading to reduced antigen-presentation), or because of immunosuppression mediated by the selected tumor variants, or more usually, due to both.

With regards to cancer immunotherapy, immunoediting mechanisms that are distinctive for the last ***e**scape* phase are in the spotlight, in particular loss of tumor detection, and immunosuppression. This is logical since diagnosed cancer patients normally bear tumors that have already progressed into the immune-escaped phase. Therefore, the big question in cancer immunotherapy is: how can we reset or provoke patient's immunosurveillance system to notice cancer? Improved understanding of the tumor immunity has implicated three main niche for therapeutic intervention: **i**) promoting the induction of antitumor effector cell (usually T-cells) responses, **ii**) promoting tumor-antigen presentation functions of dendritic cells, and **iii**) overcoming immunosuppression mediated by the tumor (Mellman et al. 2011). Indeed, for cancer immunotherapist, the ***e**scape* phase of immunoediting is definitively not considered as the final phase.

The first modern immunotherapies

In the beginning of the modern cancer immunotherapy era, several immunotherapeutic treatments were tested in clinical studies. Notably, one of the first attempts clearly followed in the foot-steps of Dr. Coley, and became a still widely used treatment: bacillus Calmette-Guerin as an immunomodulator in the treatment of bladder cancer (Morales et al. 1976). Although the specific mechanisms of this intravesical treatment are still unknown, it is believed to affect at least two of the main immunotherapeutic niche (**i, ii**): increased antigen presentation of both the foreign microbe and the tumor, and increased antitumor T-cell activation. Another well-established treatment dating back to the 1970s is allogeneic bone marrow transplantation (Weiden et al. 1979). Interestingly, this therapy form was not originally intended as an immune-based treatment but rather as re-establishment of the vital bone marrow after high-dose chemo- or radiotherapy. Nevertheless, along with the notion of the graft-versus-leukemia effect, this approach has been since successfully used to treat thousands of patients with hematological diseases as well as some solid tumors (Dougan and Dranoff 2009). The main immunotherapeutic mechanism of action here is to induce antitumor T-cells (**i**), which achieved with the graft, bypassing the need for activating endogenous antitumor immunity. Unfortunately, the method is also associated with the unwanted graft-versus-host effect, and high mortality rates due to heavy myeloablation and high risk of sepsis. Of note, various supportive treatments used for the myeloablated, neutropenic patients, such as infusion of leukocyte growth factors, granulocyte (G) or granulocyte-macrophage (GM) colony stimulating factors (CSF), were noted to increase early survival after myeloablative treatments, and were later studied in the context of immunotherapy.

Cytokine therapy

Other early immunotherapeutic studies assessed the feasibility of infusing recombinant cytokine-like molecules, e.g. interferon alpha (IFN-α), and tumor necrosis factor alpha (TNF-α), GMCSF, and interleukin-2 (IL-2), some of which were shown to mediate promising efficacy in clinical trials. These therapies were the first rationally designed biological approaches, emerged with the deepening understanding of cancer immunosurveillance mechanisms, together with isolation and

recombinant production techniques. Besides several other direct or indirect cytotoxic effects, they also possess clear immunotherapeutic features (**i, ii**):

- IFN-α increases major histocompatibility complex class I (MHC-I) antigen expression, crucial for initiation of antigen-presentation (Anderson et al. 1994), and was first approved for the treatment of hairy cell leukemia (Quesada et al. 1986), followed by several other indications such as hepatocellular carcinoma, bladder cancer and lymphoma.
- With regards to TNF-α, systemic administration proved too toxic, but isolated limb-perfusion using recombinant TNF-α together with chemotherapy showed impressive local efficacy with manageable safety profile in soft tissue sarcomas, and resulted in approval in Europe (Mocellin et al. 2005). Immunotherapeutic mechanisms of TNF-α are diverse, but they mainly involve production of inflammatory cytokines and maturation of dendritic cells for antigen-presentation (Mocellin et al. 2005).
- Granulocyte-macrophage colony-stimulating factor (GMCSF) was characterized as a cytokine with great potential as an inducer of hematopoiesis and cellular immunity (Arellano and Lonial 2008), and was soon applied as supportive treatment for neutropenic patients (see above). Meanwhile, the effects of GMCSF on dendritic cells suggested potential use as primary immunotherapy: GMCSF promotes differentiation of progenitor cells into dendritic cells and enhances their antigen-presentation. In addition, it recruits monocytes, and actives lymphocytes including natural killer (NK) cells at the site of inflammation. As a result, systemic recombinant GMCSF was tested, together with other cytokines IFN-α and IL-2, and interestingly, also with temozolomide, for treatment of advanced melanoma (Arellano and Lonial 2008). However, dose-limiting toxicities were encountered using systemic GMCSF administration, although signs of promising efficacy were also noted. Due to this, and along with the notion that high systemic levels can induce immunosuppressive myeloid-derived suppressor cells (MDSCs) (Serafini et al. 2004), GMCSF was incorporated as an adjuvant in cancer vaccines and gene therapy approaches, including oncolytic viruses, with evident success since both have resulted in positive phase III results (discussed below).
- IL-2 is a T-cell growth factor with evident immunotherapeutic effects, and high-dose IL-2 regimen was designed in the 1980s followed by series of promising animal experiments. Since then hundreds of cancer patients have been treated alone or in combination with other treatments with recombinant IL-2, with most notable success in metastatic melanoma, (Atkins et al. 1999). Many durable complete responses were observed that essentially established the curative potential of immunotherapy. Ultimately, recombinant IL-2, termed aldesleukin, was approved for treatment of metastatic renal cell carcinoma in 1993 and for metastatic melanoma in 1998. Downsides of high-dose IL-2 therapy are, however, that the curative response rates are low (ca. 6%) and hard to predict, and that the treatment contains a risk for immediate sepsis-like condition, fatal to ca. 2% of patients (Atkins et al. 1999). Of note, lower dose IL-2 regimens have also been tested, e.g. in metastatic renal cancer, which decreases the amount of serious adverse events, but this also accounts for less durable responses (Yang and Rosenberg 1997).

It soon became apparent that although potentially effective, and even curative in some patients, recombinant cytokine-like molecules administered systemically mediated serious inflammatory side-effects, and thus their production at the tumor site by means of gene therapy gained attention (Liu et al. 2010). Alternatively, researchers started investigating the use of recombinant

cytokines as adjuvant therapies, and consequently, e.g. pre-conditioning with systemic IL-2 therapy is a typical part of adoptive T-cell therapies as of now (Pellegrini et al. 2010).

Antibody therapy

More recently, the use of monoclonal antibodies as biological therapeutics has become increasingly popular. They possess a major benefit over many of the older immunotherapeutics, they are targeted against a specific antigen. Thus, theoretically all tumor-specific antigens could be targeted, however, this is not practical since tumor-antigens often resemble normal self-antigens. Currently, there are nine formally approved monoclonal antibodies for treatment of cancer, which target six tumor-associated proteins (Mellman et al. 2011): Her2/neu, VEGF, EGFR, CD20, CD33 and CD52. Although the main mechanism of action for these antibodies is considered to be inhibition of tumor-growth signaling pathways, they also mediate immune cell activations, specifically NK cell induction *via* binding to the tail-fragment of the IgG antibody when attached to the antigen on target cell. Nevertheless, the most important immunotherapeutic application in general, and for antibody therapy in particular, was the identification of an immune checkpoint protein CTLA-4 and subsequent development of an antagonistic antibody ipilimumab by Dr. James Allison and colleagues in the 1990s (Chambers et al. 2001). CTLA-4 molecule is a negative regulator of antigen-specific immune responses acting on many cell types. In brief, CTLA-4 impairs antigen-presentation in the immunological synapse between T-cells and dendritic cells, while promoting regulatory T-cells (T-reg), an immunosuppressive subtype of T-cells (Chambers et al. 2001). In addition, tumor cells can express CTLA-4 which blocks the immune responses. Thus, ipilimumab represented the first-in-class strategy to combat the (tumor) immunosuppression (**iii**). However, critic was soon raised against ipilimumab (Bakacs et al. 2012), questioning the use of drug which releases breaks of the T-cells allowing widespread immunological adverse reactions that were totally new in oncology, and unfortunately included fatalities (2.1% in a phase III trial; (Hodi et al. 2010)). In the next phase III trial involving 502 patients with metastatic melanoma, researchers were more prepared with earlier dose-reductions and effective use of steroids suggesting that most of these immune-related adverse events (ir-AEs) were actually manageable (Curran et al. 2012): As a result, no treatment-related deaths occurred, although grade 3 or 4 adverse events occurred in 56.3% of patients as opposed to only 27.5% in the standard therapy group (Robert et al. 2011). Nevertheless, efficacy was unsurpassed with significant increase in median overall survival in both studies, and for the first time in history, around 22% of patients were showing durable responses at 3-year follow-up and ongoing (Couzin-Frankel 2013). Ipilimumab was approved by the United States and European authorities for treatment of metastatic melanoma in 2011 and 2012, respectively, and is currently tested in several phase II-III studies alone or in combination with other modalities, for several tumor types including bladder cancer, lung cancer, and metastatic hormone-refractory prostate cancer.

In the wave of this encouraging immune checkpoint research, another notable antibody target strategy has emerged that potentially entails more favorable safety-profile and even better efficacy: Blocking of programmed death-1 protein, or its ligand (PD-1 or PD-L1) mediates similar release of breaks on T-cell immunity as ipilimumab, but not in the immunological synapse but instead mainly at the tumor site between the cancer cell and T-cells. Thus, PD-1L signaling that many tumors exploit to inhibit T-cell functions, is more tumor-specific. Indeed, an early clinical trial of 296 patients was associated with less serious adverse events than ipilimumab (14% grade 3–4 adverse events, 1% of fatal) and an impressive 20-25% durable response rate in patients with non-small-cell lung cancer, metastatic melanoma, or renal-cell cancer (Topalian et al. 2012).

Importantly, responses correlated with PD-L1 expression status at the tumors, suggesting specific mechanism of action combating tumor-mediated immunosuppression.

Therapeutic cancer vaccines

Decades long research on therapeutic cancer vaccines has also gained attention in the recent years, as the first cancer vaccine sipuleucel-T, a mixture of patient's peripheral blood mononuclear cells supplemented with a GMCSF cytokine and a prostate tumor-associated antigen (prostatic acid phosphatase), was approved in the United States for treatment of advanced prostate cancer in 2010 (Kantoff et al. 2010). This cell-based cancer vaccine is administered intravenously and was found to improve median survival by 4.1 months. The immunotherapeutic niche of the cancer vaccine field is to promote the tumor-antigen presentation by dendritic cells (**ii**). Several strategies have been experimented, including cell-based (tumor and dendritic cell), purified component-based, DNA-based, and tumor-peptide preparations that are administered either subcutaneously, intravenously or injected into tumor-draining lymph nodes or tumors (Aranda et al. 2013). Mechanistically, therapeutic cancer vaccines often present antitumor activity and research has provided valuable new knowledge on antitumor immunity. With the notable exception of sipuleucel-T, however, success in clinical trials has been modest. Many reasons may account for this, such as need for personalized vaccination given the unique features of each tumor, intrinsically low antigenicity of cancer cells, and tumor-mediated immunosuppression. Moreover, the emerging concept immunogenic cell death indicates that certain cytotoxic anticancer agents can provoke antitumor immune responses merely *via* release of danger signals from dying cells (Apetoh et al. 2008). Thus, it is likely that immune activation without proper danger signaling at the tumor site, coupled with distortion of the immunosuppressive circuits, is not sufficient to mediate tumor rejection. Of note, an increasingly important means of utilizing the immunological memory, and a testament to the power of immunosurveillance, is the use of prophylactic cancer vaccination; Millions of children have been vaccinated against oncogenic microbes, including hepatitis B and human papilloma virus (Dougan and Dranoff 2009), thus preventing cancer even before occurrence.

Immune-related tumor responses

As mentioned, immune-related adverse events (ir-AEs) encountered with ipilimumab therapy were a new chapter in clinical cancer research. Another peculiar characteristic of immunotherapies, especially with immune checkpoint blocking antibodies and therapeutic cancer vaccines, is the inaccuracy of radiological response evaluations in predicting survival (Wolchok et al. 2009, Kantoff et al. 2010). Survival and quality of life are naturally the most relevant endpoints for cancer patients. However, for development of treatments in clinical trials, as well as for improving cost-effectiveness and clinical decision making in oncology practice, surrogate endpoints such as radiological response evaluation are widely used. In fact, several modern trials use progression-free survival as their interim primary endpoint. Currently, contrast enhanced computed tomography (CT), is the leading method for radiological response evaluation and anatomical criteria such as Response Evaluation Criteria in Solid Tumors (RECIST) are frequently used for the characterization of responses in trials (Eisenhauer et al. 2009). These evaluation standards are suitable for many traditional treatments, such as chemotherapy and radiotherapy, where tumor shrinkage is measured as a sign of treatment benefit (Wolchok et al. 2009). However, with regards to immunotherapy, including also oncolytic viruses, responses are elicited in a more complex way: Besides tumor cell killing, there is a major inflammatory response due to danger-signals and

foreign antigens from both the virus/vaccine and the tumor, which provokes migration of immune cells coupled with extravasation. Thus, the activation of the immune system can lead to tumor swelling and initial increase in tumor diameters which might actually indicate therapeutic efficacy – an early sign of mounting an inflammatory response due to the treatment instead of progression (Wolchok et al. 2009).

In an attempt to circumvent this problem, metabolic response evaluations as assessed by e.g. positron emission tomography (PET) that measures e.g. [18F]-fluorodeoxyglucose (FDG) activity, have been developed (Wahl et al. 2009). This method registers the glucose uptake as a sign of tissue metabolism, and because increased consumption is a characteristic of most cancers, the uptake of FDG indicates tumor cell viability. In contrast, decreased signal represents reduced viability (Young et al. 1999), and could also reveal "pseudo-progression" mediated by therapeutic inflammation, because swelling is later associated with increased water content. Indeed, FDG-PET has been regarded with better biological predictive value than anatomic imaging (CT or magnetic resonance imaging [MRI]) in certain cancer types, including mesotheliomas, lymphomas, lung cancer, and esophageal cancer, especially early during therapy (Wahl et al. 2009). The metabolic response evaluation with FDG-PET might therefore be more feasible than anatomical imaging in detecting patients who are likely to benefit from immunotherapy. Another attempt to this end involves more appropriate evaluation criteria. Immunotherapy consortia and health authorities have worked together to develop immune-related response criteria (ir-RC) that take inflammatory reactions better into account. They not only allow certain initial increases in tumor diameters, but also regard development of certain new lesions in stable disease, because these may indicate immunological activity in tumor-draining lymph nodes (Wolchok et al. 2009). Besides important for response evaluations in clinical trials, these aspects have direct impact in patient care as well: If the therapeutic inflammation ("pseudo-progression") leads to premature attenuation of the therapy, it could be detrimental to the patient. Another unique characteristic in response patterns seen after immunotherapy is the durability of responses due to immunological memory. This has led to re-evaluation of statistics, particularly, of the Kaplan–Meier survival curves, to emphasize the delayed separation of the curves in order to assess impact of the treatment on long-term survival (Hoos et al. 2010).

Adoptive T-cell therapy

T-cells that have been cross-primed (activated) by dendritic cells are capable of directly recognizing their specific antigen, kill the target cell, and mediate clonal expansion, ultimately eradicating the tumor. Activated T-cells move through tissues, including tumor beds, scanning for MHC-I peptide complexes loaded with cellular antigens on the cell surfaces. Accumulating evidence suggests that the migration of antitumor T-cells is arrested in advanced tumors even if they encounter their antigens (Restifo et al. 2012), which is likely due to lack of danger-signals and/or multiple layers of immunosuppression mediated by the tumor. This is a possible explanation for typical presence of tumor-infiltrating lymphocytes in certain advanced stage tumors, such as melanoma, that can no longer control tumor growth although activation signal has been provided.

One highly anticipated form of immunotherapy is adoptive cell therapy, which generally involves the use of autologous tumor-infiltrating or circulating T-cells, which are "adopted" from the patient, genetically-modified and expanded, or only expanded *ex vivo*, and then infused back to patients. As mentioned, Dr. Rosenberg and colleagues were the first to conduct a cancer gene therapy trial in 1989 using genetically modified tumor-infiltrating lymphocytes (TILs) for treatment

of metastatic melanoma (Rosenberg et al. 1990). Since then, these techniques have been developed rigorously by a few researchers. The immunotherapeutic niche exploited here involves direct induction of antitumor T-cells (**i**). Thus, it circumvents the problem of antigen-presentation, or presumes that it has already occurred when using tumor-infiltrating lymphocytes (TILs). Presence of TILs at the tumor site, together with possibility to excise and grow them out, is therefore a prerequisite for TIL therapy. Alternatively, circulating lymphocytes can be utilized by genetically modifying them, by means of retroviral transduction, to express an artificial T-cell receptor against a tumor-antigen. Two main strategies to achieve this are T-cell receptor (TCR) modified, and chimeric antigen receptor (CAR) T-cells (Hinrichs and Rosenberg 2014). The major difference between these technologies is that CAR T-cells do not require MHC-I presentation of antigens, but can be targeted against cell surface proteins, such as CD19 in leukemia. Important factors contributing efficacy after adoptive cell transfer are also preparative lymphodepletion by chemo- or radiotherapy, and pre-conditioning of the patient with systemic IL-2 infusion. These regimens are associated with significant toxicity, while e.g. tumor-lysing and cytokine release syndromes after application of the T-cell graft pose further problems. TCR therapies can also cause autoimmune reactions due to off-target toxicity (homology between self- and tumor-antigen) (Hinrichs and Rosenberg 2014). Nevertheless, even metastatic patients who have exhausted all other available treatments have shown remarkable complete responses; In one center giving TIL therapy for example, a clinical trial series of 93 metastatic melanoma patients showed 56% objective response and 22% durable complete response rates (Hinrichs and Rosenberg 2014).

With the use of TCR and CAR T-cell technology, collection of TILs can be avoided, which has allowed clinical testing in several other malignancies, including B-cell malignancies and synovial sarcoma. The strongest testament for the curative potential of T-cell therapy was, however, seen last year when researchers announced results of an early trial using CD19-targeted CAR T-cells in pediatric patients with chemotherapy-refractory, stem cell transplantation refractory, acute lymphoid leukemia (Grupp et al. 2013): Both patients went into continuing remission, with establishment of an evident T-cell memory against leukemic cells. Very recently, another CD19 CAR T-cell trial reported corresponding results, showing complete remission in 27 out of 30 pediatric and adult patients with refractory acute lymphoblastic leukemia and a 78% overall survival rate at six months (Maude et al. 2014). Severe cytokine-release syndrome was reported in 27% of patients, but no treatment-related deaths occurred. Since adoptive T-cell therapy is more of a technology than a drug at this stage, future applications are still open but are likely to have strong impact on treatment of refractory cancer. Of note, pharmacological companies have already noted the promise of the approach and medical development is well underway (Maude et al. 2014).

The immunotherapeutic strategies colliding with contemporary knowledge on tumor immunology show that there are multiple strategies to combat even metastatic tumors, but they must be tailored and optimized for each patient. As the different immune-mechanistic niches (**i**,**ii**,**iii**), and different approaches inside each niche suggest, combinatorial immunotherapies are most likely to increase the rates of durable responses to higher percentages. However, many more challenges lie ahead because not all tumors, such as pancreatic and colon carcinoma, are yet susceptible to present clinical or experimental immunotherapy.

Unmet challenges in tumor immunology

Dendritic cells are bone-marrow derived professional antigen-presenting cells that are crucial in initiating CD8+ cytotoxic and CD4+ helper T-cell responses (Gajewski et al. 2013). Foreign antigens can be loaded onto MHC class I for cross-presentation to CD8+ T-cells, which is believed to occur after the phagocytosis of immunogenic cells, sampling of live cells, or shuttling of immunogenic peptides to dendritic cells by heat shock proteins (van der Most et al. 2006). Unfortunately, the frequency of activated CD8+ T cells or their function often remains too low to mount an effective antitumor response after immunotherapy. Reasons for this are many, and mostly due to highly evolved immunosuppressive circuits in the tumor microenvironment.

Given the enormous mutation rate of malignant tumors, all types of cancer contain significant amounts of tumor-associated or tumor-specific antigens that are subject to immunoediting by T-cells. Analogously, cancer types that best respond to current immunotherapies, such as malignant melanoma and bladder cancer, seem to fall into the highest end of mutation rate frequencies as revealed by recent cancer genome sequencing efforts (Alexandrov et al. 2013). However, some other high-mutational tumor types such as lung or colorectal cancer remain hard to control by immunotherapeutics despite the plentiful targets for T-cell responses, suggesting major immunosuppressive mechanisms. To evade immunosurveillance and eradication by the immune system, tumors have developed multiple mechanisms to hide from the immune system, many of which remain still unknown. One such mechanism is by recruiting immunosuppressive cells (**Figure 4**), such as regulatory T-cells, immature myeloid cells, and tumor-associated macrophages to the tumor site and local lymph nodes. This can lead to excessive production of chronic inflammatory mediators such as IL-6, TNF-α, and HMGB1 that leads to polarization of the cross-primed CD4+ T-cells into T-helper type 2 (Th2) cells, which are responsible for mediating mostly humoral immunity (Coussens et al. 2013, Li et al. 2013a). Th2 type immunity is ineffective in tumor eradication, and further promotes accumulation of immunosuppressive cells to the tumor microenvironment.

Regulatory T-cells are an immunosuppressive subset of CD4+ T-cells that are mainly responsible for regulating peripheral tolerance by suppressing dendritic cells and effector T-cells reactive to self-antigens (Daniel et al. 2009). Since tumor-antigens are presented similar to self-antigens on MHC class I molecules, regulatory T-cells are also effective in reducing antitumor immune responses. Advanced tumors exploit this function by secreting immunosuppressive cytokines such as transforming growth factor beta (TGF-β) (Lindau et al. 2013). In addition to recruiting regulatory T-cells, TGF-β directly inhibits cytotoxic and helper T-cells, e.g. by downregulating their cytotoxic capacity and IFN-γ production, respectively (Tian et al. 2011). Effector T-cells can be also suppressed by several other tumor-derived soluble and cell surface proteins such enzyme indolamine-2,3-dioxygenase (IDO) that deprives tryptophan causing local T-cell anergy (Muller and Prendergast 2007), CTLA-4 that inhibits the immunological synapse between dendritic cell and T-cell (Seliger et al. 2008), and PD-ligand 1 which is a co-inhibitory signal to T-cells (McDermott and Atkins 2013). Some tumors have been also shown to downregulate their MHC class I processing, presentation and tumor-antigen exposure, thus leading to decreased T-cell recognition (Leone et al. 2013). Furthermore, effector T-cells can be committed to apoptosis by tumor-derived Fas-ligand (Houston et al. 2003).

Several immature myeloid cell types mediate immunosuppressive functions, such as immature dendritic cells that can abrogate antigen cross-presentation to effector T-cells (Kusmartsev and Gabrilovich 2006). Myeloid-derived suppressor cells (MDSCs) are considered an important

suppressor mediating e.g. PD-L1, TGF-β, nitric oxide production, and recruitment of regulatory T-cells (Lindau et al. 2013). Tumor-associated macrophages with M2 phenotype provide anti-inflammatory molecules, such as IL-10 and TGF-β to promote immune evasion (Hao et al. 2012). Certain pancreatic cancer-associated fibroblasts have been suggested to de-target T-cells by secreting chemokine CXCL12 (Feig et al. 2013). Finally, there is strong clinical correlative data on the impact of different immune cell subtypes on prognosis: the presence of immunosuppressive cells accounts for poor prognosis (Chikamatsu et al. 2007, Perrot et al. 2007, Tadmor et al. 2013, Tanchot et al. 2013), whereas the effector T-cells have been linked with improved outcome (Hodi and Dranoff 2010, Nosho et al. 2010).

These aspects highlight the importance of immunosuppression for progressive human tumors. Many of the aforementioned mechanisms are overlapping and hierarchy of immunosuppression has recently been identified in e.g. pancreatic cancer *in vivo* (Feig et al. 2013), although the picture is likely to be even more complex in human tumors that develop over years if not decades. Identifying the immunosuppressive circuits that distinct tumor types typically exploit can help in developing countermeasures. Indeed, several approaches are currently being investigated to overcome each of the aforementioned challenges (Mellman et al. 2011, Loven et al. 2013, Kyi and Postow 2014, Lichty et al. 2014). Emerging clinical data suggests that rational combinations of different immunotherapeutics and immune-modulating chemotherapeutics that combat several levels of suppression can improve the efficacy even in synergistic manner (Cerullo et al. 2011, Emens 2012, Puzanov et al. 2014).

1.4 Adenovirus

Developments in cell culture and virus propagation methods in the 1950s led to rapid progress in basic virology and discovery of several new viruses, including adenovirus (Rowe et al. 1953). The name originates from the site of its discovery, the adenoid structures, which are lymphatic tissues located in the nasopharynx. The family of adenoviruses, Adenoviridae, is divided into five genera, of which human adenoviruses constitute the Mastadenovirus genus. They are further divided into seven species, A – G, based on their ability to agglutinate human erythrocytes. Using genomics as a method for typing adenoviruses, already 59 different serotypes have been discovered (Liu et al. 2012), with most of these belonging to species D. However, in this thesis and in the following chapters, we focus on serotype 5 and 3 adenoviruses, which belong to the species C and B, respectively. Both of these serotypes are endemic in most parts of the world as seroprevalence for adenovirus species C (incl. serotype 5) varies from 35% up to 97%, and for serotype 3 ranging between 40–80% (Nayak and Herzog 2010, Arnberg 2012).

Human adenoviruses are considered as low-pathogenic viruses, since they mostly cause self-limiting infections (Kunz and Ottolini 2010). However, also severe infections and complications have been reported, especially in immune-deficient individuals and infants. As epithelial cells are the primary targets for adenovirus pathology, respiratory infections, gastroenteritis, and epidemic conjunctivitis are the most common manifestations. In rare occasions, adenoviruses can also cause liver and urinary bladder infections, and even more sporadically, myocarditis and meningitis. With regards to respiratory tract infections, which are the most common, potential complications include secondary bacterial pneumonia and bacterial empyema, pleural effusion, and acute

respiratory distress syndrome (Kunz and Ottolini 2010). Different serotypes are associated with distinct pathogenicity and manifestations. Epidemic outbreaks or severe complications have been linked to serotypes 4, 7, 11, 14, 21 and 35. Notably, serotype 5 adenovirus, the most used cancer gene therapy vector, has only been associated with upper respiratory tract infections. Serotype 3 adenovirus has been identified to cause also lower respiratory tract infections, pharyngo-conjunctival fever, and conjunctivitis, which are mainly self-limiting (Kunz and Ottolini 2010).

Febrile acute respiratory diseases caused by adenoviruses are mainly encountered in infants and school-aged children, but also in military recruits. The various diseases caused by adenoviruses manifest usually relatively mildly, as acute self-limiting infections. However, for immunosuppressed patients and infants they can be severe and even life-threatening, manifesting as fulminant hepatitis, pneumonia and even meningoencephalitis. For unknown reasons different serotypes are characteristically responsible for different disease manifestations. For example Ad40 and Ad41 infections cause gastroenteritis, Ad8, Ad19 and Ad37 are typically related to epidemic keratoconjunctivitis and Ad11 and Ad21 are principal serotypes encountered in hemorrhagic cystitis infections (Kunz and Ottolini 2010). Sporadic pathological infections of pancreatic tissue, myocardium and central nervous system have also been reported.

1.4.1 Adenovirus structure

Adenoviruses are double-stranded DNA viruses surrounded by an icosahedral protein capsid, and no envelope. Virus particle measures ca. 90 nm in diameter, weights ca. 150 MDa, and shows a fiber projecting from each vertex of the capsid (**Figures 2 and 3**), which consists of seven proteins (87% of mass is protein): II (hexon, 240 units), III (penton base, 12 units), and IV (knobbed fiber, 12 units), which are the major proteins of the capsid, and IIIa, VI, VIII, and IX, the minor proteins (Smith et al. 2010) (**Table 1**). Inside the capsid lie the linear double-stranded DNA genome (13% of mass) and the associated core proteins. Genome is 36 kilo-base pairs long and, in the case of serotype 5, was fully sequenced over 20 years ago (Chroboczek et al. 1992). Core proteins provide structure, link the genome to the capsid, and are needed for packaging of the virions. In addition, the terminal core protein that attaches to the 5’ ends of the genome, which have inverted terminal repeats (ITRs), is necessary for initiation of viral DNA replication (Rekosh et al. 1977). **Figure 3** illustrates, and **Table 1** describes the main functions of the adenovirus particle proteins.

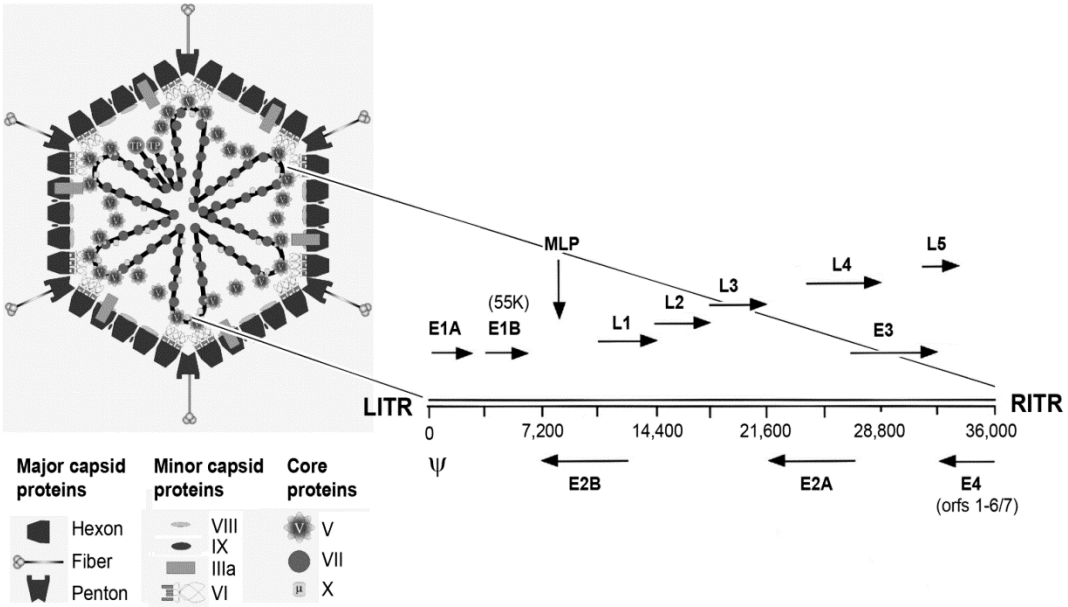

Figure 3. Adenovirus particle structure and schematic representation of the adenoviral genome. Left part: Adenovirus structure. Virus particle is composed of the outer capsid and the core. Penton base, hexon, and fiber protein with a knob, constitute the icosahedral capsid illustrated here in two dimensions. Other main proteins are depicted in roman numerals and their functions are described in Table 1. Right part: Schematics of the adenovirus genome. E = early genes, L = late genes, MLP = major late promoter, Ψ = packaging signal, which acts as a starting point for virus replication, LITR/RITR = left/right inverted terminal repeats, which are crucial for complementary DNA pairing during virus replication. Certain proteins coded by early regions, that are central for this thesis, are shown in parenthesis are (E1B55K, E4orf3, and E4orf6). Modified from: (Russell 2000).

Table 1. Adenovirus particle proteins and their main functions. Capsid proteins are bolded. References: (Saban et al. 2006, Russell 2009).

Protein	**Function**
Protease	Involved in the production of infectious virions from the procapsid by cleaving the precursors into structural proteins IIIa, VI, VII, VIII, X, and terminal protein.
Terminal protein	Required for viral DNA replication, facilitates circularization of the genome.
II = Hexon	Four types of hexons (H1–4) constitute the total of 240 hexon proteins of the capsid, providing solid icosahedral structure.
III = Penton	Provides capsid structure. Arg-Gly-Asp (RGD) motif on the penton base is central for virus internalization by interacting with α,β integrins on the cell surface.
IIIa	Stabilizes the capsid by associating with the hexon and penton proteins.
IV = Fiber	Fiber knob is the first component interacting with target receptors. Varies between different serotypes, thus altering the primary adenovirus receptor.

IVa2	Binds to DNA and is critical for the packaging process of the virus genome.
V	Provides a bridge between the core and capsid proteins.
VI	Stabilizes the virus capsid by linking the hexon and penton proteins to the core. Required for the rupture of early endosomes, releasing the viral DNA to cytoplasm.
VII	Binds to DNA and spreads along the length of the virus DNA, providing protection.
VIII	Stabilizes the inner capsid by associating with hexon and penton proteins. Links the hexons to other capsid proteins at their base.
IX	Stabilizes the outer capsid by associating with hexon and penton proteins.
X	Protein X, also known as μ, has protamine-like properties.

1.4.2 Adenovirus life cycle

The adenovirus life-cycle is typically divided into early and late phases: In the early phase, infectious virus particle enters the host cell in a process called transduction, followed by unpackaging, transfer of the virus into nucleus, and transcription and translation of the early genes. In the second phase, late adenoviral genes are expressed and then processed in the nucleus, followed by packaging and maturation of infectious virus particles, the virions. Newly formed virions then lyse the host cell and spread. Serotype 5 adenovirus expresses first its five early transcriptional units (E1A, E1B, E2, E3, and E4), after which viral DNA replication takes place, followed by expression of the second phase genes that include three intermediate/delayed units (IX, IVa2, and E2 late) and one major late unit, which is processed into five late subunits (L1-L5) (Russell 2000). Depending on the permissivity of the cell, the early phase takes ca. 6 – 8 hours, and the second phase 4 – 6 hours (Russell 2000). Adding the phases of DNA replication and cell lysing, the total time from cell entry until release of new virions takes usually 24 – 36 hours. **Figure 3** represents the schematics of the adenovirus genome.

1.4.3. Transduction and biodistribution

Adenoviruses use primarily their knob region of the capsid fiber to bind to cell surface receptors. As adenoviral transmission usually occurs *via* aerosol route on oropharyngeal, respiratory, conjunctival or gastrointestinal lining, adenoviruses have evolved multiple cell entry mechanisms to epithelial cells. For species C (serotype 5) adenoviruses, the primary high-affinity receptor was identified as the same as for coxsackie B virus, which is why the protein was termed Coxsackie-adenovirus receptor, i.e. CAR-receptor (Bergelson et al. 1997). Subsequently, CAR-receptor was found to be the prime receptor for also all other species, excluding species B (serotype 3) adenoviruses (Roelvink et al. 1998). Biologically, CAR-receptor protein belongs to the immunoglobulin superfamily and is involved in the formation of tight junctions between epithelial cells (Cohen et al. 2001). Therefore, it is basolaterally localized and it still remains unclear how adenovirus initially reaches the CAR-receptor, although other receptors and adapter molecules may play a major part here (see below). In contrast, later replication cycles have been shown to facilitate CAR-receptor reaching by producing excess amounts of fiber proteins that bind to CAR-receptors and disrupt the tight junctions (Walters et al. 2002).

With regards to species B adenoviruses, the main cell entry receptor was identified as CD46 (Gaggar et al. 2003), but some serotypes like serotype 3 seemed to require an unknown high-affinity receptor, which was only very recently identified as desmoglein 2, i.e. DSG2-receptor (Wang et al. 2011). Interestingly, binding of serotype 3 adenovirus to the DSG2-receptor was demonstrated to transiently open intercellular junctions, allowing the virus to reach other receptors. Thus, DSG2-receptor interaction of serotype 3 knob can be exploited in cancer gene therapy, by incorporating serotype 3 knob to other species adenoviruses like serotype 5 (i.e. 5/3-chimera), potentially improving binding to other non-apical receptors as well (Kanerva et al. 2002a).

Other characterized receptors for species C (serotype 5) adenoviruses besides the CAR-receptor, include MHC-I molecule alpha2 domain (Hong et al. 1997), heparan sulfate glycosaminoglycans (Smith et al. 2003), vascular cell adhesion molecule-1 (Chu et al. 2001), and scavenger receptor A, which is responsible for virus uptake and subsequent degradation in macrophages (Haisma et al. 2009). For serotype 3 adenovirus, in turn, other identified receptors besides DSG2 and CD46, are CD80 and CD86 (Short et al. 2004). In addition, several adapter molecules have been identified, which can serve as carrier molecules that the virus exploits to avoid neutralization outside the cell, or linkers between the virus and the cell surface receptors. These include coagulation factors IX and X, lactoferrin, and dipalmitoyl phosphatidylcholine for species C, and coagulation factor X for species B adenoviruses (Arnberg 2012). Importantly with regards to virology and cancer gene therapy, simple modifications of these adapter molecules *in vivo*, such as impairing coagulation factor synthesis by warfarin treatment, can reduce adenovirus transduction to unwanted organs like liver, spleen and lung, while retaining high transduction of tumors (Koski et al. 2009), thus potentially enhancing its biodistribution profile.

After binding to primary receptor, such as CAR-receptor for serotype 5, the capsid fiber bends and allows secondary binding of a penton base motif, namely Arg-Gly-Asp (RGD) motif, to αβ integrins on the cell surface (Mathias et al. 1998). Subsequently, virus is internalized from the clathrin-coated pits into endosomes (Wang et al. 1998). In addition to penton protein, also fiber shaft interactions with cell surface heparan sulfate glycosaminoglycans seem to be important for proper attachment and internalization (Dechecchi et al. 2001). Interestingly, modifications/deletions in the fiber region responsible for this binding have been shown to reduce liver transduction of serotype 5 and 5/3-chimeric adenoviruses (Bayo-Puxan et al. 2009, Koski et al. 2013b), suggesting further means by which tumor-to-liver targeting ratio could be enhanced. Of note, besides receptor and adapter molecule interactions, also several other factors impact the biodistribution of human adenoviruses in circulation. These physiological mechanisms have evolved to minimize potential for fulminant infections, by homing maximal doses of virus to liver where it can be neutralized, but this may also markedly decrease tumor transduction efficacy of adenoviral gene therapy when administered or spread intravenously. Interactions with blood cells, coagulation factors and complement, as well as natural adenoviral tropism to liver can decrease tumor transduction, while rapid clearance from bloodstream by liver sinusoidal endothelial cells and tissue macrophages, such as Kupffer cells, can in turn increase liver transduction (Khare et al. 2011). Importantly, however, when using normal treatment doses, uptake of the virus by the liver sinusoidal and Kupffer cells does not appear to mediate virus production or gene expression at the liver (Hegenbarth et al. 2000, Wheeler et al. 2001). Even if reaching tumor site, physical barriers such as intratumoral stroma and high interstitial fluid pressure can limit virus propagation. To these ends, means for intensive replication and virus arming with extracellular matrix-degrading enzymes have been tested (Smith et al. 2011). Finally, antiviral innate and adaptive immune

responses, although also contributing to immunotherapeutic efficacy, as well as newly characterized acquired tumor resistance against adenovirus pose problems, which are discussed later in more detail.

1.4.4. Early phase and DNA-repair inhibition

Infectious adenovirus particle is internalized *via* clathrin-mediated endocytosis (Wang et al. 1998). Inside the endosome, virus capsid is disrupted by acidification of the vesicle followed by conformational changes that require e.g. adenoviral protein VI leading to release of the virus from the endosome (Wiethoff et al. 2005, Campos and Barry 2007). Subsequently, virus particle surrounded by only hexon proteins attaches to the nuclear pore complex and a nuclear factor CRM1 (Strunze et al. 2005), which mediate dissociation of most of the remaining capsid proteins and transfer the viral DNA with the associated core proteins into the nucleus (Greber et al. 1997). Viral DNA highjacks the host cell's transcription machinery, for which core proteins are essential, and transcription of the early genes is initiated (Russell 2009). Viral DNA transcription takes place in the nucleus, while the viral proteins are translated in the endoplasmic reticulum, and then transported back to nucleus. Viral DNA replication and assembly of the new virions are also performed in the nucleus in so called viral factories (Chaly and Chen 1993). Adenoviral genes and their main functions are well-characterized, allowing rational genetic engineering and appliance for therapeutic purposes such as cancer gene therapy.

Early genes of the E1-E4 regions are expressed before viral DNA replication. Their main functions involve interference with the host cell cycle signaling, innate defense, and apoptotic pathways (Berk 2005). E1A and E1B coded proteins are rapidly expressed after entering the nucleus, and they activate transcription of other early genes, modulate cellular metabolism to render the host cell susceptible to virus replication, e.g. by interfering with NF-κB and p53 proteins, and promote entry of the cell cycle into S phase (Berk 2005). Notably, these functions promote apoptosis, which is counteracted by other early genes of the E1B region (see below). In addition, E1A proteins inhibit IFN-α and IL-6 responsive elements and thus play a role in counteracting innate immune responses (Anderson and Fennie 1987, Takeda et al. 1994).
One of the best-characterized and exploited functions of E1A genes is the binding of 105K protein to Retinoblastoma (Rb) family proteins (Whyte et al. 1988, Sherr 1996): this interaction releases E2F transcription factor, which in turn activates genes required for promotion of the S phase. The importance of this discovery culminates in present adenoviral cancer gene therapy, where a 24-bp deletion (Δ24) in the Rb-binding site of the E1A is often utilized, because this attenuates virus replication and most of the later gene expression in normal cells that have wild-type retinoblastoma protein, but maintains replication cancer cells with defective Rb/p16 pathway that includes almost all human tumor types (Whyte et al. 1989, Fueyo et al. 2000, Kanerva et al. 2003). Finally, direct antitumor activities and chemotherapy-sensitizing effects of adenoviral E1A region have been demonstrated in several preclinical and clinical studies (Chang et al. 2014).

E1B region is crucial for adenovirus replication in normal cells because E1B55K protein mediates inhibition of p53 protein by several means, leading to cell cycling and inhibition of apoptosis (Sarnow et al. 1982, Berk 2005). However, in p53-mutated cancer cells, these functions are dispensable (Marcellus et al. 1996), and have been utilized in the early-generation conditionally-replicating adenoviruses such as ONYX-015 (see below). Adenovirus infection, especially the double-stranded DNA genomes in the nucleus, are sensed by the same cellular mechanisms as

ionizing radiation induced DSBs (Lilley et al. 2007). Therefore, adenovirus proteins have evolved to inhibit DNA damage response signaling that would lead to cell cycle checkpoint and apoptosis. In addition to blocking p53 protein, E1B55K has been shown to associate with two other adenoviral proteins, E4orf3 and E4orf6, which disable the DSB-sensing MRN complex (Leppard and Everett 1999, Lilley et al. 2007, Schwartz et al. 2008). Consequently, E1B55K has been proposed to augment in DSB repair inhibition, although its impact has remained controversial in this regard (Hart et al. 2005). Other E1B gene products are important in eliminating other cell death mechanisms, transporting viral RNAs for translation, and initiating DNA replication (Russell 2000). The other main gene product, E1B19K, functions to attenuate p53-independent apoptotic pathway, particularly by mimicking an anti-apoptotic BCL-2 family member protein MCL-1 (Cuconati et al. 2003). Interestingly, also this mimicry is involved in DNA-damage response, because MCL-1 degradation is yet another early response to DNA damage signaling (Nijhawan et al. 2003). Nevertheless, this pathway directly induces apoptosis instead of cell cycle stop, thus rendering E1B19K less interesting with regards to radiosensitizing potential.

E2 region encodes proteins crucial for viral DNA replication (Russell 2000), whereas E3 genes are dispensable for replication *in vitro*, which has been utilized to clear room for therapeutic transgenes (E3-deleted adenoviruses). Nevertheless, these genes are needed for downregulation of host antiviral immune responses, both innate and adaptive, and also for efficient lysis of the host cell mediated by the adenovirus death protein (ADP) (Tollefson et al. 1996a). Deletion of the E3 gene product gp19K has been widely used in gene therapy since this removal does not hamper virus replication *in vitro*, because gp19K functions to inhibit expression and transportation of MHC-I molecules from the endoplasmic reticulum to the cell surface (Bennett et al. 1999). However, reduced display of viral antigens loaded onto MHC-I complex would suggest *in vivo* implications. Indeed, recent evidence in semi-permissive immunocompetent Syrian hamster model indicates that the deletion of gp19K, and another E3 gene 6.7K, can lead to faster clearance of virus by antiviral immune responses (Bortolanza et al. 2009a).

The E4 region proteins are all transcribed initially as the same mRNA, but alternatively spliced into six different open reading frames (orf). Most of these E4orf-proteins promote viral late gene expression over host gene expression, and facilitate viral mRNA metabolism, e.g. by providing nuclear export signals (Halbert et al. 1985, Weigel and Dobbelstein 2000). The third and the sixth protein, namely E4orf3 and E4orf6, have been identified to mediate important cell cycle interfering functions, often together with E1B55K, as mentioned earlier. Inhibition of the DSB-sensing protein complex MRN by these proteins has been well-characterized (**Figure 2**): E4orf3 mislocalizes the MRN complex to cytoplasmic aggresomes, while E4orf6 targets it to proteasome-mediated degradation (Stracker et al. 2002, Araujo et al. 2005). E4orf6 has also been shown to radiosensitize cells by an alternative, E1B55K-independent mechanism, not affecting the MRN levels (Hart et al. 2005). Experiments on mutant viruses have revealed that infection with E4orf3/6-deficient adenoviruses activate robust DSB repair signaling *via* ATM and ATR cascades that lead to recruitment of repair proteins to viral factories, hampering the virus DNA replication and virion production (Stracker et al. 2002, Carson et al. 2003). Notably, E4orf6 protein has also been shown to inhibit dephosphorylation of γH2AX, a histone protein involved in DSB-sensing and repair (**Figure 2**), thus paradoxically prolonging DNA damage signaling *via* other mechanisms and promoting atypical, caspase-independent cell death (Hart et al. 2007).

1.4.5. Late phase and mechanism of cell death

After transcription of the early genes, viral DNA replication takes place in the nuclear viral factories. This process requires the terminal proteins attached to the inverted terminal repeats (ITRs) of both ends of the genome, from which DNA replication begins (Rekosh et al. 1977). Intermediate genes IVa2 and IX are expressed next, and function to activate the major late promoter, promoting transition into late phase (Lutz and Kedinger 1996, Lutz et al. 1997). The late genes (L1-L5) located under the major late promoter code for proteins involved in maturation and encapsulation of the virions (Russell 2009), as well as the structural proteins (**Table 1**). In the last step of its life-cycle adenovirus triggers cell lysis, and new infectious virions are released into extracellular space.

The mechanism of cell death is important in determining the nature of subsequent immune activations, and there are indications that adenovirus mediated cell lysis is highly immunogenic. Adenovirus death protein (ADP) differs from all other E3 proteins in that although being an early gene, it is expressed in very low quantities until the activation of the major late promoter (Tollefson et al. 1992). Soon after its characterization, ADP was found to mediate atypical form of cell death, morphologically resembling to what it is now regarded as autophagic cell death (Tollefson et al. 1996b). Of note, characteristics of several death mechanisms have been later identified, including apoptotic, autophagic and necrotic cell death, but especially with regards to cancer virotherapy, autophagy appears to gain support (Rajecki et al. 2009, Tazawa et al. 2013). Under physiological conditions, autophagy is a catabolic process that is activated during starvation and provides energy by degrading cytoplasmic organelles in autophagosomes (Mizushima and Komatsu 2011). However, several chemotherapeutics, such as TMZ, and oncolytic viruses have been shown to induce autophagic cell death (type II programmed cell death), which is characterized by increased turnover of cellular organelles beyond reversibility (Chen and Karantza 2011). Importantly, autophagic cell death has been regarded highly immunogenic (Guo et al. 2014). While a “silent” form of cell death, such as apoptosis, can lead to immunological tolerance (Green et al. 2009), immunogenic cell death activates dendritic cells leading to increased cross-presentation of antigens to effector T-cells (Hannani et al. 2011). Immunogenic cell death is characterized by exposure of calreticulin on the plasma membrane, followed by release of other danger-associated molecular patterns (DAMPs), adenosine-triphosphate (ATP) and nuclear protein high-mobility group box-1 (HMGB1), which has been also regarded for oncolytic adenovirus in this thesis and elsewhere (Diaconu et al. 2012). Each of these DAMPs are needed to activate the nearby dendritic cells and they also function to attract other immune cells (Hannani et al. 2011, Guo et al. 2014). In addition to releasing potent DAMP signals, activation of autophagy also leads to upregulation of MHC-I and II complexes that mediate antigen-presentation (Dengjel et al. 2005). Indeed, functional autophagy has been regarded as a prerequisite for activation of antitumor immune responses (Michaud et al. 2011, Guo et al. 2014).

Prompt danger-signaling, MHC complex upregulation, and the fact that no specific autophagy-inducing genes have been identified from adenovirus, may suggest that autophagy is actually a host defense and alert mechanism against this intracellular intruder. This may hold true in normal cells, but with regards to cancer cells that are devoid in apoptotic mechanisms, several oncolytic viruses seem to benefit from autophagy (Guo et al. 2014). Specifically with regards to adenovirus, autophagy may be exploited to generate nutrients needed for building viral progeny particles, and it has positive effect on virus replication (Jiang et al. 2011, Rodriguez-Rocha et al. 2011). Growing body of evidence indicates that autophagy accounts for improved antitumor efficacy in the context

of oncolytic virotherapy (Tazawa et al. 2013): 12 out of 14 recent preclinical publications reported that autophagy coincided with, or contributed to improved anticancer efficacy. Oncolytic immunotherapy in an immunocompetent host would be expected to benefit even further from this immunogenic type of cancer cell death.

1.4.6 Host antiviral defense mechanisms

Humans have evolved in close contact with plethora of infectious agents and parasites, which is why sophisticated immune defense mechanisms and extensive inter-individual variation are necessary for us to survive. As previously discussed, there is a delicate balance between non-self and self when immune system determines between activation and tolerance. Thus, the existence of autoimmune diseases, where overactive immunity destroys healthy tissues, and immune-escaped tumors, where body's own transformed cells have tamed the immune system, is not actually surprising. Human immune system consists of innate (i.e. natural) immunity and adaptive (i.e. acquired) immunity. The innate immunity forms the first-line defense against novel pathogens, and is mostly mediated by cells that are always present in the body. In fact, most of the human tissues, with the notable exception of central nervous system, possess some degree of innate immune responsiveness. For example epithelial cells produce type I interferons soon after virus infection, which mediate direct antiviral effects, but also recruit immune cells such as macrophages and natural killer cells. Innate immune components act very rapidly, usually within minutes after assault, but responses are unspecific and can eradicate only sporadic pathogens without the help from adaptive immunity. In contrast, adaptive immunity is based on targeted responses mediated mainly by T and B-lymphocytes, which are potent in eradicating infections and tumors, but require cross-presentation from innate immunity, maturation, and lack of suppressive signals. However, once established, immunological adaptive memory maintains the prompt responsiveness against possible recurrent encounters. With regards to oncolytic viruses, immune system plays a pivotal role: Antiviral immune responses may lead to rapid clearance of the virus and poor oncolytic efficacy. However, oncolytic virus replication may also provide danger-signals and tumor-antigen spreading necessary to induce antitumor immune responses, and thus helps in breaking the tumor-induced immune tolerance. The latter notion has led to the development of oncolytic immunotherapy concept (Lichty et al. 2014). Of note, different characteristics of genetically-modified adenoviruses, such as capsid-modifications and deletions of the E3 region genes, alter the host immune responses, and therefore identified mechanisms in basic virology and immunology are not always directly applicable for oncolytic immunotherapy (Zaiss et al. 2009, Thaci et al. 2011). Furthermore, tumor immunology, as previously discussed, will undoubtedly further confound the picture (Gajewski et al. 2013), which is why experimental approaches studying both the innate and adaptive arms of the immune system are crucial for development of oncolytic immunotherapy.

Innate antiviral immunity

Innate immunity composes mainly of autocrine and paracrine signaling of the infected cell, epithelial barriers, mast cells, phagocytic neutrophils and macrophages, natural killer (NK) cells, and the complement system. Dendritic cells are often regarded as innate immune cells as well, although they function in borderlands between innate and adaptive immunity together with other antigen-presenting cells.

Adenovirus is often encountered by the epithelial cells of the respiratory or gastrointestinal tract, and in the case of oncolytic virotherapy, by the tumor or tumor endothelial cells. Outside the cell, adenovirus is susceptible to neutralizing effects of the complement system, as well as neutralizing antibodies, if pre-existing from a previous encounter with the same serotype virus (Zaiss et al. 2009). As discussed, intravenous administration leads to major elimination of the virus by liver sinusoidal endothelial and Kupffer cells, in which the virus does not replicate, demonstrated by ca. 90% decrease in the originally administered virus DNA during the first 24 hours by the liver innate immune system in mice (Worgall et al. 1997).

Primary task of the innate immunity is to recognize the virus, because many physiological processes utilize the same endocytotic cell entry mechanisms. Indeed, already at the binding of adenovirus to its CAR-receptor on cell surface, viral capsid proteins are sensed as foreign, which triggers initial innate immune responses (Tamanini et al. 2006). Other early receptors include toll-like receptor 2 (TLR-2) on the cell surface, the α,β integrins that function as secondary adenovirus entry receptors, and toll-like receptor 9 (TLR-9) that is located in the endosome. Once the viral DNA is released from the endosome into cytosol, it can be further sensed by DNA-dependent activator of IFN-regulatory factors (DAI) and nucleotide oligomerization domain (NOD)-like receptors (Thaci et al. 2011). TLRs belong to the pattern-recognition receptor family of proteins that function to detect pathogen-associated molecular patterns (PAMPs). TLR-9 recognizes unmethylated CpG dinucleotide sites that appear to be more prominent in adenoviral genomes than in normal cellular DNA (Hemmi et al. 2000), although this varies by serotype, and species C serotype 2, and thus probably also serotype 5 adenovirus, seem to be less immunogenic in this regard (Krieg et al. 1998). Interestingly, TLR-2 appears to be activated also by the endogenous HMGB1 protein that is released from dying tumor cells in immunogenic cell death (Curtin et al. 2009, Li et al. 2013a). HMGB1-TLR-2 interaction was shown to mediate antitumor immune responses in a glioma model, when treated with oncolytic adenovirus, TMZ, and radiotherapy. Notably, all treatments alone lead to HMGB1 release as well. In summary, it appears that DAMPs that are secondary danger signals, are crucial in potentiating the immune responses *via* similar mechanisms as PAMPs in order to produce effective antitumor response (Li et al. 2013a).

After activating innate immune receptors, signals are transduced *via* several different adaptor proteins, such as MyD88 and TRIF, and mitogen-activated protein kinases (MAPKs), to effector proteins including transcription factors, NF-κB and IRF3/7, and signal transducer and activator of transcription 1/2 (STAT1/2) that induce cytokine and/or interferon (IFNs) production, and hinder cell cycling (Kawai and Akira 2006, Zhu et al. 2007). Thus, receptor signals lead to rapid alteration of host gene expression and metabolism, as well as paracrine signaling to the neighboring cells and associated immune cells. In principle, characteristics of the innate immune response depend on the cell type, and on the integrative actions of different PAMP signals. There are several independent receptors and associated downstream mediators in innate immunity, but the two main distinct signaling pathways are interleukin-1 receptor (IL-1R) and interferon α receptor (IFN-αR) mediated pathways. Downstream effector proteins of these two pathways attempt to block virus replication in separate ways: the interleukin pathway triggers inflammatory response that calls innate immune cells for help to eliminate the virus, whereas the IFN pathway strives for shutdown of cellular mechanisms both on autocrine and paracrine levels (Thaci et al. 2011).

IL-1R signaling leads to inflammatory response, release of chemokines and cytokines, aimed at controlling the infection locally by recruiting neutrophils, macrophages, and NK-cells, to phagocytose and lyse the infected cells and further amplify the response (Thaci et al. 2011). The

pattern of different inflammatory cytokines, in conjunction with e.g. IFN response, danger signals and immune cells, dictates whether the response is pro- or anti-inflammatory (Hendrickx et al. 2014). In general, IL-10 has been regarded as anti-inflammatory, whereas IL-8 and TNF-α are examples of mainly pro-inflammatory cytokines, but again depending on the context and kinetics (Muruve 2004). Many cytokines of acute innate response, especially interleukins, act in the borderlands between innate and adaptive immunity, and can either stimulate or inhibit e.g. dendritic cells and effector lymphocytes. For example, IL-12 and 18 stimulate NK T-cells to produce IFN-γ (see below) that is needed for effective helper T-cell type 1 (Th1) adaptive immune responses (Taniguchi et al. 2003).

IFNs are classified according to the receptor through which they signal: Type I IFNs (mainly IFN-α/β) are the main antiviral innate immune signals that activate the IFN-αR, while type II IFNs (-γ) are part of the adaptive immune system secreted by lymphocytes during infection and signal through IFN-γR. In addition, more recently discovered type III IFNs (-λ) comprise another part of innate immunity with many similarities to the type I IFN response, but are activated in response to different viruses, such as rhino- and influenza A virus (Hermant and Michiels 2014). To date, no evidence of IFN-λ response in adenovirus infection exists. Hence, type I IFNs play the key role in innate defense against adenovirus: They can be divided into IFN-α (includes 13 subtypes), IFN-β, IFN-κ, IFN-ε, IFN-ο, IFN-τ and IFN-δ. Type I IFN response is initiated by interaction of adenoviral DNA with some of the aforementioned intracellular receptors, followed by an attempt to block adenoviral replication in an autocrine manner (Thaci et al. 2011). In addition, type I IFN production by the infected cell leads to a more rapid IFN-αR-mediated signaling in the neighboring cells, which activates JAK-STAT pathway and leads to formation of IFN-stimulated gene factor 3 (ISGF3) transcriptional complex that results in expression of more than 300 IFN-stimulated genes (ISGs) (Thaci et al. 2011). Thus, the surrounding uninfected cells can produce extensive defense mechanisms that prevent possible replication attempts. Notably, if adenovirus already manages to express its E1A genes, the ISG production is nearly abolished *via* inhibition of ISGF3 (Anderson and Fennie 1987, Kalvakolanu et al. 1991). However, some ISGs, such as protein kinase R (PKR) and Myxovirus resistance protein A (MxA), that can be induced even during infection, are able to limit adenovirus replication to certain degree (Shi et al. 2007). In particular, MxA protein is located at a critical intersection between several interferon-mediated antiviral signaling pathways (Randall and Goodbourn 2008). It has been shown to block viral replication at early stages by trapping viral proteins and preventing viral protein synthesis, although its impact on adenovirus infection remains unknown (Staeheli and Pavlovic 1991, Kochs and Haller 1999, Haller et al. 2007). Critical role of the type I IFN response in adenovirus infection is underlined by the finding that breast cancer initiating/ stem cells that have dysfunctional toll-like receptor signaling, show increased susceptibility to oncolytic adenoviruses (Ahtiainen et al. 2010). Ultimately, a complex interplay between IFN and inflammatory cytokine responses is needed to clear adenoviral infections.

Cytokine and IFN responses can control adenovirus infection locally, by recruiting NK-cells, granulocytes, macrophages, and dendritic cells to phagocytose the infected cells (Hendrickx et al. 2014). These innate immune cells also amplify and modulate the response by secreting more cytokines. As their name indicates, NK-cells are natural born killers capable of rapidly eliminating infected cells based on their innate immune stress-response (Ferlazzo and Munz 2004), and activate other immune cells by e.g. secreting IFN-γ, GMCSF, and TNF-α (Ferlazzo and Morandi 2014). Dendritic cells and macrophages are the main professional antigen-presenting cells (APCs) that have a similar capacity to phagocytose without pre-stimulation or MHC class I presentation, and they also internalize cellular fragments and proteins *via* endocytosis (Nayak and Herzog 2010).

Dendritic cells recognize the internalized adenovirus or viral DNA fragments as pathogenic by their endosomal TLR-9 receptor (Hendrickx et al. 2014), after which they process the engulfed viral antigens and present them *via* MHC-II to CD4+ and CD8+ T-cells (Muruve 2004), acting as the first step of adaptive immune involvement. This antigen-presentation occurs in local lymph nodes, but requires several stimulatory signals, such as IFN-γ, TNF-α, DAMPs, PAMPs, and/or antigen-antibody immune complexes, for proper activation of APCs (Li et al. 2013a, Platzer et al. 2014). In contrast to stimulating immune cells, certain cytokine profiles and their interplay with local immune cells can alternatively lead to immune tolerization of dendritic cells, which is exploited by advanced tumors (Green et al. 2009). APCs can be also inhibited, edited, or stimulated by other immune cells at later stages, such as regulatory T-cells, MDSCs, and NK-cells that provide additional control over engagement of the potent adaptive immunity (Lindau et al. 2013, Ferlazzo and Morandi 2014). Thus, the innate immune mechanisms are intertwined with adaptive immunity which is described later. Tipping of the scales between the two opposing outcomes, i.e. immune activation and tolerance, as well as the nature of the response (i.e. Th1 *versus* Th2-type response, see below), depends on the previously discussed mechanism of cell death, the immunogenicity of the pathogen, and on the predominant immune status at the infection site. With regards to the latter, extracellular HMGB1 protein has gained attention as a central cytokine for all immune cells, bridging the gap between innate and adaptive immunity.

HMGB1 as a multi-faceted modulator of immunity

HMGB1 was first identified as a nuclear chromatin protein, but after its identification as a late mediator of sepsis in 1999 (Wang et al. 1999), the multifunctional extracellular role of HMGB1 has gained much attention. HMGB1 is a central player in local inflammation, where it can be passively released by virtually any cell type undergoing immunogenic cell death (discussed above), while being actively secreted by innate immune cells such as macrophages, monocytes and dendritic cells (Sims et al. 2010). In principle, the former release as a DAMP accounts for immune activation, whereas the latter chronic production promotes immunosuppression, for example by recruiting suppressive cell types and by inhibiting dendritic cell functions and maturation (**Figure 4**). To this end, high levels of HMGB1 can also prevent macrophages from effectively phagocytosing dying cells, which further hinders antigen-presentation (Liu et al. 2008, Friggeri et al. 2010). Of note, also NK cells can produce HMGB1 as a means of impacting the inflammatory milieu in dendritic cell editing (Semino et al. 2005). HMGB1-mediated modulation of immunity is not, however, limited to innate immune cells (see below for adaptive immunity): Immunogenically released HMGB1 acts as a chemotactic substance attracting T and B-cells to the site of tissue damage, and also induces CD4+ and CD8+ T-cell proliferation (Li et al. 2013a). Under chronic HMGB1 stimulation, however, IFN-γ production of T-cells is suppressed, while immunosuppressive regulatory T-cells are promoted *via* RAGE-receptor (receptor for advanced glycation end-products).

The fate between the two opposing outcomes is dictated by different post-translational modifications of the HMGB1 molecule, its redox status, spatiotemporal changes in concentration, and other concurrent cytokine and molecular patterns, all of which affect the receptor interactions on immune cells (Kang et al. 2014). Moreover, one peculiar characteristic of HMGB1 is that it interacts with several different receptors that may have completely opposing effects, not only on different cells, but also on the same cell type. Thus far, various TLRs and RAGE have been identified as the main receptors (**Figure 4**), while many others like TIM-3 (T-cell immunoglobulin domain and mucin domain 3) and CXCR4 (chemokine receptor type 4) have been also proposed (Li et al. 2013a). In a recent comprehensive review, Kang et al. covered the diverse biological roles of

HMGB1, and its involvement in the pathogenesis of over 50 diseases (Kang et al. 2014). The authors paid heed to the potential of HMGB1 as a clinical biomarker and a therapeutic target in a wide range of conditions, and listed over 200 therapeutic strategies employed to target HMGB1 either specifically or indirectly; Remarkably, around three-thirds of these experimental approaches have been published quite recently since 2010, indicating that the research field is currently blooming and many applications are awaited. In the context of clinical cancer research, vast majority of studies have implicated HMGB1 as a marker of poor prognosis in many tumor types (Li et al. 2012, Fahmueller et al. 2013, Stoetzer et al. 2013, Wittwer et al. 2013). However, certain adjuvant chemotherapies in gastric and breast cancer were associated with improved outcome and smaller tumor burden, respectively, when the tumors showed high HMGB1 expression (Bao et al. 2010, Yamazaki et al. 2014), probably reflecting the capacity to release HMGB1 in response to immunogenic treatments. To summarize, depending on the local microenvironment, source and dynamic changes, as well as coordination with other stimuli, HMGB1 can either promote or suppress immune reactions, which appears crucial for most if not all immune cells.

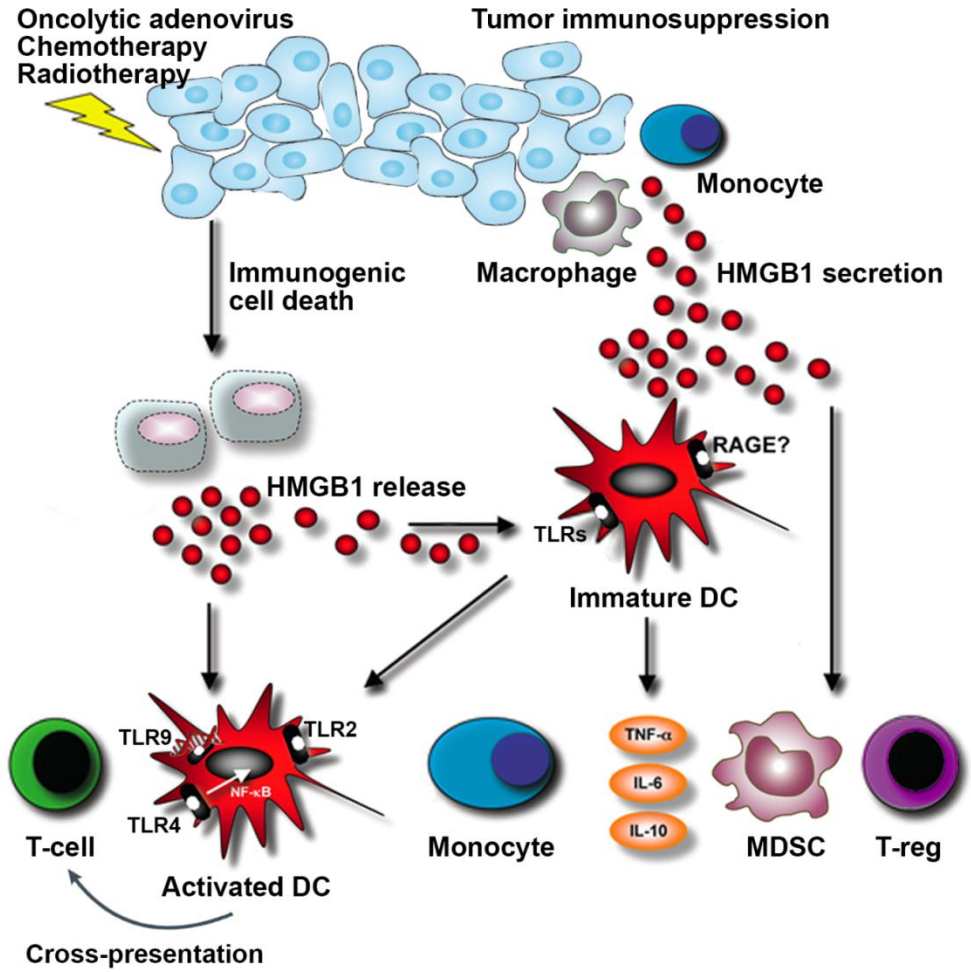

Figure 4. Dual roles of extracellular high-mobility group box 1 (HMGB1) protein in regulating immune functions. HMGB1 is passively released in immunogenic cell death, which can be triggered

by certain chemotherapeutics, radiotherapy, and oncolytic adenoviruses (left part). In cooperation with other danger-associated molecular patterns (DAMPs), dynamically released HMGB1 stimulates dendritic cells (DCs) by binding to toll-like receptors (TLRs), specifically TLR-2 and TLR-4, or as an immune complex with DNA fragments to the endosomal TLR-9. Subsequently, TLR-signaling leads to modulation of gene transcription *via* NF-κB, resulting in enhanced antigen-presentation and T-helper type 1 polarization of adaptive immunity. In a striking contrast, constitutive secretion of HMGB1 by tumor-associated macrophages and monocytes can results in immunosuppression (right part): High levels of oxidized HMGB1 leads to direct inhibition of DC maturation, probably *via* binding to Receptor for Advanced Glycation End-products (RAGE), followed by production of chronic inflammatory mediators such as IL-6, IL-10, and TNF-α that further suppress cell-mediated immunity and have tumor-promoting functions. HMGB1 possesses also chemokine and cytokine activities that attract immune cells and regulate their activity: Immunogenic HMGB1 release enhances migration and activation of effector T-cells, whereas chronic HMGB1 exposure leads to accumulation of immunosuppressive myeloid-derived suppressor cells (MDSCs) and regulatory T-cells (T-regs). Monocytes are also attracted but fall in between these two main phenotypes, because once in the periphery they can mature either into suppressive or antigen-presenting macrophages/DCs, depending on the local microenvironment. Modified from: (Hagemann et al. 2007).

Adaptive antiviral immunity

Infected cells express the viral antigens together with their endogenous antigens on the cell surface MHC class I molecules, which are recognized by mobile T-cells that constantly screen for antigens loaded onto MHC I (Muruve 2004). Once encountering its specific antigen, cytotoxic T-cell (CD8+) eliminates the cell and signals for other cytotoxic T-cells, T-helper (CD4+) cells, and antibody secreting B-cells to expand and activate against the recognized pathogen (Zaiss et al. 2009). However, the adaptive immune cells require priming by innate immunity in order to recognize antigens in the first place, and even if encountered before, the resting effector cells need co-stimulatory signals for reactivation. APCs mediate this cross-presentation by forming an immunological synapse with a T-cell in lymph nodes, where the APCs not only present their antigens on MHC I and II molecules to the CD8+ and CD4+ T-cells, respectively, but also needs to provide co-stimulatory signals, such as B7 and CD40 molecules, in order to activate the T-cell (Seliger et al. 2008, Smith 2014). Once correctly primed, the immune response leads to organized interplay between T-helper, cytotoxic T-cells and B-cells, inducing activation of cellular (T-cells) and humoral (B-cells) immunity (Zaiss et al. 2009, Nayak and Herzog 2010). Ultimately, these cells mediate system-wide, specific immune responses capable of eradicating the infected cells *via* cytotoxic T-cell responses, and eliminating free adenoviruses and boosting further antigen-specific immunity by secreted antibodies.

Both arms of the adaptive immunity, cellular and humoral response, are necessary for clearing infections, but with regards to anticancer immunotherapy, engagement of the cellular immunity is more desirable. Following dendritic cell mediated cross-presentation at the lymph nodes, polarization of the cross-primed CD4+ T-cells either into T-helper (Th) type 1 or type 2 phenotype leads to preferential induction of cytotoxic CD8+ T-cells or antibody-producing B-cells, respectively. These adaptive immune cell phenotypes (polarized CD4+ T-cells and their associated effector cells) can be distinguished by their secreted cytokine profiles: type 1 responses are dominated by production of IFN-γ and IL-2, while type 2 responses are characterized by production of IL-4, IL-5, and IL-10 (Morel and Oriss 1998). Even though the Th1 and Th2 cytokine profiles and

their immunological consequences always show considerable overlapping, the dominance of either response can downregulate the other, resulting in systemic polarization of the adaptive immunity. Immune-escaped, advanced tumors are able to repress both arms, but also preferentially misdirect the mounted immune responses towards Th2 pathway, for example by secreting IL-10, TGF-β and prostaglandins, because humoral immunity is ineffective in eradicating cancer cells (Ichim 2005). This is demonstrated by the systemic Th2-skewed balance observed in many cancer patients (Sato et al. 1998, Lauerova et al. 2002, Zanussi et al. 2003). In contrast, most viral infections and adenovirus vectors have been found to result mainly in Th1-type immunity (Miller et al. 2002, Lee et al. 2008, Cox et al. 2013), which is logical since cellular immunity is more potent in eliminating infected cells. Of note, adenovirus as well as other virus infections increase also anti-inflammatory IL-10 levels, reflecting the natural (or tumor-induced) peripheral tolerance, which is crucial in limiting the inflammation (Reid et al. 2002b, Cox et al. 2013). Finally, the feature of adenovirus vectors to induce Th1-type immunity has been adapted and further enhanced in the context of oncolytic immunotherapy by arming the virus with immunostimulatory transgenes such as GMCSF and CD40-ligand, which have shown promise in redirecting the ineffective Th2 cytokine profile towards Th1-type responses, as will be discussed later (Malmstrom et al. 2010, Cerullo et al. 2011, Pesonen et al. 2012).

The recruitment of adaptive immunity takes usually around 4–7 days, while the innate immune responses are instant, underlining the need for both arms to control the infection (Muruve 2004). The mounting of an effective adaptive response can be observed as a second peak in inflammatory cytokines in blood, resulting from cytotoxic T-cells killing the infected cells. B-cell activation and antibody production is even slower in primary infections, partly because they need two signals for activation, one from the antigen and another from the Th2-type CD4+ cell. Thereafter, B-cell activation leads to antibody production against adenoviral proteins that peaks in 2–4 weeks in humans (Pesonen et al. 2010). Majority of the antibody responses to adenovirus vectors is directed towards the hexon protein (Sumida et al. 2005). With regards to adenoviral cancer gene therapy, conflicting reports have been published about the role of neutralizing antibodies: A phase I clinical trial using repeated intrapleural injections of a replication-deficient adenovirus vector coding for IFN-β reported high levels of IFN-β in pleural effusion after the first dose, but significantly lower levels after subsequent treatment doses (Sterman et al. 2010); Here, the lack of IFN-β expression was correlated with induction of virus-neutralizing antibodies that most likely hampered therapeutic transgene production. In contrast, efficient transgene expression was reported after intratumoral administration of a replication-deficient adenovirus coding for IL-24, despite the presence of neutralizing antibodies (Tong et al. 2005). Nonetheless, the impact of pre-existing immunity is likely to be the opposite when considering oncolytic immunotherapy where the oncolytic virus is actually desired to induce potent immune responses against the host tumor cell. Indeed, in this setting the existence of neutralizing antibodies has not been correlated with reduced antitumor efficacy (DeWeese et al. 2001, Reid et al. 2002a), but rather in a striking contrast, even more pronounced antitumor immune responses have been noted in pre-immunized animals and patients (Tuve et al. 2009, Kanerva et al. 2013), which has been further associated with improved therapeutic outcome (Alemany and Cascallo 2009, Tuve et al. 2009). Finally, neutralizing antibodies appear to be important from a safety perspective in oncolytic adenovirus treatments (Pesonen et al. 2010).

Adaptive immunity is capable of creating an immunological memory after primary virus infection, which is long-lasting and allows for faster and more specific responses against potential re-infections (Nayak and Herzog 2010). As discussed here and previously, these principles can be

adapted to antitumor immunity as well. In summary, the nature of adaptive immune response relies on several factors, such as cytokine responses, immunogenicity and dose of the antigen, presence of immunosuppressive cell types, and genotypic characteristics such as immune-receptor polymorphisms (van Sorge et al. 2003, Nayak and Herzog 2010, Lindau et al. 2013). Immune system can also become tolerant to antigens, which is crucial for limiting autoimmune reactions, but can be devastating with regards to cancer (Smith 2014). Adaptive antiviral immunity is essential for effective eradication of infected cells and circulating viruses, but it has a dual role with regards oncolytic immunotherapy: On one hand antiviral responses can limit the replication in tumors and restrict the vector distribution to distant metastases, but on the other hand robust antiviral immunity towards infected cancer cells, coinciding with immunogenic oncolysis, danger-signaling, release of tumor-specific antigens, and skewing of the immunity towards Th1 type responses, can lead to enhanced antitumor immunity (Melcher et al. 2011, Kanerva et al. 2013). As recent advances in clinical oncolytic immunotherapy suggest, the latter scenario clearly seems to have an upper hand when properly designed and applied (Andtbacka et al. 2013, Lichty et al. 2014).

1.5 Cancer-therapeutic adenovirus vectors

Growing numbers of different oncolytic viruses have been tested in clinical I-III phase trials during the past two decades (Pol et al. 2014). As of June 2014, there were 1331 registered cancer gene therapy trials, of which replication-deficient or oncolytic adenovirus accounts for 26% either alone or in combination with other modalities (Journal of Gene Medicine, http://www.abedia.com/wiley/index.html). Thus, adenovirus is an important vector in clinical development for several tumor types. Moreover, this translates into large body of safety data, indicating overall good tolerability with the approach. Other promising vectors include herpes simplex virus, reovirus, vaccinia virus, and Newcastle disease virus (Pol et al. 2014). The earliest clinical trials date back to the 1950s, when certain non-attenuated virus strains, such wild-type adenovirus, hepatitis B virus, and West Nile virus were tested in advanced cancer patients (Kelly and Russell 2007). The modern era of cancer gene therapy began, however, in the early 1990s after the genetic modification techniques became available to design safer tumor-targeted virus vectors.

Two major classes of adenoviruses used in experimental and clinical settings are replication-deficient and replication-competent, i.e. oncolytic adenoviruses. Replication-deficient viruses have been rendered incapable of replicating by producing large genomic deletions, and are used to carry therapeutic genes to the target tissues, i.e. tumors. In contrast, oncolytic viruses entail only minor genetic deletions or modifications that modify them selective for cancer cells, where they can replicate, causing oncolysis, and spread to other cancer cells (Russell et al. 2012). Importantly, oncolytic viruses have either natural selectivity to cancer cells, such as oncolytic reovirus, or have been genetically modified to prevent replication in normal cells, as in the case of oncolytic adenovirus (Fukazawa et al. 2010, Kyula et al. 2012). In this thesis we have used adenoviruses as tumor-selective oncolytic agents, and therefore the preclinical and clinical focus is on adenoviruses, although landmark studies with other oncolytic viruses are discussed as well.

The main advantages of using adenoviruses as gene therapy vectors are that adenoviruses can infect both dividing and non-dividing cells, adenoviral DNA does not naturally integrate into the host genome, relatively high levels of transgene expression can be achieved, and adenovirus has

reasonably high transgene capacity (depending on the vector generation; see section 1.6) (Russell 2000, Alba et al. 2005). Practical benefits include the facts that adenovirus biology is well-characterized, adenoviral genome is relatively easy to modify, adenovirus particle and its DNA are stable to storage even for prolonged periods, production of new virions is straightforward, and as a result, high viral titers can be produced (Smith et al. 2010).

Adenovirus has also several constrains that limits its use. The main limitation for many gene therapy approaches is the transient nature of gene expression, rendering the use of adenoviruses insufficient in certain diseases that require long-lasting expression of the genetic material (Russell 2000). Natural tropism to liver can also increase liver toxicity while decreasing target tissue transduction (Arnberg 2012). Since adenoviruses are common human pathogens, the immune system can eradicate the virus rather efficiently and many individuals also possess pre-existing immunity, which are problematic to therapeutic transgene expression (Hendrickx et al. 2014). However, this immunogenicity can also account for treatment efficacy when considering cancer gene therapy with oncolytic viruses, as will be discussed later (Lichty et al. 2014). Further specifically with regards to cancer gene therapy, low expression of CAR receptors on many tumor types can reduce cancer cell transduction (Arnberg 2012). In order to circumvent these problems, adenoviruses have been genetically modified to increase gene expression, to improve tumor targeting over liver tropism, and finally, to evade or further induce immune responses depending on the utility. Strategies to achieve these goals are next discussed in more detail.

1.5.1 Transductional targeting

Viral selectivity to cancer cells can be enhanced by various genetic alterations that reduce biodistribution to normal tissues such as liver, improve homing to tumors, and reduce replication in normal cells, while increasing oncolysis of cancer cells. These modifications can ultimately enhance both treatment efficacy and safety of adenoviral cancer gene therapy. Two main ways to achieve improved targeting are transductional and transcriptional targeting. The previous technique involves modification of the viral capsid or coating of the virus, in order to enhance and target viral entry to cancer cells. The latter one, in turn, comprises of engineering the viral genome to allow and enhance replication and gene expression to occur only in cancer cells.

Adenovirus serotype 5 is the most used cancer gene therapy vector (Khare et al. 2011). Given the preclinical importance of CAR-receptor for its cell entry (Bergelson et al. 1997), much effort has been focused on re- or co-targeting the virus to other receptors in order to increase its transduction to cancer cells with low CAR-receptor expression (Rauen et al. 2002). In fact, low CAR-receptor expression has been shown to correlate with more aggressive disease, possible due to metastatic characteristics of the cancer, which may be a central reason for its downregulation in tumor cells (Matsumoto et al. 2005). Nevertheless, the importance of CAR-receptor for serotype 5 binding in human gene therapy is less clear (Hall et al. 2010). The most utilized means of increasing adenovirus transduction is fiber pseudotyping, where the entire knob is replaced with its structural counterpart from another serotype. This results in so called chimeric adenovirus, of which the first example is adenovirus serotype 5 replaced with a serotype 3 knob (Krasnykh et al. 1996). As a consequence, this 5/3 chimeric adenovirus is able to transduce cells *via* serotype 3 receptors, which were much later identified, and the main receptor appearing desmoglein-2 (Short et al. 2004, Fleischli et al. 2007, Wang et al. 2011). The chimeric serotype 5/3 adenovirus has since shown favorable efficacy in several preclinical and clinical therapy settings (Kanerva et al. 2003,

Koski et al. 2010, Hemminki and Hemminki 2014). One distinct example of transductional targeting is to extend the use of adenoviruses to other serotypes. As mentioned, serotype 3 adenovirus utilizes different receptors for transduction, and due to different capsid conformation, is not abolished by neutralizing antibodies directed against serotype 5 (Smith et al. 2010). An oncolytic adenovirus based on serotype 3 has been engineered, and was shown to mediate efficient antitumor inhibition *in vivo* without being neutralized by anti-serotype 5 antibodies (Hemminki et al. 2011). Moreover, the serotype 3 oncolytic virus was well-tolerated with potential signs of efficacy seen in refractory cancer patients (Hemminki et al. 2012). Interestingly, pre-existing serotype 5 immunity did not seem to abrogate the functions of the serotype 3 virus in patients, suggesting that serotype switching might be a feasible approach for prolonged treatment periods.

Ligand incorporation is another means by which cancer cell transduction can be increased, at least preclinically. Besides downregulating multiple cell surface proteins, progressing tumors also upregulate some adhesion molecules that allow rational targeting by incorporating their ligands to the virus capsid (Nicklin et al. 2005, Hall et al. 2010). Thus, various fiber knob modifications have been tested: For example, adding an RGD-motif to the HI loop of the knob can redirect and increase the virus attachment to cell surface integrins that are plentiful in majority of the tumors, as demonstrated in pancreatic cancer and malignant glioma (Bilbao et al. 2002, Tyler et al. 2006). A similar infectivity-enhanced conditionally-replicating adenovirus Ad5-Δ24-RGD was tested in a phase I clinical trial in 21 progressing ovarian cancer patients (Kimball et al. 2010): Treatments were well-tolerated, seven patients had prolonged virus genomes in circulation, and 71% of the patients showed disease control after one month of follow-up. Incorporation of polylysine motif (pk7) that binds to heparan sulfate proteoglycans on is another experimental example, although more controversial due to natural presence of the receptor on also normal cells (Zheng et al. 2007). Furthermore, novel ligands can be incorporated also to adenoviruses lacking the natural fiber knob. The replacement of the knob by human CD40-ligand, for example, showed increased virus transduction to cancer cells expressing CD40 (Belousova et al. 2003, Izumi et al. 2005).

In addition to genetic transductional modifications, also adapter molecules have been tested preclinically that cross-link the adenovirus capsid proteins with specific cell surface molecules. As an early example, the folate receptor, which is ubiquitously expressed in cancer cells, was targeted by using a folate ligand conjugated to an anti-fiber antibody (Douglas et al. 1996). In another attempt, an EpCAM tumor-antigen was targeted in a similar approach because of its high expression in several tumor types including breast, ovarian, colon and lung cancer (Haisma et al. 1999). However, these attempts have not been yet tested clinically, mostly due to safety concerns regarding the use of a separate chemical linker molecule besides the well-studied adenovirus (Verheije and Rottier 2012).

1.5.2 Transcriptional targeting

Tumor-targeted viral gene expression and replication are the most important means by which gene therapy vectors are rendered cancer selective. There are two main approaches for transcriptional targeting: genetic deletions and incorporation of tumor-specific promoters. Genetic deletions are much utilized for targeting adenoviruses, owing to their ability to express viral proteins in cancer cells that complement the missing gene functions (Berk 2005). E1A and E1B regions are essential for adenovirus replication and for transcription of the later genes, as previously discussed (Stillman 1986, Whyte et al. 1988). Thus, deletion of these regions renders

the virus replication-deficient in normal cells, but tumor-specific pathways that complement these defects allow conditional replication in cancer cells. The most known example, the matriarch of conditionally-replicating adenoviruses, is ONYX-015 that has deletions in the gene coding for E1B55K, which is responsible for inhibition of p53 protein. This attenuates replication in normal cells with intact p53, but allows ONYX-015 to replicate in p53-deficient cancer cells (Bischoff et al. 1996). ONYX-015 has been tested perhaps the most extensively both preclinically and in clinical trials (discussed in section 1.7). Early on it was shown to specifically replicate in p53-mutated cells (Heise et al. 2000), but the E1B55K deletion also reduced the cell killing potential, given the several important actions of E1B55K (Dix et al. 2001). Instead of deleting the whole adenoviral early gene, a partial genomic deletion has been introduced with promising results: Protein products of the E1A gene normally bind to the retinoblastoma protein (Rb) that releases the E2F transcription factor, which is required for activating genes that promote the S phase and consequently virus replication (Whyte et al. 1988). However, in cancer cells that feature dysregulated Rb/p16 pathway, free E2F is constantly available, rendering this function of E1A dispensable (Sherr 1996). Thus, a targeted deletion to the Rb binding site of E1A region, such as 24 base-pair deletion (Δ24), makes the virus transcriptionally cancer-selective by attenuating replication in normal cells (Fueyo et al. 2000). These Δ24 oncolytic adenoviruses have been widely used due to their improved oncolytic capacity over the early-gene deleted adenoviruses (Kanerva et al. 2003, Hakkarainen et al. 2009), and their clinical use is discussed later.

Incorporation of tumor-specific promoters to viral genome controlling its gene expression is another way to target replication to cancer cells. Prostate-specific antigen (PSA) targeted adenovirus was one of the first attempts where prostate-specific enhancer was inserted to control the E1A region, and resulted in selective replication in PSA-expressing prostate tumors and showed promising efficacy *in vivo* (Rodriguez et al. 1997). Carcinoembryonic antigen (CEA) specific suicide gene (thymidine kinase mediated pro-drug conversion) expression approach was tested in colorectal cancer mouse model with fair efficacy and reduced liver toxicity as compared to irrelevant cytomegalovirus promoter (Brand et al. 1998). Given the natural tropism of human adenovirus to liver, as earlier discussed, treatment of liver carcinoma seems appealing. To this end, a tumor-specific α-fetoprotein driven E1B55K-deficient adenovirus was shown to mediate regression of hepatocellular carcinoma after intravenous injections in mice, although virus replication was slow probably due to E1B55K-deletion (Ohashi et al. 2001). Furthermore, a carcinoid tumor specific Chromogranin A was utilized to control E1A region of a conditionally-replicating adenoviruses that did not show viral gene expression in normal hepatocytes, but mediated selective carcinoid tumor inhibition *in vivo* (Leja et al. 2007).

Aforementioned examples of promoters are very tumor-type specific, which may increase their specificity but often limits applicability since they have been designed for a very narrow indication. Moreover, as the common tumor resistance against small-molecular inhibitors suggests, some cancer clones of a progressing tumor are likely to be resistant or become resistant to a selective pathway inhibition or targeting (Hojjat-Farsangi 2014). Therefore more recent attempts have focused on designing pan-tumoral selective promoters. A logical extension to the Rb de-targeting of the Δ24-deleted adenovirus is an E2F-specific promoter. This approach utilizes the same Rb/p16 pathway dysregulation entailed by virtually all tumors (Sherr 1996), in that it requires free E2F that binds to the promoter controlling the E1A region. Since normal cells halt their cell cycle when infected by an adenovirus, they lack free E2F transcription factor thus attenuating virus replication (Rojas et al. 2009, Hemminki and Hemminki 2014). Interestingly, these double transcriptionally targeted adenoviruses harboring both E2F-promoter and Δ24-deletion seem to benefit from the

double-control of E1A due to positive feedback loop of free E2F binding in cancer cells. Another example is the cyclooxygenase 2 (Cox-2) promoter, which exploits the ubiquitous expression of Cox-2 in various cancer types (Wang and Dubois 2006). Accordingly, Cox-2 promoter is also used to control E1A expression together with Δ24-deletion and has been tested preclinically and in refractory cancer patients with good safety and potential signs of efficacy (Bauerschmitz et al. 2006, Pesonen et al. 2010). In addition, vascular endothelial growth factor (VEGF) promoter has been evaluated preclinically, and together with Cox-2 promoter, the nature of these promoters allowed external control by using common anti-inflammatory drugs such as dexamethasone and salicylic acid (Kanerva et al. 2008). Finally, one emerging notable promoter is the human telomerase reverse transcriptase (hTERT) promoter controlling E1A, which is based on a fundamental hallmark of cancer that high telomerase levels can maintain telomere lengths and promote tumor growth (Hanahan and Weinberg 2011). Indeed, this promoter has been tested in a variety of preclinical and clinical settings with favorable results (Ito et al. 2006, Hemminki et al. 2011, Diaconu et al. 2012, Hemminki et al. 2012, Pesonen et al. 2012).

1.6 Replication-deficient adenoviruses and their clinical use

Replication-deficient adenovirus vectors have been improved over the years owing to the achievements in virus- and immunobiology. These vectors are widely used for both cancer therapy and to correct various monogenic diseases and inherited enzyme deficiencies (Ginn et al. 2013). Adenoviruses have a limited capacity to carry additional DNA, very alike other vectors, which is why construct of novel vectors has been based on deleting viral genes; Adenovirus can become unstable if the genomic load is more than 105% of the wild-type cargo (Bett et al., 1993). Thus, the first generation adenovirus vectors were deleted of the E1 gene region and often also E3 region, while a therapeutic transgene was typically inserted in the place of E1 region (Hall et al. 2010). These viruses can accommodate fairly large transgenes of up to 8.2 kilo-base pairs (Alba et al. 2005). A major set-back for the first generation adenoviruses is that the transgene expression, although typically potent at start after a successful transduction, remains transient due to inability of the vector to replicate, and immune responses efficiently eradicating the vector (Muruve 2004). Nevertheless, these viruses have been utilized to deliver various anticancer transgenes such as anti-angiogenic proteins (Im et al. 2001), monoclonal antibodies (Jiang et al. 2006), cytokines (Sung et al. 2002), tumor-suppressor proteins (Nielsen et al. 1998), and prodrug converting enzymes (Tyynela et al. 2002). One notable clinical success story among the first-generation vectors is the Ad-p53 (Gendicine), which was approved for the treatment head and neck cancer in China in 2003, becoming the first gene therapy modality in clinical use (Peng 2005). Ad-p53 is an E1-deleted serotype 5 adenovirus coding for p53 tumor-suppressor protein under a rous sarcoma virus (RSV) promoter. Combination of Ad-p53 together with radiation showed even synergistic efficacy in head and neck cancer (Peng 2005), and was later studied with radiotherapy also in liver cancer with promising results (Yang et al. 2010). In addition, Ad-p53 was reported to mediate antitumor efficacy when combined with other conventional treatments, such as cisplatin and 5-fluorouracil in advanced lung cancer, and chemoembolization in liver cancer (Peng 2005). The reasons why this first-generation adenovirus vector has been clinically so successful particularly in China are likely many, ranging from patient availability to different regulatory authorities (Pearson et al. 2004). Nevertheless, as 10% of the over two million head and neck cancer patients in China are estimated to receive the treatment annually, large bulk of safety and efficacy data should be generated in the

years to come. Ultimately, large cohorts of patients are needed to reliably evaluate treatment efficacy and to further develop clinical practice (administration route, dosing, fractionation, etc.).

Second generation adenovirus vectors were designed to overcome the limited transgene expression capacity by targeting deletions also to viral genes in the E2 and E4 regions that are highly immunogenic, thus decreasing antiviral immune responses (Hall et al. 2010). These adenoviruses have been utilized in similar approaches as the first generation vectors (Alba et al. 2005, Murugesan et al. 2009), but still suffer from the same problem of rapid immune eradication (O'Neal et al. 1998). As a result the third generation vectors were designed: They are called gutless vectors, devoid of all natural viral genes except the packaging signal and inverted terminal repeats (see **Figure 3**), which also cleared more room for therapeutic transgenes, and can carry up to 36 kilo-base pairs of foreign genetic material (Alba et al. 2005). Gutless adenoviruses are helper-dependent, because they require co-infection with a helper adenovirus that provides it with essential genes to produce new virions. These last generation of replication-deficient adenovirus vectors have the advantage of evoking a minimal immune response, thus resulting in longer gene expression. Indeed, these vectors have been actively tested for various gene replacement therapies (Ginn et al. 2013).

1.7 Oncolytic adenoviruses and their clinical use

Oncolytic viruses are defined as viruses that selectively replicate in tumor cells rather than normal cells. The modern use of oncolytic viruses stems from the fact that replication-deficient virus vectors proved safe but therapeutically insufficient in cancer gene therapy trials (Rein et al. 2006). Conditionally replicating adenoviruses were the first selectively replicating, i.e. oncolytic, adenoviruses developed: By applying tumor-specific transductional and transcriptional targeting, they mediate both transgene expression and virus replication in the infected cancer cell, followed by lysis of the host cell and release of new virus progeny that are able to further spread and infect other cancer cells (**Figure 5**). Thus, virus replication also leads to improved transgene expression in tumors, further potentiating treatment efficacy. The discussed strategies to control the expression of E1A region, along with subsequent gene regions and viral DNA replication, have been found effective in confining oncolytic adenovirus replication to cancer cells (Fueyo et al. 2000, Reid et al. 2002a, Bauerschmitz et al. 2006). Oncolytic adenovirus dl1520, better known as ONYX-015, was the first to employ this approach: Deletion of the early E1B55K gene rendered the virus selective for cancer cells with dysfunctional p53 gene (Bischoff et al. 1996). Given the many functions of E1B55K, however, it was later discovered that defects in viral mRNA export also accounted for the attenuation of virus replication in normal cells (O'Shea et al. 2004). ONYX-015 was the first oncolytic virus tested in clinical trials for treatment of advanced head and neck cancer in 1996 (Ganly et al. 2000), and since then ONYX-015 has been studied in phase I-II clinical trials for treatment of various tumor types such as head and neck cancer, advanced sarcoma and ovarian cancer (Nemunaitis et al. 2000, Vasey et al. 2002, Galanis et al. 2005). Safety of the virus was good throughout, but efficacy was modest in most of the trials: As a single agent, ONYX-015 showed ca. 15-21 % response rate in head and neck cancer (Ganly et al. 2000, Nemunaitis et al. 2000), while lack of notable efficacy was reported in pancreatic, ovarian, and colorectal cancer (Vasey et al. 2002, Hamid et al. 2003, Hecht et al. 2003). Importantly, ONYX-015 was very early on noticed to synergize with conventional treatment modalities preclinically. Therefore it was tested together with cisplatin and 5-fluorouracil for treatment of head and neck cancer: while ONYX-015 as a single

treatment reach only 21% tumor response rate (partial response of >50%) in advanced head and neck cancer patients (Nemunaitis et al. 2000), combining it with chemotherapy increased the 6-month durable response rate up to 63%, and 8 out of 30 patients showed complete response (Khuri et al. 2000). However, another early combination therapy trial, assessing ONYX-015 together with mitomycin, doxorubicin and cisplatin for the treatment of advanced sarcomas showed again less than 20% response rate, indicating that only certain combinations and/or tumor types can lead to clinical benefit. These data underline the importance for rational combinations and indications: Some of the potential mechanisms underlying combinatorial benefit with conventional therapies have been previously discussed, and are studied in this thesis.

Another oncolytic adenovirus studied extensively in China, H101 (Oncorine), is very similar to ONYX-015 but it also lacks the complete E3 region (Garber 2006, Yu and Fang 2007). This deletion may result in more rapid clearance of the virus by the immune system, given the importance of E3 region proteins in counteracting host immune responses (Bortolanza et al. 2009a). It has also been linked to improved oncolytic potency *in vitro* (Suzuki et al. 2002). Nevertheless, H101 has shown good safety and efficacy in clinical trials (Yu and Fang 2007), and it became the first oncolytic adenovirus approved for treatment of cancer in 2005, indicated for head and neck cancer therapy in China (Garber 2006). In fact, E3-deletion may serve a paradoxical benefit of increased immune activation against the virus in patients, which can lead to enhanced immunotherapeutic antitumor activity. Unfortunately, the initiated phase III clinical trial in head and neck cancer using ONYX-015 that has the intact E3-region was never accomplished due to funding issues, and therefore cross-comparison between H101 and ONYX-015 is difficult. The fact that clinically evaluated E1B55K-deleted adenoviruses, H101 and E1-deleted Ad-p53, appear to synergize with radiotherapy suggests that E1B55K protein would not be crucial for its radiosensitizing effects. However, its impact on tumor radiosensitation has not been previously assessed in comparison to other proposed radiosensitizing adenoviral proteins (**Figure 2**). Approval of H101 was based on a randomized phase III trial where intratumoral injection of H101 together with cisplatin/adriamycin and 5-fluorouracil was compared to chemotherapy alone (Xia et al. 2004): H101 showed significantly higher overall response rate of 78.8 % as compared to 39.6 %, in cisplatin chemotherapy, whereas adriamycin-based regimen failed to show difference (50.0 % in both groups). Survival data was, however, not reported due to difference in regulations between the health authorities: China relied at the time on objective responses, while the Western countries have always based their approval on survival benefit. Other examples of clinically tested oncolytic adenoviral constructs with promising data include an oncolytic adenovirus based on the E1B55K-deleted backbone of ONYX-015, which is armed with a cytosine deaminase/HSV-tk fusion suicide gene, for treatment of prostate cancer (Freytag et al. 2007), a PSA tumor-antigen targeted oncolytic adenovirus CG7870 for the treatment of metastatic prostate cancer (Small et al. 2006), and a hTERT promoter-driven oncolytic adenovirus, Telomelysin, in advanced solid tumors (Nemunaitis et al. 2010).

Reovirus is an interesting exception to other oncolytic viruses in that the type 3 (Dearing) naturally prefers to replicate in tumor cells that have an activated Ras signalling pathway (Kyula et al. 2012). It has been studied extensively for treatment of e.g. metastatic head and neck cancer with promising efficacy, and has reached phase III evaluation in combination with carboplatin/paclitaxel. Furthermore, a very recent phase II randomized trial studying reovirus for treatment of advanced pancreatic cancer failed to meet its primary endpoint of progression-free survival in the total patient population in this very dismal cancer type, but showed a trend for improved survival (39% increase in median progression-free survival) in a sub-analysis where only

KRAS-mutated patients were included (ClinicalTrials.gov Identifier: NCT01280058). Thus, a rational approach of exploiting the altered Ras-pathway as a "natural" targeting strategy seemed to work in this disease.

1.7.1. Oncolytic immunotherapy

Tumor-selective adenoviruses initiate immune reactions when entering and replicating in cancer cells. While the classical view of gene therapy is to minimize the antiviral immunity, oncolytic immunotherapy exploits it, and aims at further boosting and redirecting immune reactions against the tumor (**Figure 5**). Adenovirus is a highly immunogenic human pathogen, owing to the lack of efficient immune-escape mechanisms by the virus, and lack of immunological tolerance by the host (Tuve et al. 2009). This leads to multilevel cross-talk between the host and the virus, and acute immune response described earlier. Oncolysis caused by the virus releases danger-signals and tumor-associated antigens that augment in breaking the immunotolerance of the advanced tumors, which can lead to mounting of antitumor immune responses (Melcher et al. 2011). Thus, immune activation at the tumor site was soon discovered to associate with treatment benefits (Lichty et al. 2014). The main components of this effect include virus-triggered immunogenic cell death that leads to release of danger-signals (DAMPs), spreading of tumor-specific antigens that are taken up by antigen-presenting cells, processing and cross-presentation of these tumor-antigens to effector T-cells at local lymph nodes, and sufficient stimulus (lack of immunosuppression) to activate antitumor T-cell responses (**Figure 5**). In addition, infection and oncolysis can alter the cytokine milieu and skew the immunosuppressive environment at the tumor, thus further attracting and activating immune cells (Lichty et al. 2014). Viruses have also direct effects on the infiltrating immune cells. This has been demonstrated preclinically in various tumor models and for many different oncolytic viruses (Choi et al. 2006, Diaz et al. 2007, Fukuhara and Todo 2007, Prestwich et al. 2009, Diaconu et al. 2012, Parviainen et al. 2014). Specifically with regards to adenovirus, intratumoral treatment given to syngeneic mice that were immunologically tolerant to *neu*-positive mammary tumors were shown to induce potent immune responses both at the tumor site and at the tumor-draining lymph nodes (Tuve et al. 2009). Treatment with replication-deficient adenovirus led to generation of both adenovirus- and *neu*-specific effector T-cells in the lymph nodes, while at the tumor site, there was an expansion of adenovirus-specific T-cells, despite the presence of immunosuppressive regulatory T-cells. Indeed, these antiviral T-cells could efficiently kill virus-infected cancer cells and restrict tumor-growth. Moreover, the authors showed that pre-existing immunity against adenovirus actually increased antitumor efficacy that was mediated by CD4+ helper and CD8+ cytotoxic T-cells, despite the induction of neutralizing antibodies. These data suggest that both antiviral and antitumor immune responses can be generated by tumor-selective adenoviruses, and that antiviral immunity may overcome the tumor-mediated immunosuppression, potentially unmasking also tumor-specific T-cell responses. Accumulating preclinical and clinical evidence supports these findings (Choi et al. 2006, VanOosten and Griffith 2007, Kanerva et al. 2013, Hemminki and Hemminki 2014).

It should be noted, however, that direct cytotoxicity *via* oncolysis can result in significant therapeutic efficacy as well, and appears an important component of efficacy. Historical data on the first oncolytic virus trials suggested feasibility of direct tumor cell killing, i.e. tumor debulking (Kelly and Russell 2007): Several non-attenuated virus strains such as mumps virus were tested clinically, and some showed remarkable responses often in immunocompromised patients. These cases showed unrestrained infections, but were often associated with severe side-effects, and with the emergence of advanced chemotherapeutic regimens, the approach of using non-targeted

oncolytic viruses was largely abandoned. Nevertheless, these historical data demonstrate the capacity of direct oncolysis. In the light of newer evidence, the effective oncolysis and tumor debulking is not necessarily counterintuitive to immune activation. A recent preclinical study using intravenously delivered oncolytic vesicular stomatitis virus to treat murine multiple myeloma, reported a single-shot cure by debulking all tumor lesions in less than 72 hours (Naik et al. 2012). Importantly, this approach also generated antimyeloma T-cells that were reported to eradicate the minimal residual disease. Generation of immunological memory against the cancer is the most notable perk of immunotherapy that allows prolonged disease-free survival, which has been demonstrated even in patients with metastatic treatment-refractory cancer (Kyi and Postow 2014, Maude et al. 2014). Hence, the field of oncolytic virotherapy has shifted towards oncolytic immunotherapy and several strategies have been developed to further boost the antitumor immunity (Lichty et al. 2014). Supporting antitumor immunity over immunosuppression with the use of immunomodulatory low-dose chemotherapy has been employed by the cancer vaccine field as well as oncolytic immunotherapy (Curtin et al. 2009, Cerullo et al. 2011, Sistigu et al. 2011). This strategy has been discussed earlier, and is further studied in this thesis.

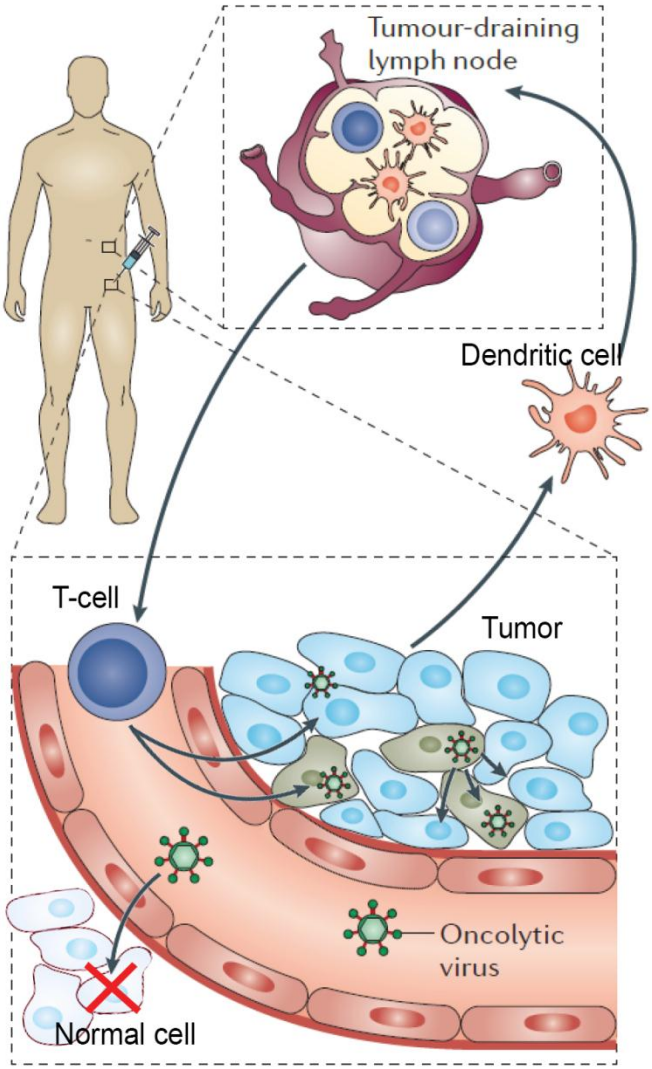

Figure 5. Schematic mechanism of action of oncolytic immunotherapy. Intratumoral injection of oncolytic adenovirus leads to transduction and selective replication in cancer cells (bottom box), but not in normal cells because of targeted genetic modifications done in the virus genome (transcriptional targeting). Thereafter, oncolytic adenovirus triggers immunogenic cell death, releasing danger-signals and new virions that are ready to infect surrounding cancer cells and spread *via* circulation to distant metastases. Simultaneously, tumor-associated antigens are spread from the lysed cancer cell that are picked up and processed by dendritic cells, which are activated by danger-signals. This augments in breaking the immune tolerance, leading to migration of dendritic cells to tumor-draining lymph nodes (top box) where they cross-present the virus- and tumor-antigens to effector T-cells. Primed and activated T-cells then travel *via* circulation to the site of infection, eradicating the virus-infected tumor cells and other unmasked tumor cells. Modified from: (Lichty et al. 2014).

1.7.2. Virus arming with immunostimulatory transgenes

Infusion of recombinant cytokines, such as previously discussed IFN-α, TNF-α, GMCSF, and IL-2, have been tested clinically, and are still used as an immunotherapeutic approach to treat certain malignancies (Lejeune et al. 1998). Nevertheless, most of these treatments were too toxic due to systemic effects of pro-inflammatory mediators. Arming of oncolytic viruses with immunostimulating molecules is appealing due to local production at the tumor site, thus providing spatiotemporal stimulatory signals, while minimizing the systemic side-effects. A plethora of different cytokine-like molecules have been investigated preclinically, while only a handful has entered clinical testing. In the context of adenovirus vectors, arming strategies have included the aforementioned TNF-α, IFN-α, and IL-2, as well as IFN-β, IFN-γ, IL-4, IL-12, IL-15, CD40-ligand, and GM-CSF, and T-cell co-stimulatory molecule B7-1 (Li et al. 2005). As interferons mediate antiviral responses, the incorporation of IFN-α and IFN-β in virus vectors may seem controversial. However, several tumor types and cancer stem-like cells have been found deficient in interferon signaling (Critchley-Thorne et al. 2009, Ahtiainen et al. 2010), suggesting the downregulation as a means of immune evasion. An acute type I IFN production may therefore induce immune reactions at the tumor site. Indeed, a replication-deficient adenovirus coding for IFN-β was shown to eradicate mesothelioma tumors by enhancing antitumor immunity in mice (Odaka et al. 2002). IFN-β coding adenoviruses have been since studied in clinical trials, which have implied antitumor immune inductions also in patients (Chiocca et al. 2008, Sterman et al. 2010). TNF-α armed adenovirus vectors have been extensively studied in laboratory (MacGill et al. 2007), where promising results have led to clinical testing of a replication-deficient adenovirus expressing TNF-α under the control of a radiation-inducible promoter (Senzer et al. 2004). Intriguingly, a phase I clinical trial reported no dose-limiting toxicities in combination with radiotherapy, and objective tumor responses were seen in 70% of patients with various advanced solid tumors (Senzer et al. 2004). Another phase I trial assessing the combination for treatment of advanced soft tissue sarcomas reported equally well-tolerated profile and showed 85% response rate (Mundt et al. 2004). Supported by further encouraging results in phase I/II dose-escalation studies (Chang et al. 2012, Hecht et al. 2012), randomized controlled trials using the approach are underway (Hernandez et al. 2010).

Several cytokines have shown promise as arming molecules in the context of oncolytic adenoviruses as well. IL-12 activates NK cells and T-cells and is naturally produced by antigen-presenting cells. A replication-deficient adenovirus vector coding for IL-12 was tested in a phase I trial of 21 patients with advanced digestive tract tumors (Sangro et al. 2004): While the treatment was well-tolerated, and showed 29% disease control rate together with significant increases in effector immune cells in the post-treatment biopsies, the effects were transient probably due to low production of the cytokine. Therefore, IL-12 expressing oncolytic adenoviruses are being investigated in immunocompetent murine models for clinical testing (Bortolanza et al. 2009b). Anti-CTLA4 antibody ipilimumab, which has been approved for treatment of melanoma, is an appealing arming molecule given its function of releasing the immunosuppressive breaks of the T-cells (Hodi et al. 2010). Results of a combination therapy trial with oncolytic herpes simplex virus T-VEC have been recently reported with impressive signs of preliminary efficacy and manageable safety profile in metastatic melanoma patients (Puzanov et al. 2014). Nevertheless, local production of the antibody might further improve the safety. Also given the virus-induced accumulation of tumor-infiltrating lymphocytes and their local suppression in the tumor

microenvironment, local production ipilimumab from a vector combined with immune activation by the virus would seem feasible. This approach has been tested preclinically: Both a replication-deficient and an oncolytic adenovirus coding for ipilimumab showed increased antitumor immune responses and high efficacy *in vivo* (Liu et al. 2011a, Dias et al. 2012).

Oncolytic adenoviruses expressing CD40-ligand

Another attractive candidate to stimulate T-cells is CD40-ligand (CD40L), a transmembrane protein expressed mostly on CD4+ helper T-cells, which binds to the CD40-receptor on antigen-presenting cells such as macrophages and dendritic cells (Grewal and Flavell 1998). Activation of CD40 by its ligand enhances antigen presentation and IL-12 production by these cells, resulting in more potent innate immune response and increased T-cell priming (van Kooten 2000). Thus, CD40 is a critical costimulatory signal that can trigger T-cell expansion and polarize effector functions towards Th1-type response, both of which are required for effective antitumor cytotoxic T-cell responses (Loskog et al. 2005). In addition, CD40L can induce apoptosis of tumor cells, further directly activates cytotoxic T-cells, and reduces immunosuppression (Grewal and Flavell 1998, Loskog et al. 2005). These characteristics render CD40L an appealing arming molecule. Similar to many cytokines, systemic side-effects limit the use of recombinant CD40L. In the context of adenoviral cancer gene therapy, however, CD40L has been tested preclinically and in cancer patients: Phase I-II clinical trials studying a replication-deficient adenovirus for treatment of invasive bladder cancer (Malmstrom et al. 2010) and chronic lymphocytic leukemia (Wierda et al. 2010), resulted in good safety and evidence of biological activity but modest efficacy. Furthermore, an oncolytic adenovirus coding for CD40L has shown promising antitumor efficacy in syngeneic murine models, together with evidence of antigen-presenting cell induction, IL-12 production, Th1 polarization and effector T-cell infiltration to the tumor site (Diaconu et al. 2012). Accordingly, the CD40L-coding oncolytic adenovirus was assessed in patients with treatment-refractory solid tumors, and showed good safety, Th1 type immune responses in majority of the patients, antitumor T-cell inductions in peripheral blood, and promising signs of efficacy (Pesonen et al. 2012).

Oncolytic adenoviruses expressing GM-CSF

GMCSF is a potent cytokine and a leukocyte growth factor that enhances the function of antigen-presenting cells. Local increase in GMCSF levels stimulates recruitment of monocytes and induces their maturation into dendritic cells and macrophages (Chang et al. 2004a). In addition, GMCSF activates several types of innate immune cells, including NK-cells, dendritic cells, and macrophages, thus leading to enhanced tumor cell killing, and subsequent tumor-antigen processing and presentation to effector T-cells (Arellano and Lonial 2008). However, similar to many other cytokines, systemic use of recombinant GMCSF is compromised by serious inflammatory side-effects, while the efficacy has remained modest due to low levels in tumors (Arellano and Lonial 2008). Moreover, high systemic GMCSF levels have been correlated with induction of immunosuppressive MDSCs (Serafini et al. 2004), whereas local production and low systemic levels have not been associated with the effect. In fact, GMCSF mobilizes myeloid cells which is a logical reason also for MDSCs accumulation after systemic exposure, while locally it acts as a chemoattractive molecule and a local activator of immune cells (Arellano and Lonial 2008). Therefore, GMCSF has been widely used as an adjuvant in various immunotherapeutic strategies (Mellman et al. 2011), along with evident success as in the case of approved cancer vaccine sipuleucel-T (Kantoff et al. 2010). In cancer gene therapy, GMCSF has been perhaps the most used arming cytokine. Replication-deficient adenovirus vector expressing GMCSF was shown to increase

efficacy of an oncolytic adenovirus in an adjuvant manner *in vivo* (Burroughs et al. 2004). Furthermore, oncolytic adenoviruses coding for GMCSF have been tested in several mouse and semi-permissive hamster models including head and neck, liver, bladder, and pancreatic cancer with impressive efficacy and evidence of antitumor immune activations (Koski et al. 2010). Moreover, the GMCSF-coding oncolytic adenovirus has been recently assessed in cancer patients: Intratumoral treatments were well-tolerated in advanced head and neck cancer patients in a phase I trial, together with signs of systemic biological activity and local objective responses mainly in the injected lesions (Chang et al. 2009). In another clinical report, patients with advanced solid tumors who received a GMCSF-expressing serotype 5 oncolytic adenovirus showed good overall safety, antiviral and antitumor immune activations, and possible signs of efficacy with disease stabilization in 50% of patients (Cerullo et al. 2010). Moreover, a chimeric serotype 5/3 adenovirus coding for GMCSF was assessed in a similar setting, and presented corresponding safety, signs of biological and immunological activity, and disease stabilization in 67% of patients (Koski et al. 2010). The latter treatment agent is currently being investigated in phase I-II clinical trials.

Other promising GMCSF-coding oncolytic viruses include a vaccinia and a herpes simplex virus. Vaccinia virus is a highly immunogenic virus of the pox-family of viruses, rendering it attractive for cancer immunotherapy. Certain strains of vaccinia have been genetically modified selective for cancer, and have been studied preclinically and clinically. JX-594 is an oncolytic vaccinia genetically engineered cancer-selective and inserted with GMCSF transgene, which has shown promising results in clinical trials: JX-594 was well-tolerated after intratumoral injections in phase I trial together with signs of immunological and antitumor efficacy (Mastrangelo et al. 1999, Park et al. 2008, Hwang et al. 2011). A recent randomized phase II dose-finding trial in liver cancer, showed good tolerability and intrahepatic disease control rate of 50%, which included both injected and non-injected lesions (Heo et al. 2013). In addition, virus replication and GMCSF expression were shown to precede the induction of antitumor immunity. The most notable clinical example of GMCSF-expressing viruses is, however, talimogene laherparepvec (T-VEC), a GMCSF-coding oncolytic herpes simplex virus, which has been extensively tested in clinical trials (Senzer et al. 2009, Harrington et al. 2010). A major breakthrough for the field of oncolytic immunotherapy was achieved in 2013, when T-VEC was reported to improve 6-month progression-free survival over subcutaneous GMCSF for the treatment of 295 patients with advanced melanoma (Andtbacka et al. 2013): Objective response rate in the T-VEC arm was 26% and durable response rate 16%, both of which were significantly higher than the 6% and 2% in the GMCSF arm, respectively. Serious adverse reactions occurred in 26% of T-VEC and 13% of GMCSF-treated patients, but over grade 3 reactions were less frequent than 3% in both arms. Thus, the treatment was well-tolerated and the study met its primary endpoint, and T-VEC is expected to receive approval by the health authorities in the near future. Conclusive long-term overall survival data has not yet been reported, as of November 2014.

2. AIMS OF THE STUDY

The aim of this thesis is to improve efficacy while maintaining low toxicity by combining oncolytic immunotherapy with conventional treatment modalities, and to identify novel predictive and prognostic factors for better patient selection. Thesis studies are depicted with roman numericals (I-IV). Specific aims of the studies I to IV are detailed below.

• Study I: To evaluate the potency and study the mechanisms behind radiosensitizing effects of adenoviral proteins E1B55K, E4orf3 and E4orf6 in prostate cancer preclinically

• Study II: To identify signalling pathways and marker proteins relevant for acquired resistance against oncolytic adenoviral immunotherapy in ovarian carcinoma xenografts

• Study III: To study the safety, efficacy and immunological effects of oncolytic immunotherapy combined with low-dose chemotherapeutics temozolomide and cyclophosphamide preclinically, and in patients with advanced metastatic cancer

• Study IV: To study the potential and role of serum HMGB1 protein as a novel predictive and prognostic biomarker for oncolytic immunotherapy in cancer patients with advanced solid tumors

3. MATERIALS AND METHODS

Materials and methods are described in more detail in the original publications.

3.1 Cell lines

In all preclinical studies, virus production was done in human lung adenocarcinoma cell line A549 (American Type Culture Collection [ATCC], Manassas, VA) and tittering in human E1-transformed embryonal kidney cell line 293, obtained from Microbix (Toronto, Ontario, Canada). In studies I and III, metastatic human prostate cancer cell line PC3-MM2 (ATCC, Manassas, VA) was used in subcutaneous mouse models *in vivo*, and primarily used *in vitro*. In addition in study I, we used another prostate cancer cell line DU-145, a breast cancer cell line M4A4-LM3, cervical cancer Henrietta Lacks (HeLa) cells (all from ATCC, Manassas, VA), and head and neck cancer primary explant UT-SCC8 cells , and in study III, a breast cancer cell line MDA-MB-436 was used (ATCC, Manassas, VA). In study II, we used human ovarian adenocarcinoma SKOV3 derived cell lines: SKOV3.ip1, generated by passaging *via* mouse peritoneal cavity, was provided by Dr. Price (M. D. Anderson Cancer Center, Houston, TX), and transgenic firefly luciferase expressing SKOV3-Luc cells were provided by Dr. Negrin (Stanford Medical School, Stanford, CA). Summary of cell lines is presented in **Table 2**.

Table 2. Cell lines used in thesis studies.

Used in study	Cell line (name)	Description	Source or reference
I, III	PC-3MM2	human prostate cancer cells	ATCC
I	DU-145	human prostate cancer cells	ATCC
I	UT-SCC8	head and neck squamous cell carcinoma cells	provided by Dr. Erjala
I	HeLa	human cervical cancer cells (Henrietta Lacks)	ATCC
I	M4A4-LM3	breast cancer cells	ATCC
II	SKOV3.ip1	human ovarian carcinoma cells	provided by Dr. Price
II	SKOV3-Luc	human ovarian carcinoma cells	provided by Dr. Negrin
II	A549	human lung adenocarcinoma cells	ATCC
II	HEK293	transformed human embryonic kidney cells	Microbix
III	MDA-MB-436	human breast cancer cell line	ATCC

3.2 Adenovirus constructs

Virus cloning and production, if not stated otherwise, is described in the original publications and/or references. In order to characterize and titer the produced viruses, constructs were tested for the presence of transgenes and genetic modifications/deletions, and for the absence of wild-type virus by polymerase chain reaction (PCR). Virus particle concentration (VP/ml) was determined spectrophotometrically (l = 260 nm), and the amount of infectious particles per ml (pfu/ml) was measured by a standard tissue culture infectious dose 50 ($TCID_{50}$) assay on 293 cells

(see below). For patient treatments, virus production was done according to the current good manufacturing practice by Oncos Therapeutics, Inc. (Helsinki, Finland), which was regulated by the Gene Technology Board. Adenoviruses in used studies I-IV are summarized in **Table 3**.

3.2.1 Replication-deficient adenoviruses

Recombinant replication-deficient serotype 5 adenoviruses rAdE4orf6 (Querido et al. 1997), rAdE4orf3 (Araujo et al. 2005), and rAdE1B55K used in study I were provided by Dr. Matthew D. Weitzman (Laboratory of Genetics, The Salk Institute for Biological Studies, La Jolla, CA). These viruses are E1- and E3-deleted and the transgene (E4orf6, E4orf3 or E1B55K) is located in the deleted E1 region under the cytomegalovirus (CMV) promoter. In addition, rAdE4orf3 virus encodes green fluorescent protein (GFP) under the same promoter for visualization of transgene expression. Ad5(GL) control virus is a similar replication-deficient construct but expresses GFP and luciferase as transgenes (Wu et al. 2002). As a replication-competent control in study I, we used a wild-type serotype 5 adenovirus Ad300wt, obtained from ATCC (Manassas, VA). In neutralizing antibody assays of study III, we used a matching capsid harboring replication-deficient adenoviruses expressing luciferase: Ad5Luc1 (Krasnykh et al. 2001), Ad5/3Luc1 (Kanerva et al. 2002a), Ad3Luc1 (Fleischli et al. 2007), and Ad5lucRGD (Kanerva et al. 2002b).

3.2.2 Oncolytic adenoviruses

Oncolytic adenovirus Ad5/3-Δ24 used in preclinical experiments in study II (Kanerva et al. 2003), and Ad5/3-Δ24-GMCSF (Koski et al. 2010) in study III, were both produced in A549 cells and purified on double cesium chloride gradients. Ratio of VP/infectious units was 6 for Ad5/3-Δ24, and 18 for Ad5/3-Δ24-GMCSF. All oncolytic adenoviruses used in patient treatments (III, IV) have been previously published and are described in **Table 3**.

For increased transductional targeting, 5/3 chimeric viruses are serotype 5 adenoviruses modified with a serotype 3 knob (Kanerva et al. 2003), whereas RGD coding viruses are capsid-modified at the RGD-4C motif in the HI-loop of the fiber (Dmitriev et al. 1998). Serotype 3 knob replacement extends the cell entry repertoire to serotype 3 receptor, which has recently been proposed to be desmoglein 2 (Wang et al. 2011), while the RGD modification improves virus binding to integrins that are found on cancer cell surface (Hemminki et al. 2001) and tumor vasculature (Arap et al. 1998). The fully serotype 3 oncolytic adenovirus Ad3-hTERT-E1A (Hemminki et al. 2011), in turn, may circumvent neutralizing antibody response mounted against serotype 5, while still boosting antitumor immune responses in patients (Hemminki et al. 2012).

With regards to transcriptional targeting, the Δ24 viruses harbor a 24 base-pair deletion in the retinoblastoma (Rb) binding site of E1A region, which attenuates replication at an early phase in normal cells that have wild-type retinoblastoma protein (Fueyo et al. 2000). As a consequence, Δ24-viruses replicate only in cells with Rb/p16 pathway defects, including nearly all human tumor types (Sherr 1996). Alternatively or in addition, a tumor-specific promoter is utilized to target virus replication: human telomerase (hTERT) promoter controlling the E1A restricts virus replication to immortalized cells with high telomerase activity (Fujiwara et al. 2008). The E2F-promoter, featured in ICOVIR-7 and Ad5/3-E2F-Δ24-GMCSF viruses, works hand in hand with the Δ24 deletion by activating E1A transcription in cells where free E2F is available, owing to the dysregulation of the

Rb/p16 pathway (Rojas et al. 2009). Finally, the cyclooxygenase 2 (Cox-2) was utilized as a promoter controlling E1A (Pesonen et al. 2010), since high Cox-2 expression is a hallmark of various tumor types (Wang and Dubois 2006).

The purpose of immunogenic transgenes is to stimulate antitumor immunity: Granulocyte-macrophage colony-stimulating factor (GMCSF) and CD40-ligand (CD40L) were featured in most of the viruses. GMCSF mobilizes immune cells and enhances type 1 dendritic cells to initiate cytotoxic immune responses. It has been widely used in cancer immunotherapy as an immune stimulant (Arellano and Lonial 2008). CD40L, on the other hand, is a co-stimulatory signal that binds to its CD40 receptor on antigen-presenting cells, mainly macrophages and dendritic cells, and leads to efficient antigen presentation, cytokine production, and eventually T-cell priming (van Kooten and Banchereau 2000). Soluble CD40L (Vonderheide et al. 2001) or gene therapy with CD40L (Malmstrom et al. 2010, Pesonen et al. 2012) has been used in cancer immunotherapy to a lesser extent, but early phase trials have demonstrated safety and evidence of antitumor activity in humans. Besides these immunogenic transgenes, a sodium iodide symporter protein (hNIS) was used as a radiotherapy adjuvant and for virus tracking purposes (Hakkarainen et al. 2009).

3.3 Preclinical in vitro experiments

3.3.1 Efficacy and synergy experiments

Combination efficacy experiments with external beam radiotherapy or chemotherapeutics in studies I and III, respectively, were performed on prostate cancer PC3-MM2 and DU-145, head-and-neck cancer explant UT-SCC8, and breast cancer MDA-MB-436 and M4A4-LM3 cells. The latter were cultured in RPMI-1640 (Lonza, Basel, Switzerland), and the others in Dulbecco's modified Eagle medium (DMEM; Lonza), both supplemented with 10% fetal bovine serum (2% during virus infections to avoid virus neutralization by serum), 1% L-glutamine, and 1% penicillin-streptomycin. The cells were maintained at humidified 37°C and 5% CO^2 incubators, and on a slow rocker during virus infections. Studies were performed in triplicates and readings/results were compared to untreated (mock) cells.

In study I, cells were seeded into 96-well plates, the next day infected with virus(es) for 2 h followed by 24 h incubation, and then irradiated with 0, 2 or 8 Gy depending on the relative sensitivity of each cell line (Rajecki et al. 2009, Dias et al. 2010). External beam irradiation was administered by a linear accelerator (Clinac 600C/D, Varian Medical Systems, Palo Alto, CA, USA) using a 6 MV photon beam and a dose rate of 400 MU/min (ca. 4 Gy/min) through a 1-cm thick plastic phantom bottom containing 1 cm of water. Cell viability was assessed by CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI), measuring optical density with spectrophotometer at 490 nm. In clonogenic assays, the treatments were carried out similarly in 24-well plates using PC3-MM2 cells, but the treated cells were then transferred into 6-well plates at 1000 cells/well and incubated for 10 days. Colony formation was assessed by fixing the cells with 10% paraformaldehyde, and staining with 1% crystal violet in 70% ethanol, followed by counting colonies under a light microscope.

In study III, cancer cells (PC3-MM2 or MDA-MB-436) were seeded on 96-well plates and (co-)treated with oncolytic adenovirus Ad5/3-D24-GMCSF, temozolomide (TMZ; MSD, Espoo, Finland), and/or 4-hydroperoxycyclophosphamide (4-HPCP), which is an active metabolite of cyclophosphamide (D-18864; NIOMECH, Bielefeld, Germany). Treatments were performed in a total volume of 100 µl of growth medium per well, adding 100 µl after 24 h for incubation. When cytopathic effect was observed (ca. day 4 post-treatment), all comparable plates were simultaneously measured for cell viability by using CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega) with spectrophotometer at 490 nm.

3.3.2 Immunofluorescence and protein analyses

Visualization of DNA double strand breaks in study I was performed by phospho-γH2AX immunofluorescence: PC-3MM2 cells were fixed on slides at 24 h post-treatment (infection and/or irradiation) with 4% paraformaldehyde for 20 min, permeabilized with ice-cold 70% ethanol, and blocked with 0.5% bovine serum albumin (BSA) supplemented with 0.05% sodium azide in phosphate-buffered saline (PBS) for 1 h. Slides were incubated with primary mouse monoclonal phospho-γH2AX antibody (Upstate, clone JBW301, Boston, MA; 1:1000) for 1 h, washed twice, and then incubated with secondary Alexa Fluor 594 antibody (Invitrogen, Carlsbad, CA; 1:500). For detection of adenovirus capsid in the same study, treated cells were permeabilized with 0.1% Triton (x100) supplemented with 0.5% BSA in PBS, and blocked for 1 h with an immunomix containing 5% normal donkey serum, 0.2% BSA, and 0.05% sodium azide in PBS. We used primary goat polyclonal anti-adenovirus (anti-hexon) antibody Virostat #1401 (Virostat, Portland, ME; 1:200) and secondary antibody Alexa fluor 594 (Invitrogen; 1:500). Slides were mounted using Vectashield with DAPI counterstaining (H-1200, Vector Laboratories, Peterborough, UK) and analyzed by confocal microscopy (see below). In addition, GFP expression signal of the rAdE4orf3 virus treated cells was also visualized by immunofluorescence.

In study II, induction of Myxovirus resistance protein A (MxA) expression was studied by immunofluorescence: To simulate the effect of stromal cells, SKOV3.ip1 cells were pretreated with or without recombinant universal type I interferon-α (IFN-αA/D, Sigma-Aldrich, St Louis, MO; 100 IU/ml) 16 h before and again during infection, and infected with 100 VP/cell of Ad5/3-Δ24 adenovirus for 30 min on ice (on coverslips). Following 0 min, 30 min, 1 h and 2 h incubation, cells were washed, and fixed with 4% paraformaldehyde for 10 min. We used primary rabbit polyclonal anti-MxA antibody (H-285, Santa Cruz Biotechnology, Dallas, TX; 1:100) and primary anti-adenovirus antibody Virostat #1401 (Virostat; 1:50), and then Alexa fluor 405 and Alexa fluor 594 secondary antibodies (Invitrogen), respectively. In all immunofluorescence studies, cells were visualized using a laser scanning confocal microscope Zeiss LSM 5 Duo (Jena, Germany). Captured images were processed with Adobe Photoshop and Illustrator softwares (Adobe Systems, San Jose, CA).

In addition to immunofluorescence in study I, we also analyzed virus transgene production and quantity of DNA double strand breaks by Western blot. For virus transgene analysis, PC-3MM2 cells were infected with recombinant viruses and 24 h later harvested, lysed with CelLytic M (Sigma-Aldrich), and protein normalized. For γH2AX immunoblotting, PC-3MM2 cells were harvested at 30 min or 24 h post-treatment (infection and/or irradiation), sonicated, and acid-extracted overnight with 0.2M hydrochloric acid to detach H2AX histone protein from DNA. We used ultraviolet-B irradiated (ca. 150 J/m^2) HeLa cells as a positive control for DNA double strand

breaks as published (Halicka et al. 2005). Primary antibodies were mouse polyclonal anti-E1B55K (2A6; 1:20), rat polyclonal anti-E4orf3 (6A11; 1:25), mouse polyclonal anti-E4orf6 (Rsa#3; 1:20), and rabbit monoclonal anti-phospho-H2A.X (#9718; Cell Signaling Technology, Danvers, MA; 1:1000). The 2A6 (E1B55K) and the Rsa#3 (E4orf6) antibodies were provided by Dr. David A. Ornelles (Wake Forest University School of Medicine, Winston-Salem, NC), and the 6A11 (E4orf3) antibody was provided by Dr. Thomas Speiseder (Heinrich-Pette-Institute for Experimental Virology, University of Hamburg, Germany). In addition, mouse monoclonal anti-GAPDH antibody (#39-8600, Invitrogen; 1:2000) was used for visualization of normalized gene expression.

3.3.3 Immunogenicity of cell death experiments

Immunogenic cell death is an increasingly well-documented concept, where certain DAMPs, i.e. calreticulin, adenosine triphosphate (ATP) and high-mobility group box 1 (HMGB1), are released/exposed from dying cells, and subsequently activate the associated immune cells (Hannani et al. 2011). Immunogenicity of cell death in study III was assessed by measuring calreticulin exposure on the cell surface, and quantitating the released ATP and HMGB1 protein in the supernatant, as previously described (Diaconu et al. 2012). PC3-MM2 cells were mock-treated or treated in triplicates with 100 VP/cell of Ad5/3-Δ24-GMCSF virus, and 12 h later treated with/without TMZ (c = 0.0025 mg/ml) and/or 4-HPCP (c = 0.00208 mg/ml). After 24 h, intact cells were harvested, washed and incubated in anti-calreticulin antibody (ab2907; Abcam, Cambridge, UK; 1:1000) for 40 min at 4 °C, and then similarly in secondary antibody Alexa fluor 488 (A21206; Invitrogen; 1:100). Calreticulin on surface of intact cells was analyzed by FACSAria flow cytometer (BD Biosciences, San Diego, CA), and FlowJo software (Tree Star, Ashland, OR). For extracellular ATP and HMGB1 analysis, supernatants of identically treated cells were collected on ice at 36 h post-treatment (12 h later than in calreticulin analysis), and analyzed with ATP Determination Kit (A22066; Molecular Probes/Invitrogen) and HMGB1 ELISA Kit (ST51011; IBL International, Hamburg, Germany) according to the manufacturer's recommendations, and in the case of HMGB1, using a normal range procedure.

3.4 Preclinical in vivo studies

All animal experiments were approved by Experimental Animal Committee of the University of Helsinki and the Provincial Government of Southern Finland (ESAVI-2010-09782/Ym-23). The health of the mice was followed daily, and treatments and bioluminescence imaging were performed either in medetomidine-ketamine-0.9% saline (1:2:7) anesthesia or in isoflurane gas anesthesia. In study I, external beam radiotherapy was given as whole-body irradiation to avoid the need for prolonged anesthesia. Mice were euthanized according to the humane end-point guidelines.

3.4.1 Animal models and efficacy experiments

For subcutaneous prostate tumor models (I, III), male Nude/NMRI mice were used, provided by Scanbur BK (Sollentuna, Sweden) in study I, and by Taconic (Ejby, Denmark) in study III. Four (III) or five (I) million PC3-MM2 cells were inoculated into both flanks of the mice and tumor growth was

measured using the formula "length x width^2 x 0.5". When tumors reached injectable size (day 7 post-implantation), mice were randomized, and treated with intratumoral injections of adenovirus or growth medium (total of $4 \times 1 \times 10^9$ VP/tumor in study I, and $2 \times 2 \times 10^{10}$ VP/tumor in study III). Fractionated external beam radiotherapy in study I was given every other day (4 × 2 Gy): Mice remained in standard plastic cages and received whole-body irradiation delivered by Clinac 600C/D linear accelerator. In study III, mice received intraperitoneal injections of metronomic low-dose cyclophosphamide (CP; 20 mg/kg in saline) or saline on days 0, 4, and 7, and low-dose pulse of TMZ (10 mg/kg in saline) or saline for five consecutive days starting on day 3 post-CP-treatment. Viruses used in study I were rAdE1B55K, rAdE4orf3, rAdE4orf6, Ad5(GL), and Ad300wt, and in study III Ad5/3-Δ24-GMCSF.

In study II, we established two intraperitoneal ovarian tumor models. Here, female C.B-17 SCID mice were used (Taconic) that were xenografted intraperitoneally with ten million SKOV3.ip1 cells in a survival experiment, or with five million SKOV3-Luc cells in a tumor volume follow-up model. In the survival experiment, mice were 10 days later injected intraperitoneally with 3×10^7 VP of Ad5/3-Δ24 virus or growth medium, and health of the mice was followed until guideline symptoms and then euthanized. Tumor masses in the peritoneal cavity were surgically collected on ice and snap-frozen to -80 °C for later analyses. In the tumor growth follow-up experiment, mice were treated on days 3, 7, and 10 intraperitoneally with 1×10^9 VP of Ad5/3-Δ24 virus or growth medium, and imaged noninvasively by bioluminescence imaging to monitor tumor growth: During isoflurane gas anesthesia, 150 mg/kg of D-luciferin (Promega, Madison, WI) was injected intraperitoneally and 10 min later imaged by IVIS 100 (Xenogen, Alameda, CA), as described (Hemminki et al. 2011). Images were overlaid with Living Image 2.50 (Xenogen). Total flux (photons/s) was measured by drawing regions of interest around the peritoneal area of the mice and background was subtracted. When treated tumors had relapsed on day 27, mice were euthanized, peritoneal tumors were surgically removed and two blocks of 0.3 cm^3 intact tumor with stroma were freshly transplanted in laparotomy into anesthesized new SCID mice. In addition, two groups of new mice received five million naïve SKOV3-Luc cells intraperitoneally. Then the mice with transplanted (relapsed/mock-treated) or naïve tumors were treated on days 5, 8, and 11 intraperitoneally with 1×10^9 VP of Ad5/3-Δ24 virus or growth medium, and again, imaged noninvasively for tumor growth.

3.4.2 Determination of functional virus

To determine the amount of functional adenovirus particles in tumor tissue in study II, control and virus-treated ovarian cancer xenografts were homogenized mechanically, freeze/thawed three times, and centrifuged to collect supernatants, which were used for a tissue culture infectious dose 50 ($TCID_{50}$) assay: 293 cells seeded on 96-well plates at the density of 10000 cells/well were infected with supernatant in increasing ten-fold dilutions per row of wells, and the cytopathic effect (CPE) was recorded under a light microscope on day 10. Formula to determine the $TCID_{50}$-titer ($TCID_{50}$/100 µl), and the converted pfu-titer ($[TCID_{50} \times 10]^{-0.7}$), was $TCID_{50} = 10^{1+d(S-0.5)}$, where d = Log 10 of the dilution (= 1 for ten-fold dilutions), S = the sum of ratios (starting from the first 10^{-1} dilution), with ratio meaning the number of CPE wells /total number of wells per dilution. Similar tittering was performed for all constructed adenoviruses to determine pfu-titers.

3.4.3 Gene expression and microarray analysis

We investigated the gene expression patterns of adenovirus-treated relapsed SKOV3.ip1 tumors *versus* mock-treated tumors in study II. First, total cellular RNA of homogenized tumors was reverse-transcribed into cDNA using Qiagen QuantiTect Reverse Transcription Kit (205311; Hilden, Germany), including the genomic DNA wipeout procedure. Next, concentrations of the cDNAs were balanced by NanoDrop 2000 Spectrophotometer (Thermo Scientific, Waltham, WA), and samples were stored in -80 °C. We confirmed microarray results (see below) of certain differentially-expressed key genes both by semi- (described in original publication) and fully quantitative reverse transcriptase-PCR (described here): cDNA samples were amplified by LightCycler 480 real-time PCR with SYBR Green I Master mix (Roche, Mannheim, Germany) and using specific primers for human interferon α-inducible protein 16 (IFI16) (5-ACTGAGTACAACAAAGCCATTTGA-3 and 5′-TTGTGACATTGTCCTGTCCCCAC-3′), interferon α-inducible protein 27 (IFI27) (5′-ACCTCATCAGCAGTGACCAGT-3′ and 5′-ACATCATCTTGGCTGCTATGG-3′), and Myxovirus resistance protein A (MxA) (5′-ACCTACAGCTGGCTCCTGAA-3′ and 5′-CGGCTAACGGATAAGCAGAG-3′). Human β-actin (5′-TCACCCACACTGTGCCCATCT-3′ and 5′-GTGAGGATCTTCATGAGGTAGTCAGTC-3′) was used for normalization to human genomic mRNA. ΔΔ-comparative threshold method was used to calculate the relative amounts of the indicated mRNA (Livak and Schmittgen 2001), and results were expressed as mean fold-change in expression levels.

For microarray analysis, total RNA from xenografted tumors was extracted as described above. Microarray data obtained with Affymetrix GeneChip HG-U133A (Affymetrix, Santa Clara, CA) was pre-processed using Bioconductor in R (Durinck 2008). Hs133P_Hs_ENSG annotation library version 9 was used to bind the probes to transcripts. Normalization was performed by robust multiarray average normalization (Irizarry et al. 2003).

3.4.4 Immunohistochemistry analyses

Tissue level analyses in studies II and III concentrated on assessing protein levels of a proposed adenovirus resistance marker MxA and an established autophagy marker LC3B, respectively. MxA immunohistochemistry (II) was performed on five adenovirus-resistant, and five mock-treated frozen SKOV3.ip1 xenografts, together with normal SKOV3.ip1 cells as a control. Frozen sections were boiled in 10 mmol/l citrate buffer pH 6 for 30 min, covered with 3% hydrogen peroxide for 10 min, and then treated with Protein Blocking Agent (Novocastra Laboratories, Newcastle, UK) for 10 min. MxA antibody (sc-50509; Santa Cruz Biotechnology, Santa Cruz, CA; 1:1000) was applied for 60 min at room temperature. After washing, sections were treated with NovoLink Polymer Detection System (Novocastra Laboratories) according to manufacturer's instructions and counterstained with hematoxylin. Immunohistochemistry for microtubule-associated protein light chain 3 isoform B (LC3B) is widely used as a marker of autophagy for its involvement in the formation of autophagic vacuoles (autophagosomes), of which a punctate LC3B expression pattern is a strong indicator. PC3-MM2 xenografts were harvested on day 12 post-treatment (III), fixed with 4% paraformaldehyde, and embedded in paraffin. Cut sections were boiled in 10 mmol/l citrate buffer pH 6 for 15 min, covered with 3% hydrogen peroxide for 5 min, and stained with rabbit polyclonal LC3B antibody (ab48394; Abcam; 1:1500) in Dako Antibody Diluent (S0809; Dako, Carpinteria, CA) for 60 min at room temperature. Sections were then washed, treated with LSAB+ System-HRP Kit (K0679; Dako) according to manufacturer's instructions, and counterstained with

hematoxylin. In both studies, slides were analyzed under a Leica DM LB microscope (Leica Microsystems, Wetzlar, Germany) and images were captured using an Olympus DP50 color camera (Olympus, Münster, Germany) and Studio Lite 1.0 software (Pixera, San Jose, CA). To determine the extent of autophagy (III), we calculated the average of five 40x visual fields of LC3B punctate-positive cells (>3 dots/cell).

3.4.5 Electron microscopy

Electron microscopy was used to study autophagy in mouse xenografts and to confirm virus production (III). Immediately after euthanizing the mice, PC3-MM2 xenografts were surgically removed and fixed with 2.5% glutaraldehyde in 0.1 mol/l phosphate buffer pH 7.4. Samples were post-fixed with 2% osmium tetroxide for 1 h, dehydrated in series of ethanol, and embedded in LX-112 resin. Ultra-thin sections were cut at 50–60 nm, and stained with uranyl acetate and lead citrate in Leica EMstain automatic stainer (Leica microsystems) according to manufacturer's recommendations. Electron microscopy was performed by Jeol JEM-1400 (Jeol, Tokyo, Japan) at 80 kV accelerating voltage, and digital microphotographs were captured using Olympus-Sis Morada digital camera (Olympus).

3.5 Patient series

3.5.1 Advanced Therapy Access Program (ATAP)

Altogether 290 patients with advanced solid tumors refractory to conventional treatment modalities were treated with oncolytic adenoviruses in the context of an ISRCTN registered Advanced Therapy Access Program (ATAP) between Nov 2007 and Jan 2012 at Docrates Hospital, Helsinki, Finland (ATAP: a treatment for refractory cancer with oncolytic adenoviruses, ISRCTN: 10141600). ATAP was regulated by Finnish Medicines Agency (FIMEA) as determined by the European Committee Regulation No 1394/2007 on advanced therapy medicinal products, amending Directive 2001/83/EC and Regulation No 726/2004. ATAP was in compliance with European Union and Finnish regulations and was evaluated by The Gene Technology Board and Medicolegal Department of the Finnish Ministry of Social Affairs and Health. All patients voluntarily contacted the clinic and the suitability of each patient was evaluated before treatment decisions. Inclusion criteria for ATAP were: advanced solid tumors progressing after and refractory to conventional therapies, and patients' WHO performance score ≤ 3 at baseline. Exclusion criteria were: major organ dysfunction, organ transplant, known brain metastasis, HIV or other major immunosuppression, elevated bilirubin, alanine transaminase (ALT) or aspartate transaminase (AST) increased over x3 upper limit of normal, severe thrombocytopenia, and other severe disease.

All patients gave a written informed consent after the principles of treatments, including possible side-effects were explained verbally and in writing. Treatments were performed according to Good Clinical Practice and based on Article 35 of the Helsinki Declaration of World Medical Association. ATAP was a personalized therapy program, not a clinical trial, and treatment decisions were based on individual characteristics of the patients, their tumors, and what had been learned from earlier

patients. After receiving treatments described in studies III and IV, patients were free to receive other cancer therapies, including additional virus treatments. All retrospective clinical-epidemiological research conducted in this thesis including patient sample analyses (III, IV), have been approved by the Helsinki University Central Hospital Operative Ethics Committee (HUS 62/13/03/02/2013). In addition, we obtained a separate permission, written informed consents from the patients and ethics committee approval (Dnro HUS 368/13/03/02/2009), for patient biopsies and biopsy analyses, since this data could be useful for developing more effective patient treatments. Data relating to these analyses are reported in the context of study II in this thesis. Clinical data for studies were collected from medical records and population registry.

3.5.2 Patient selection, treatments and follow-up

Patient selection criteria for our clinical-epidemiological analyses were based on the given ATAP treatments (III) or available baseline serum samples (IV). Particularly, study III focused on patients treated at earlier phase with the combination of oncolytic adenovirus and low-dose chemotherapeutics cyclophosphamide (CP) and temozolomide (TMZ) (N = 17). Study IV, in turn, included all patients with available non-hemolytic baseline serum sample for assessment of circulating HMGB1 level (N = 202). In addition in study III, nonrandomized matched control patients (N = 17) were selected in order to estimate the effect of TMZ adjuvant therapy on overall survival; these patients were treated similarly in the ATAP, but did not receive TMZ. Matching was based on known prognostic factors (percentage of successful matches in parenthesis): tumor type (100%), concomitant low-dose CP administration (yes/no 94%), exact same round of virus treatment (71%), treatment with the same oncolytic adenovirus (52%), WHO performance status at baseline (48%). Self-controls were not allowed.

Patients received oncolytic adenovirus intratumorally (primary tumor and/or any injectable metastases) in ultrasound or CT guidance, when applicable. In case of a peritoneal or pleural disease, the intratumoral injection was performed intracavitary. Typically, one fifth of the virus dose was given intravenously in an attempt to achieve virus transduction of uninjectable lesions as well (Nemunaitis et al. 2001, Reid et al. 2002b). Some patients that lacked injectable lesions, however, received the whole virus dose intravenously. Viruses used in patient treatments are described in **Table 3**. Virus doses ranged from 1×10^{10} to 4×10^{12} VP in study III, and from 2×10^{9} to 4×10^{12} VP in study IV. In the absence of contraindications, patients received low-dose cyclophosphamide in order to reduce regulatory T-cells (Lutsiak et al. 2005, Cerullo et al. 2011), which was administered perorally 50 mg daily in a metronomic manner starting one week before the virus treatment and continued until progression, or as a bolus infusion of 1000 mg intravenously on the day of the virus treatment, or as a combination of these (Cerullo et al. 2011). Both the metronomic oral and the intravenous infusion administration of CP have been shown to induce similar immunological effects (Cerullo et al. 2011). All patients in study III (excluding matched controls), and a subset of patients in study IV, received oral low-dose pulse of TMZ (100 mg/day), which was administered according to three different dosing schedules, investigated in study III: 5 days before the virus (group 1), 5–7 days before and two weeks after the virus (group 2), or 7–10 days after the virus treatment (group 3). Chemotherapeutic doses were adjusted for pediatric patients (N = 2 in III, and N = 5 in IV). 29 % of patients in study III, and 51 % in study IV, received the studied virotherapy as a serial treatment of three consecutive virus treatment cycles at 3-4 week intervals, due to emergence of evidence that multiple injections of oncolytic adenovirus could enhance immunologic responses (Kanerva et al. 2013).

Patient follow-up started on the day of the virus treatment. Patients were monitored for 24 h in the hospital and 4 weeks as outpatients, with intermittent recordings of clinical status and laboratory data. Adverse reactions (ARs) were reported according to Common Terminology Criteria for Adverse Events (CTCAE) v3.0 criteria. Pre-existing symptoms were listed only if worsened, and in that case they were scored according to final severity. In both studies (III, IV), ARs were further classified as either leading, or not leading, to patient hospitalization, malformation, life-threatening condition, or death; Any of these conditions constituted a serious adverse event (SAE), which were also reported, together with treatment results, to the FIMEA as requested. Of note, in study III we considered transient lymphocytopenia as an AR, whereas in study IV we excluded it from our analyses due to accumulating evidence indicating that it is compatible with lymphocyte redistribution, which is commonly seen after both virus infections and immunotherapy and does not appear as an actual adverse reaction but rather a phenomenon contributing to treatment efficacy (i.e. trafficking of lymphocytes) (Reid et al. 2002b, Brahmer et al. 2010, Kanerva et al. 2013, Hemminki and Hemminki 2014).

Table 3. Adenoviruses used in thesis studies.

	Used in study	Virus	Transduct. targeting	Transcript. targeting	Transgene	Source or reference
Oncolytic	I	Ad300wt	5	-	-	ATCC
	II	Ad5/3-Δ24	5/3	Δ24	-	Kanerva et al. 2003
	III, IV	ICOVIR-7	5, RGD	Δ24, E2F	-	Nokisalmi et al. 2010
	III, IV	Ad5-Δ24-RGD-GMCSF	5, RGD	Δ24	GMCSF	Pesonen et al. 2012
	III, IV	Ad5/3-Cox2L-Δ24	5/3	Δ24, COX2	-	Pesonen et al. 2010
	III, IV	Ad5-Δ24-GMCSF	5	Δ24	GMCSF	Cerullo et al. 2010
	III*, IV	Ad5/3-Δ24-GMCSF	5/3	Δ24	GMCSF	Koski et al. 2010
	III, IV	Ad5/3-hTERT-E1A-CD40L	5/3	hTERT	CD40L	Diaconu et al. 2012
	III, IV	Ad3-hTERT-E1A	3	hTERT	-	Hemminki et al. 2011
	IV	Ad5/3-E2F-Δ24-GMCSF	5/3	Δ24, E2F	GMCSF	Ranki et al. 2012 Mol Ther. Suppl.1
	IV	Ad5/3-Δ24-hNIS	5/3	Δ24	hNIS	Rajecki et al., 2012
Non-replicating	I	rAdE1B55K	5	-	-	Marcellus et al., 1996
	I	rAdE4orf6	5	-	-	Querido et al., 1997
	I	rAdE4orf3	5	-	GFP	Araujo et al., 2005
	I	Ad5(GL)	5	-	GFP, LUC	Wu et al., 2002
	III	Ad5Luc1	5	-	LUC	Krasnykh et al., 2001
	III	Ad5lucRGD	5, RGD	-	LUC	Kanerva et al., 2002b
	III	Ad5/3Luc1	5/3	-	LUC	Kanerva et al., 2002a
	III	Ad3Luc1	3	-	LUC	Fleischli et al., 2007

* Virus was used both in preclinical experiments and in patient treatments. Other oncolytic viruses used in studies III and IV were only used in patient treatments, while non-replicating viruses (III) were used for neutralizing antibody titer determination of serum samples.

3.5.3 Response evaluation and survival analysis

Tumor assessment in the ATAP was performed by contrast-enhanced computed tomography (CT), positron emission tomography-computed tomography (PET-CT), or magnetic resonance imaging (MRI), which was performed before and typically 3–6 weeks after a single treatment. In case of a serial treatment, post-treatment imaging was performed after the complete treatment series, typically 9-14 weeks after the first treatment. Response evaluations were performed by professional radiologists by applying modified Response Evaluation Criteria in Solid Tumors (RECIST) v1.1 for CT and MRI scans (Eisenhauer et al. 2009), and modified PET Response Criteria in Solid Tumors (PERCIST) for PET-CT as previously described (Koski et al. 2013a). Evaluations applied to overall disease status including injected and non-injected lesions, and the following classification was used: CR, complete response (disappearance of all tumors); PR, partial response (≥ 30% reduction in the sum of the longest diameters of all measured lesions); MR, minor response (MR, 10-29% reduction in the sum); PD, progressive disease (≥ 20% increase in the sum, or appearance of new metastatic lesions); SD, stable disease (tumor measurements not fulfilling the criteria for response or progression). For PET-CT, the same percentages were used, but evaluations based on [(18)F]-fluorodeoxyglucose activity within target lesions as published (Koski et al. 2013a). In addition in study III, we assessed tumor marker responses, if elevated at baseline, by applying the same percentages to the change between best response and baseline value.

Baseline tumor load score assessment in study IV, was based on the whole-body radiographic evaluations of 95 patients: Tumor masses in lungs, liver, peritoneal cavity, bones, lymph nodes, and other sites were graded from 0 to 3 (none to high tumor burden), with bulky tumor at any location giving an additional 3 points, and the sum was calculated (possible range: 0-21 points). Presence of pleural/ascites effusion was also recorded, but it did not affect the solid tumor load score. Overall survival (III, IV) was calculated from the day of the first virus treatment (in study III: day of the first TMZ-combined treatment) until death or study conduction. Patient status (dead/alive information) was obtained from medical records and the population registry.

3.5.4 Quantification of viral DNA in serum

Patient blood samples were collected at normal hospital visits before and after virus treatments. Samples were centrifuged to separate clots, and the resulting serum (supernatant) and clots were stored at -20°C. As a surrogate of virus replication, we analyzed viral DNA in serum and blood clots at multiple timepoints by quantitative PCR (III, IV). Total DNA was extracted from serum using carrier DNA (polydeoxyadenylic acid; Roche) with QIAamp DNA mini kit (Qiagen, Hilden, Germany), which was then eluted in 60 µl nuclease-free water and measured by spectrophotometry to determine DNA concentration. Quantitative PCR using specific primers for serotype 5 oncolytic adenoviruses was performed as previously described (Cerullo et al. 2010, Escutenaire et al. 2011, Pesonen et al. 2012). For serotype 3 adenovirus, method and primers are described in the original publication (III) and reference (Hemminki et al. 2012). The viral loads were calculated using a regression standard curve based on serial dilutions of adenoviruses in normal human serum.

3.5.5 Protein analyses on patient samples

Protein level analyses on patient samples included immunohistochemistry for LC3B protein on patient ascites samples (III), immunohistochemistry for MxA on patient tumor biopsy samples (reported in the context of study II), serum inflammatory cytokine measurements by cytometric bead array (III, IV), and serum HMGB1 protein measurements by human HMGB1 ELISA (III, IV).

In order to study autophagy in patient ascites tumor cells, whole ascites samples were centrifuged to collect cells, which were then fixed with methanol and assessed for LC3B immunohistochemistry as described above. LC3B primary antibody (ab48394; Abcam; 1:1500) was applied for 120 minutes. MxA immunohistochemistry analyses on patient tumor biopsies were performed as described for *in vivo* experiments in study II. Briefly, sections from paraffin blocks of tumor biopsies were cut on glass slides, assessed for MxA immunohistochemistry using anti-MxA antibody (sc-50509, Santa Cruz Biotechnology; 1:1000), and mounted under cover slips. Biopsy stainings were evaluated and scored (from 0+ to 3+) by an independent pathologist, who had no information about the pre-specified hypotheses. Hematoxylin eosin stainings were used as technical controls and for interpretation of tissue and cellular morphology.

For serum cytokine analysis, 50 µl of serum sample was used for BD Cytometric Bead Array (CBA; BD Biosciences, San Diego, CA) performed using BD CBA Human Soluble Protein Master Buffer Kit and BD CBA Human IL-6, IL-8, IL-10, TNF-α, and GM-CSF Flex Sets (BD Biosciences, San Diego, CA) according to manufacturer's instructions for serum samples on 96-well plates. BD FACSArray Bioanalyzer, BD FACS Array System software, and FCAP Array v1.0.2 software (BD Biosciences) were used for data analysis.

Serum HMGB1 protein concentration was assessed by HMGB1 ELISA Kit (ST51011; IBL International, Hamburg, Germany) according to manufacturer's instructions, using a high sensitive range protocol; Mircotiter plates were incubated with samples/controls for 23 h. Multipipetting was used when applicable, and the plates were analyzed immediately with spectrophotometer at 450 nm. Hemolytic serum samples were considered unsuitable for analysis. An identical HMGB1 ELISA protocol was used for ascites/pleural effusion samples. HMGB1 concentration was calculated from raw values plotted on the high sensitive range standard curve (standards on the same plate), and occasional negative values were considered as undetectable levels and regarded as zero. Change in serum HMGB1 (ΔHMGB1) was assessed by subtracting individual baseline concentration from post-treatment values. Since the same plate with same conditions was used for every sample of a respective patient, technical replicates proved unnecessary for serum ELISA and cytokine analyses due to negligible variance in readings.

3.5.6 Neutralizing antibody titer determination

Serum neutralizing antibody titer was determined by measuring serum-mediated blocking of gene transfer by a capsid-matched non-replicating adenovirus. First, 293 cells were seeded at 10000 cells/well on 96-well plates and incubated overnight. Serum samples were incubated at 56°C for 90 min to inactivate complement, and a four-fold dilution series was prepared in serum-free growth medium (1:1 to 1:16384). Non-replicating Ad5Luc1, Ad5LucRGD, Ad5/3Luc1, and Ad3Luc1 viruses were used for assessing serotype 5 capsid, RGD-modified serotype 5 capsid, 5/3-chimeric capsid, and serotype 3 capsid oncolytic viruses, respectively (see **Table 3**). Non-replicating virus was mixed

with the serum dilutions and incubated at room temperature for 30 min, followed by infection of 293 cells at 100 VP/cell in triplicates. Growth medium with 10% FCS was added 1 h later, and 23 h later cells were lysed with 1x Reporter Lysis Buffer (Promega) and luciferase activity was measured using Luciferase Assay System (Promega) and TopCount Luminometer (Perkin-Elmer). Raw values were plotted relative to gene transfer achieved with the respective non-replicating virus alone, and the neutralizing antibody titer was determined as the lowest dilution that blocked gene transfer for over 80%.

3.5.7 Enzyme-Linked ImmunoSpot (ELISPOT) assay

Peripheral blood mononuclear cells (PBMC) were extracted from collected whole blood samples by Percoll gradient centrifugation, and PBMCs were immediately stored in CTL-CryoABC serum-free medium (Cellular Technology Ltd., Cleveland, OH) at -140°C. In studies III and IV, T-cell reactivity against a ubiquitous tumor-epitope Survivin was measured, while in study III, also adenovirus-specific responses were studied by interferon-γ Enzyme-Linked ImmunoSpot (ELISPOT) assay. In order to avoid artificial or incorrect signals, we performed all ELISPOT assays without pre-stimulation or clonal expansion of PBMCs, and thus results represent the actual frequency of these cells in blood. Since T-cell responses can take time to establish after immunotherapy, in study IV we analyzed PBMC samples following consecutive treatment cycles as well. Assays were performed according to manufacturer's instructions using the h-INF-γ ELISPOT PRO 10 plate kit (MABtech, Stockholm, Sweden). To specify parts of protocol, viable cells were manually counted using Trypan Blue under a light microscope, and blocking medium contained 10% FCS as serum. For tumor-specific antigen responses, PBMCs were stimulated in triplicates with a tumor-associated BIRC5 PONAB peptide Survivin (ProImmune, Oxford, UK), and for adenovirus-specific responses with human adenovirus serotype 5 penton or serotype 3 hexon peptide pools (HAdV-5 or HAdV-3; ProImmune) for 20 h. Dried plates were analyzed with AID-ELISpot reader (Autoimmun Diagnostika, Strassberg, Germany), and the results were expressed as means of triplicates. In both studies, unspecific interferon-γ T-cell responses were also observed that might include T-cell reactivity against unknown tumor epitopes, and these were therefore not subtracted (Kanerva et al. 2013). We used a threshold of ≥ 20% change in spot forming colonies (SFC) from baseline together with an absolute count of ≥ 10 SFCs (per 1 million cells) as a true positive T-cell activity (induction/decrease), and otherwise considered it as anergy.

3.6 Statistics and in silico analyses

3.6.1 Preclinical statistics and pathway analyses

Statistical analyses were performed using two-tailed Student's t-test for *in vitro* data, and nonparametric Mann-Whitney U test, one-way analysis of variance repeated measures, and Kaplan-Meier method with Log-Rank test for *in vivo* data (SPSS 15.0 – 18.0, Chicago, IL). In order to determine synergism between adenovirus, TMZ, and 4-HPCP in study III, the cytotoxicity interactions were analyzed by CompuSyn software (ComboSyn, Paramus, NJ) using the Chou–

Talalay median effect principle (Chou 2010). For microarray data in study II, the normalized pre-processed raw data were analyzed with statistical tests in order to identify differentially expressed genes, which were deemed as such if the ratio of medians of normalized intensities of cases and controls were either less than 1/1.7 or greater than 1.7 and the standard deviation between all samples was at least 0.4. Data were next analyzed with two different *in silico* platforms: Ingenuity Pathways Analysis (IPA) (Ingenuity Systems, Mountain View, CA) and Moksiskaan analysis (Laakso and Hautaniemi 2010). In IPA, canonical pathway analysis was used to identify the most significant altered pathways according to the IPA library. The significance of the association between the data set and the canonical pathway was studied by ratio of data-set genes that map to a pathway divided by the total number of genes mapping to that pathway, and by Fisher's exact test. Moksiskaan is a data integration platform containing gene, pathway and drug information obtained from Ensembl (Hubbard et al. 2009), Gene Ontology (Ashburner et al. 2000), KEGG (Kanehisa et al. 2010), SPIA (Tarca et al. 2009), SNPs3D (Yue et al. 2006), PathwayCommons (Cerami et al. 2006), PINA (Wu et al. 2009) and COSMIC (Forbes et al. 2010), which we used to identify connections between the differentially expressed genes, and related them to existing drugs, diseases, and known cancer mutations.

3.6.2 Patient series statistics and multivariate analyses

In study III, adverse reactions (ARs) were analyzed with two-tailed Student's t-test, while in study IV ARs and imaging responses were compared by χ^2 (chi-squared) test. Further in study IV, patient characteristics were compared by χ^2 and Student's t test, tumor load data was analyzed by linear correlation and Mann-Whitney U test, and the frequency of pleural/ascites effusion was compared by χ^2 test. Overall survival data (III, IV) were analyzed by Kaplan–Meier method with Log-Rank tests. With regards to patient sample analyses, virus titers in blood were studied by Kruskal-Wallis test, serum cytokine data by Kruskal-Wallis test and one-way analysis of variance (ANOVA) with multiple comparison tests, and neutralizing antibody and HMGB1 serum data (III) with two-tailed Student's t-tests. Correlations of serum HMGB1 with T-cell responses or survival were assessed by two-tailed Fisher's exact test in study III, where only nine cases were evaluable. In contrast in study IV, correlations of baseline HMGB1 with survival or imaging responses were first studied by Log-Rank or χ^2 tests, respectively, and then verified in separate multivariate analyses (Cox proportional hazards and logistic regression models, respectively) using SPSS v.21.0 (SPSS, Chicago, IL). Assumption of proportional hazards for each factor (candidate confounding variables) was tested by evaluating parallelity of lines in log minus log survival plot, and for uncertain factors, by counting the Schoenfield's partial residuals and linearly regressing them against natural logarithm of survival time. To avoid overfitting, Cox and logistic regression models were also tested with fewer number of parameters, which rendered similar results. Predicted probabilities for each case were used to calculate a Receiver Operating Characteristic (ROC) and cross-validate the logistic regression model. Statistical analyses were performed using Microsoft Excel (Microsoft, Redmond, WA), GraphPad software (GraphPad Software Inc., La Jolla, CA) and SPSS software, all tests were two-sided, and values of $P < 0.05$ considered statistically significant.

3.7 Ethical considerations

Patient samples and radiological evaluations in ATAP, which were used in our clinical-epidemiological studies III and IV, were collected and performed for safety and response monitoring purposes. All patients gave written informed consent, which included permission for analysis of collected sample material and anonymous scientific publication of patient data. All clinical-epidemiological data and patient sample analyses in studies III and IV were approved by the local ethics committee (HUS 62/13/03/02/2013). Blood sample collection was intended for monitoring safety (e.g. liver transaminases) and treatment efficacy (e.g. tumor markers), causing additional but acceptable harm for patients. Ascites and pleural effusion samples were obtained during effusion removal in order to relieve patients' symptoms. Tumor biopsies (in the context of study II), which were designed for development of patient treatments, caused additional but acceptable harm to patients, and were conducted after written informed consents, and approved by the local ethics committee (Dnro 368/13/03/02/2009). Radiological imaging with CT and PET-CT were used for monitoring treatment efficacy, which caused additional but acceptable ionizing radiation exposure, whereas MRI scanning caused no biological harm (used always for pediatric patients). Samples were analyzed and data was processed anonymously and used for intended purposes only. For ethical aspects regarding animal experiments, see preclinical *in vivo* studies.

4. RESULTS AND DISCUSSION

4.1 Improving efficacy of radiotherapy by recombinant adenoviruses expressing radiosensitizing proteins

Radiotherapy is widely used and important treatment of prostate, breast, and head and neck cancer, among other types. However, high irradiation doses carry the risks for side-effects to normal tissues, and thus lower curative doses would be desirable. One promising multi-modal approach is adenoviral cancer gene therapy combined to radiotherapy. While radiosensitizing transgenes such as p53 have been successfully utilized in combination treatments using replication-deficient adenovirus vectors (Pan et al. 2009, Yang et al. 2010), preclinical evidence indicates that cancer cells can be sensitized to irradiation by serotype 5 adenovirus replication *per se* (Kim et al. 2009, Rajecki et al. 2009), although the mechanisms behind this combinatorial benefit remain unknown. Data from cellular studies suggests radiosensitizing potential for innate adenoviral gene products: early region 1B 55-kDa (E1B55K) protein, and early region 4 proteins 11-kDa (E4orf3) and 34-kDa (E4orf6). The normal function of these proteins is to maintain effective virus replication in the host cell by preventing cell cycle arrest, which is induced in response to the adenovirus genomes (viral double-stranded DNA is interpreted as DNA double-strand breaks [DSB]) (Lilley et al. 2007). Adenoviral proteins E4orf3 and E4orf6 interact directly with a MRN complex (Mre11, Rad50, and NBS1), which is crucial for DSB recognition (Williams et al. 2007), mislocalizing or targeting it for degradation (Stracker et al. 2002), while besides other functions E1B55K protein may enhance the activity of E4orf3 and E4orf6 in MRN complex inhibition (Lilley et al. 2007, Jayaram et al. 2008). To study these aspects and their applicability for cancer virotherapy, we assessed the radiosensitizing potential of replication-deficient adenoviruses expressing the proteins E4orf3, E4orf6, or E1B55K *in vitro* and *in vivo*, and revised the mechanisms behind the effect.

4.1.1 Virus characterization and *in vitro* efficacy combined with radiotherapy

Recombinant viruses rAdE4orf3, rAdE4orf6, and rAdE1B55K as well as wild-type control (Ad300wt) and replication-deficient control (Ad5[GL]) viruses transduced prostate cancer PC3-MM2 cells at over 90% rate (> 85% for rAdE1B55K) and showed high levels of transgene expression at 24 h post-infection (**Study I, Fig. 1**). Combination treatment with each recombinant adenovirus followed 24 h later by radiotherapy was able to increase cancer cell killing as compared to combination treatment with Ad5(GL) control virus or radiotherapy alone. rAdE1B55K mediated the effect only in prostate cancer DU-145 cells, while rAdE4orf3 and rAdE4orf6 viruses increased killing of prostate cancer DU-145 and PC3-MM2, and breast cancer M4A4-LM3 cells, and rAdE4orf6 of also head and neck cancer UT-SCC8 cells (**Study I, Fig. 2A**). Furthermore, when combined with radiotherapy, rAdE4orf6 virus was the most potent of recombinant adenoviruses in mediating cell killing at 10-fold lower titer (**Study I, Fig. 2B**), and decreasing colony formation of PC3-MM2 cells (**Study I, Fig. 4**). Since molecular level studies suggest that E4orf6 and E1B55K (Stracker et al. 2002, Schwartz et al. 2008), as well as E4orf3 and E1B55K (Leppard and Everett 1999), might work in complexes to inhibit DSB repair, we studied the co-infections with multiple recombinant viruses together with radiotherapy in prostate, breast and head and neck cancer cells. Surprisingly, double or triple co-infections failed to improve cancer cell killing over the most effective virus component

alone in any tested cell line (PC3-MM2 shown in **Study I, Fig. 3**; M4A4-LM3 and UT-SCC8 not shown), suggesting that either E4orf6 or E4orf3 is sufficient in sensitizing a cancer cell to irradiation, and that E1B55K is not required for the synergistic effect. Of note, wild-type control virus Ad300wt, exhibiting oncolysis and expression of each studied protein (from inherent serotype 5 genes) mediated superior cell killing in all *in vitro* experiments (**Study I, Figs. 1, 2, and 4**).

4.1.2 Improved *in vivo* efficacy by combination therapy

Efficacy of recombinant and control viruses together with or without fractionated radiotherapy was assessed in an aggressive model of prostate cancer PC3-MM2 subcutaneous xenografts in Nude mice. When combined with radiation, rAdE4orf3 was the most effective recombinant adenovirus *in vivo*, by virtually halting the tumor growth at the level of 400% from original and significantly inhibiting it as compared to Ad5(GL) control virus with radiation ($P < 0.01$; **Study I, Fig. 5**). Similarly, rAdE4orf6 mediated significant tumor growth inhibition in combination with radiotherapy ($P < 0.05$, as compared to Ad5[GL] + radiation), whereas, consistent with our *in vitro* results, rAdE1B55K together with radiation failed to inhibit tumor growth. Also in line with *in vitro* data, the replicative control virus Ad300wt presented superior inhibition and halting of tumor growth when combined with radiotherapy ($P < 0.001$ as compared to Ad5[GL] + radiation), but not alone (data not shown). It should be noted that human wild-type adenovirus does not replicate in mouse cells, which renders the use of Ad300wt in our experimental setting as an "oncolytic" control virus, since only PC3-MM2 xenografts are of human origin allowing replication. Interestingly, if compared to Ad5(GL) alone or mock-treated animals, both of which developed rapidly growing tumors and had to be sacrificed by day 12, recombinant viruses showed some antitumor efficacy in the absence of radiotherapy (**Study I, Fig. 5**), which has been indicated by *in vitro* results of us and others (Brand et al. 1999).

4.1.3 Persistent DNA damage in cancer cells after combination therapy

Mechanisms behind radiosensitizing effects of recombinant and control adenoviruses were assessed in PC3-MM2 cells by studying the persistence of DSBs after irradiation. Immunofluorescence and Western blot against DSB-binding histone protein phospho-H2AX (gamma-form) was used to visualize DSBs at 30 min and 24 h after irradiation (**Study I, Fig. 6**). As expected from earlier reports indicating DSB repair inhibition as the main mechanism behind radiosensitizing effects of replicative adenoviruses (Stracker et al. 2002, Rajecki et al. 2009), wild-type control virus Ad300wt infection prior to radiotherapy showed strong persistence of DSBs at 24 h post-irradiation in both immunofluorescence and Western blot. Similarly, both rAdE4orf6 and rAdE4orf3 virus treatment prior to radiotherapy lead to inhibition of DSB repair as indicated by persistent γH2AX foci (in 40-50% of cells) and high protein levels still at 24 h. In contrast, rAdE1B55K treatment caused weak DSB repair inhibition since only ca. 25% of cells showed γH2AX foci at 24 h post-irradiation. As expected, the replication-deficient control virus Ad5(GL) prior to radiotherapy or radiotherapy alone, although inducing DSBs immediately after irradiation (at 30 min timepoint), failed to inhibit DSB repair as seen at 24 h timepoint when the majority of the DNA breaks were repaired.

In summary, rAdE4orf6 and rAdE4orf3 viruses mediated strong inhibition of DSB repair, leading to radiosensitation and increased cancer cell killing *in vitro* and *in vivo* when combined with radiotherapy. Our results suggest that either E4orf6 or E4orf3 are sufficient in enhancing cancer cell killing by radiotherapy, whereas E1B55K does not significantly contribute to the effect. If comparing their relative radiosensitizing potentials, E4orf6 proved more effective *in vitro*, while E4orf3 seemed to have an upper hand *in vivo*, suggesting a more complex scenario in real therapy setting. Nevertheless, as indicated by the superior potency of the replicative "oncolytic" control virus Ad300wt, effective oncolysis combined to radiotherapy leads to improved antitumor efficacy, which cannot be achieved by gene transfer and DSB repair inhibition alone. This intrinsic ability of adenoviruses to sensitize cells to irradiation could be harnessed against cancer cells by selective targeting.

Since the early phase of adenoviral cycle takes usually 6 – 8 hours, during which also DSB-repair inhibiting E4orf-proteins are expressed, it would be interesting to assess whether radiosensitation occurs optimally at this time. Nevertheless, for full combinatorial effects completion of oncolytic virus replication cycle would be desirable as well. On the other hand, also irradiated cells could be infected, followed by adenoviral gene expression and DSB repair inhibition, and as demonstrated by Rajecki *et al.* a schedule where radiotherapy was given 24 h prior to infection, provided optimal synergistic efficacy in their study (Rajecki et al. 2009).

In addition to DSB repair inhibition, radiation-induced DSB damage mediates also other beneficial effects with regards to oncolytic immunotherapy: Nokisalmi *et al.* showed in several cell lines and using different adenoviruses that ionizing radiation induces increased production of mRNA and proteins, including adenoviral transgene products (Nokisalmi et al. 2012). This phenomenon may have accounted for improved efficacy in study I as well, although it remains controversial since Nokisalmi *et al.* also administered adenoviral gene therapy 24 h after irradiation. Recent evidence in radiobiology has proposed that ionizing radiation promotes immunogenic type of cell death (Apetoh et al. 2007, Golden et al. 2012). Moreover, autophagic cell death was observed as the primary mechanism underlying the synergy between radiotherapy and oncolytic adenovirus (Rajecki et al. 2009). Promotion of alternative cell death pathways that are immunogenic, by using the combination of oncolytic adenovirus and radiotherapy, may therefore further increase efficacy. This could lead to antitumor immune activations in an immunocompetent host: It is intriguing to speculate that such combinations could provide enough danger-signals and antigen-spreading together with direct oncolysis to skew the immunosuppressive environment of human tumors, and mount tumor-specific immune responses. To this end, a multimodal study addressing the combination of adenoviral gene therapy, radiotherapy, and temozolomide (TMZ) in the treatment of glioma *in vivo*, demonstrated that such antitumor immune responses are achievable, and are dependent on HMGB1 release, which is a key DAMP molecule released in immunogenic cell death (Curtin et al. 2009).

Emerging evidence thus suggests that combination benefits are mediated, at least, by intrinsic adenoviral proteins E4orf6 and E4orf3, increased transgene expression, and enhanced immunogenicity. As the study I and previous data demonstrate that replicative adenoviruses mediate superior radiosensitizing effects *in vivo* [Ad300wt in study I; (Rajecki et al. 2009)], an oncolytic adenovirus armed with either E4orf6 or E4orf3 as a transgene and delivered 24 h after radiotherapy, might mediate further enhanced antitumor efficacy. Alternatively, given the immunogenicity of cell death, an oncolytic adenovirus with intact intrinsic E4orf-genes armed with an immunostimulatory transgene, such as GMCSF, that would be administered 24 h after

radiation, could increase both cytotoxic synergy and adaptive antitumor immune responses. The combination treatment with radiotherapy and oncolytic immunotherapy is therefore a promising way to minimize the curative irradiation dose, consequently reducing the harmful side-effects, while increasing therapeutic efficacy and activating immune system to detect the cancer.

4.2 Upregulation of interferon signaling mediates acquired tumor resistance to oncolytic adenovirus in vivo

Preclinical and clinical data suggests that initially sensitive tumors can become resistant to virotherapy (Strauss et al. 2009, Koski et al. 2010, Kanerva et al. 2013), which is not surprising given the tremendous transforming capacity of advanced tumors. With regards to resistance to chemotherapy and targeted therapies, many resistance mechanisms have been identified, which has allowed development of countermeasures (Ross et al. 2009, Cathcart et al. 2012). For oncolytic adenoviruses, however, such data has been missing. Several mechanisms behind anti-adenoviral innate immunity have been identified in normal cells (Sung et al. 2001, Nociari et al. 2007, Zhu et al. 2007), while with regards to cancer, two human pancreatic cancer cell lines were found to entail different permissivity to adenoviral vectors and characterized by differential interferon gene signature and myxovirus resistance protein A (MxA) expression (Monsurro et al. 2010). The latter *in vitro* studies did not, however, assess the causality between actions of the virus and the upregulation of interferon pathways. In an attempt to identify underlying mechanisms and gain insights into developing countermeasures, we studied acquired tumor resistance against oncolytic adenovirus Ad5/3-Δ24. We developed two orthotopic mouse models of acquired resistance using ovarian carcinoma SKOV3.ip1 and SKOV3Luc xenografts, where initially responding tumors gain resistance and relapse, and used them to investigate the phenomenon on mRNA, protein and tissue levels.

4.2.1 Animal model of acquired resistance and characterization of virus-resistant phenotype

We showed in mice that intraperitoneal ovarian carcinoma SKOV3.ip1 tumors treated with single intraperitoneal injection of oncolytic virus Ad5/3-Δ24 are initially inhibited, but eventually relapse after a prolonged disease-free period of 120 days (**Study II, Fig. 1A**). The relapsed tumors were surgically removed, virus was extracted and used for replication assays, that revealed presence of functional virus (**Study II, Fig. 1B**). As comparison, also virus from naïve responding SKOV3.ip1 tumors collected at day 4 post-infection was extracted and similarly assessed for functional tittering: Interestingly, the recurring 120-day old tumors showed even higher amounts of functional replicative virus, which had apparently been rendered ineffective by relapsing tumors. Next, we performed microarray analysis of five relapsed and five untreated tumors: More than hundred genes were found downregulated in the adenovirus-resistant tumors, whereas only a handful were significantly upregulated (**Study II, Table 1 and Suppl. Table S1**). Expression results regarding some of the key genes were further confirmed by semi and fully quantitative PCR (**Study II, Fig. 3A and Suppl. Fig. S2**). Two separate *in silico* analyses were performed on microarray data to investigate the gene expression signature: Moksiskaan analysis indicated activation of innate immunity in response to virus (**Study II, Suppl. Fig. S1 and Suppl. Table S2**), whereas Ingenuity Pathway analysis revealed upregulation of interferon signaling related genes in relapsed tumors (**Study II, Fig. 2**), suggesting tolerance on tumor microenvironment level.

4.2.2 Identification of potential therapeutic targets and a virus-resistance marker

In addition to studying connections between differentially expressed genes, we used Moksiskaan analysis to relate them to existing drugs, diseases and known cancer mutations. This approach was aimed at finding novel druggable targets that could be utilized during oncolytic virotherapy as virus-sensitizers, or alternatively, as therapeutic targets after the emergence of virus resistance. Interestingly, we identified a downregulated gene in virus-resistant cells that might be an upstream molecule accounting for the resistant phenotype, which had existing agonists: nuclear receptor subfamily 3 group C member 2 (NR3C2) could be stimulated by desoxycorticosterone acetate, desoxycorticosterone pivalate, and desoxycortone (mineralocorticoids without glucocorticoid activity) (**Study II, Suppl. Fig. S1c and Suppl. Table S1**). This group of drugs are used, together with glucocorticoids, for replacement therapy of adrenocortical insufficiency and Addison's disease, and for treatment of salt-losing adrenogenital syndrome. Importantly, these drugs lack glucocorticosteroid activity that would be highly immunosuppressive and unfavourable for immunotherapy. We did not find any existing inhibitors for the upregulated genes, however, we identified several upregulated genes that code for membrane proteins (**Study II, Table 2 and Suppl. Data**), which could be useful targets for development of new drugs, i.e. monoclonal antibodies or small molecular inhibitors, for combination therapy with oncolytic adenoviruses.

Myxovirus resistance protein A (MxA), a key downstream protein acting at an intersection of all interferon signaling pathways, was identified as one of the most significantly upregulated genes in virus-resistant tumors. Thus in line with previous studies, MxA seems to reflect the virus-resistant phenotype (Monsurro et al. 2010), and we hypothesized we could utilize it as a resistance biomarker. Unlike the *in vitro* results on the virus-resistant, MxA-positive pancreatic carcinoma cell lines by Monsurro et al. suggested, we found very low levels (5%) of *in vitro* MxA positivity in ovarian carcinoma SKOV3.ip1 cells, indicating virus permissivity. Indeed, after initially responding to virotherapy (**Study II, Fig. 1**), the relapsed tumors containing ineffective virus showed very high levels of MxA staining, while nontreated tumors retained the MxA-low phenotype (**Study II, Fig. 3B-C**). Thus, we presume that killing of MxA-negative (virus-permissive) cells would result in selection of MxA-positive cells that would re-grow, eventually resulting in tumor relapse (**Study II, Fig. 4**). Alternatively, although not mutually exclusively, adenovirus treatment might also induce resistance and MxA-upregulation in initially sensitive cells which then cause relapse. Corroborating with the latter scenario, we showed *in vitro* that MxA expression can be induced in SKOV3.ip1 cells by infection with oncolytic adenovirus (**Study II, Fig. 3D and Suppl. Fig. S3**).

Interestingly, an important pathogen recognition receptor, TLR-2 expression was found upregulated in the virus-resistant tumors (**Study II, Table 2**). Since there is evidence that majority of adenovirus-mediated immune responses are dependent on TLR-2 and 9 activation (Appledorn et al. 2008), it is possible that virus-resistant tumors feature constitutive activation of toll-like receptor signaling. TLR-2 mediates mainly interleukin production from immune cells, which is the other main arm of innate immunity. Thus, the upregulation of type I IFN and toll-like receptor signaling seem to complement the antiviral phenotype, although further experiments are needed to assess the role of TLRs. For example, it would be interesting to study if TLR-2 stimulation, similar to the observed IFN-α-receptor stimulation, would lead to MxA upregulation.

It is noteworthy that also favourable consequences of immunogenic cell death (see below) leading to activation of antitumor immunity may be connected to the antiviral innate immune responses triggered by oncolytic adenoviruses. For instance, an endogenous DAMP molecule HMGB1 has been shown to mediate antitumor immune responses in a glioma model exactly *via* TLR-2 on dendritic cells (Curtin et al. 2009). It is possible that TLR-2 upregulation observed in the virus-resistant tumors could also reflect dendritic cell mediated DAMP signalling that is however attenuated in the SCID mice lacking effector T cells. Furthermore, an alkylating chemotherapeutic cyclophosphamide (CP), which is commonly used as an adjuvant drug, poses another example of potential benefits of the innate immune response: Standard dose chemotherapy with CP exerts activation of endogenous type I IFN response that can lead to increased dendritic cell activation, cross-presentation and antitumor efficacy (Schiavoni et al. 2011). Interestingly, these beneficial effects were synergized by co-administration of mouse type I IFNs. Nevertheless, low-dose CP mediates rather opposite immunological effects, and has not been linked to type I IFN response (Sistigu et al. 2011). Since low-dose chemotherapy has several favourable immunological effects over high-dose in the adjuvant setting with immunotherapy (Loven et al. 2013), current knowledge would not support promotion of type I IFN signalling, especially in the context of oncolytic adenovirus where antiviral innate immunity may dampen the oncolytic effect. Nonetheless, dual role of innate immunity should be considered when designing methods to counteract the antiviral resistance phenotype. For instance, it may be feasible to allow some degree of initial type I IFN response after oncolytic immunotherapy, which associates with danger-signaling and immune cell activations at the tumor site, and only later apply drugs that counteract emerging anti-viral state.

4.2.3 Role of tumor stroma in maintaining resistance

After showing that MxA expression can be induced in SKOV3.ip1 cells by virus infection *per se*, we evaluated whether it can be further boosted by external interferon stimulus. To simulate the effect of stromal cells, which are the major source of type I interferons in tumors, we pre-treated the SKOV3.ip1 cells with recombinant universal type I interferon-α: Virus infection after IFN-α treatment resulted in further upregulation of MxA expression at 1 h after infection (**Study II, Fig. 3D and Suppl. Fig. S3**). Next we evaluated the impact of tumor stroma, and confirmed the emergence of virus-resistance, in another orthotopic ovarian cancer mouse model using SKOV3-Luc cells. These cells allowed us to monitor growth of the intraperitoneal tumors by bioluminescence *in vivo* imaging. After three intraperitoneal injections of oncolytic adenovirus Ad5/3-Δ24, tumors initially responded to treatment, but relapsed after two weeks suggesting acquired virus resistance (**Study II, Fig. 5A**). When tumors reached considerable size, mice were killed and both the untreated and the relapsed tumors were surgically removed and freshly transplanted, together with tumor stroma, into new SCID mice in laparotomy. Concurrently, two new groups of mice with naïve intraperitoneal SKOV3-Luc tumors were established. Then the mice were treated as previously, and tumor growth was again monitored: As expected, the naïve tumors initially responded to treatment followed by relapse. Of the surgically transplanted tumors, the previously untreated tumors responded to the virus treatment, whereas the previously virus-treated tumors could not be inhibited ($P < 0.001$) (**Study II, Fig. 5B**). Importantly, replication assays demonstrated again the presence of functional virus in the resistant and relapsed tumors (average 1.0×10^8 pfu/g, not shown). Hence, the transplanted tumors retained the resistant phenotype when transferred together with tumor stroma into new mice. Interestingly, cancer cells derived from ascites of the untreated mice were able to grow and form colonies on cell culture plates, whereas cancer ascites cells from the relapsing virus-treated mice were instantly killed *ex vivo*

(data not shown). This further indicates the role for tumor stroma in maintaining resistance, because the relapsed cancer cells had become depend on their tumor microenvironment: While the whole-tumor transplantation was successful in naïve mice, single cell cultures failed to grow, probably due to retained susceptibility to latent virus or stress signals.

In summary, we have identified an innate resistance mechanism that tumors can acquire to protect against oncolytic adenoviruses. Our data indicates that functional adenovirus is rendered ineffective by acquired resistance of ovarian tumors, which is characterized by upregulation of interferon signalling, as indicated by MxA protein expression, and involve crucial interactions with tumor stroma. Corroborating with our results, breast cancer initiating /stem cells have been found sensitive to oncolytic adenovirus due to their impaired toll-like receptor signalling and dysfunctional type I IFN response (Ahtiainen et al. 2010). Our microarray data and *in silico* analyses may help in developing countermeasures to reverse/counteract the virus-resistant phenotype, while MxA protein could be utilized as a simple biomarker correlating with the resistant phenotype (see below for preliminary translation into human data).

Based on our results in study II indicating that acquired resistance against adenovirus in relapsed tumors is mediated by upregulation of type I interferon signalling, we have started to study chemical compounds that could reverse the antiviral phenotype. While our results are pending at this point, we have for instance tested a small-molecule inhibitor, termed virus-sensitizers 1 (VSe1), which was identified as a potent enhancer of oncolytic vesicular stomatitis virus (VSV) mediated cancer cell killing *in vitro* and *in vivo* (Diallo et al. 2010). VSe1 has a narrow histone-deacetylase inhibitor-like activity on gene expression representing closely to our adenovirus-resistant SKOV3.ip1 gene expression signature, rendering it attractive to test in our models. Intriguingly, the “pharmacoviral” approach using small-molecular inhibitor VSe1 was shown to selectively enhance virus growth in tumor cells but not in normal tissues (Diallo et al. 2010). This type of potential safety issues are important to assess carefully given the central role of IFN responses for normal antiviral immunity as well.

4.2.4 Translational data on identified virus-resistance marker MxA in cancer patients

MxA protein expression was found a potentially useful marker correlating with the adenovirus-resistant phenotype *in vivo*. To date, no direct evidence of acquired resistance against oncolytic adenovirus in human tumors exists, and also utility of antiviral resistance markers remains unknown. Therefore translation into patients would serve as valuable evidence and starting point for developing countermeasures and perhaps selecting patients for oncolytic adenoviral therapy. In a preliminary attempt to assess this, we received pre-treatment tumor biopsies from 15 patients with refractory solid tumors, who were thereafter treated with oncolytic adenoviruses in the context of the ATAP. Biopsies were taken shortly before the first oncolytic adenovirus treatment. In addition, two of these patients had available post-treatment tumor biopsies, which were used to study the acquired resistance against oncolytic adenovirus. We performed immunohistochemistry with MxA antibody, and correlated tumor staining scores with possible signs of treatment efficacy, in order to assess if MxA phenotype correlates with therapy outcome.

We found that most of the 15 pre-treatment biopsies had missed the tumor tissue or proved otherwise unrepresentative for MxA assessment (e.g. necrotic tissue). As a result only nine biopsies were evaluable for scoring of MxA positivity and localization: four were scored as 3+,

other four as 2+, and one case as 1+. Patients with the highest MxA score (3+) showed a median overall survival of only 53 days, patients with MxA score 2+ had median survival of 204 days, and the one patient with MxA score 1+ showed survival of 123 days. When MxA scores 1+ and 2+ were grouped together, there was a trend for increased overall survival over the highest MxA score 3+ patients (P = 0.107, Log-Rank test). Since overall survival is an endpoint affect by multiple prognostic factors, we next assessed imaging and marker responses as possible signs of treatment benefit. However, only three cases were evaluable by these parameters. One metastatic breast cancer patient with cytoplasmic MxA staining scored as 2+ showed progressive disease in both post-treatment PET imaging and tumor markers. The other two interesting cases are discussed next in more detail.

For the other two patient cases MxA immunohistochemistry could be applied on both pre- and post-treatment tumor biopsies. Moreover, facilitating comparison between these cases, both patients had ovarian carcinoma tumors progressing at baseline, both received their first oncolytic adenovirus treatment on the same week with Ad5/3-Δ24-GMCSF virus together with metronomic cyclophosphamide as a serial treatment, and were imaged and biopsied again either on day 76 or 78 post-treatment. Patient n:o 1 showed sustained intense nuclear MxA staining 2+, while presenting much less cytoplasmic MxA staining, in all biopsies (**Figure 6A**). Interestingly, this patient seemed to benefit from oncolytic adenovirus treatment, showing a minor metabolic response in PET imaging (-13.6% decrease), stabilization of Ca12-5 tumor markers, and improvement in symptoms. She had an overall survival of 336 days. On the contrary, the patient n:o 2 showed intense membranous (perinuclear) and cytoplasmic MxA staining 3+ in the pre-treatment biopsy, and strong membranous/cytoplasmic MxA 3+ but progressive loss of nuclear staining in the post-treatment biopsy (**Figure 6B**). This patient showed a stable disease in PET imaging (+2.4% increase), but a progressive disease in Ca12-5 tumor markers accompanied with worsened symptoms, and had an overall survival of 101 days.

Taken together, while based on only three case reports, there is room for speculation that membranous/cytoplasmic as opposed to nuclear MxA localization might indicate antiviral phenotype and poor therapy responsiveness. Especially the pattern of progressive loss of nuclear MxA and accumulation of cytoplasmic MxA, in the patient with relatively worse outcome is noteworthy (**Figure 6B**). Indeed, human MxA protein has been reported to mediate antiviral activities mainly in the cytoplasm, endoplasmic reticulum and perinuclear region, although its specific role in adenovirus infection has remained unknown (Haller et al. 2007, Wisskirchen et al. 2011). Interestingly, as seen in **Fig. 3C of study II**, an almost identical cytoplasmic/perinuclear staining pattern that lacks nuclear MxA, can be observed in the virus-resistant recurrent ovarian carcinoma SKOV3.ip1 xenografts, as compared to the potentially virus-resistant patient biopsy (**Figure 6B**). The reason why MxA was highly expressed already in the pre-treatment biopsies is unknown. One explanation could be wild-type adenovirus infections during the course of tumor evolution, because tumor lesions can initially present fertile ground for replication but thereafter acquire resistance. Alternatively, MxA could be involved in other unknown tumor-promoting or resistance mechanisms as well.

We set to study whether MxA positive or negative phenotype in patient tumors would correlate with signs of treatment efficacy, or lack thereof, following oncolytic virotherapy. Our preliminary analysis was hampered by several unrepresentative biopsies and lack of assessed endpoint parameters. Nevertheless, case reports of strong 3+ and cytoplasmic MxA expression in tumors were potentially associated with antiviral resistance and weaker efficacy, while nuclear MxA

staining 2+ linked to a patient case with promising signs of efficacy. Accordingly, patients with lower MxA staining scores trended for longer overall survival, although based on only nine cases. Therefore, corroborated by our preclinical data, these results of MxA as a candidate marker for adenovirus-resistance hint towards possible translational relevance, although further studies are needed to draw any conclusions.

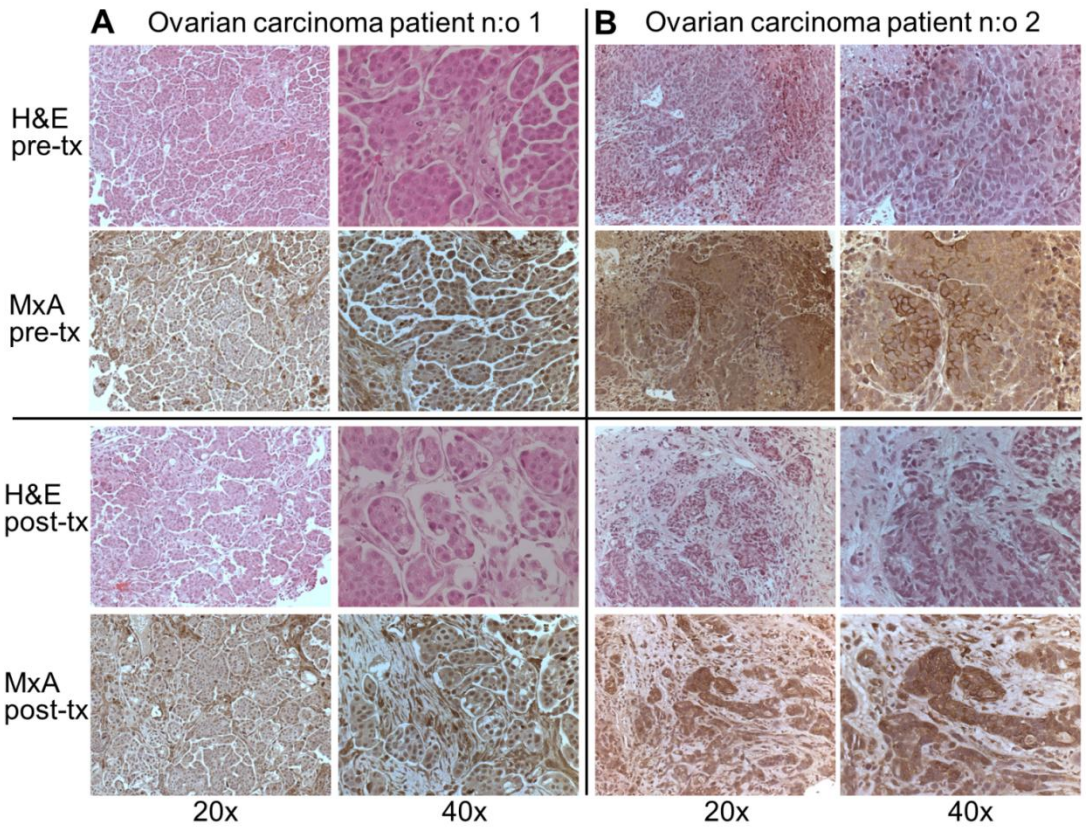

Figure 6. MxA immunohistochemistry on pre- and post-treatment tumor biopsies in two ovarian carcinoma patients. Two ovarian carcinoma patients were treated during the same time period with three consecutive intratumoral injections of oncolytic adenovirus Ad5/3-Δ24-GMCSF (a serial treatment) together with metronomic cyclophosphamide. Tumor biopsies were taken before, and 76 or 78 days after the first treatment of the series, in **A** and **B**, respectively. **A**) Patient n:o 1 featured intense MxA staining in nuclei, scored as 2+, in both pre- and post-treatment biopsies. In addition, a third biopsy taken on day 98 after the first treatment showed similar nuclear staining pattern (not shown). Objective signs of possible benefit were observed for patient n:o 1: radiological PET evaluation showed minor response (MR) in total metabolic activity, tumor markers were stabilized, and symptoms were improved. **B**) Patient n:o 2 presented strong membranous, perinuclear MxA staining, scored as 3+, in both biopsies, but lacked nuclear staining especially in the post-treatment biopsy. Patient n:o 2 showed a stable disease in PET evaluation, but a progressive disease in Ca12-5 tumor markers and worsened symptoms. Hematoxylin eosin stainings were used as technical controls and for interpretation of cell and tissue morphology. Slides were scored by an independent pathologist. H&E, hematoxylin eosin staining; MxA, myxovirus resistance protein A; tx, treatment (Liikanen et al., unpublished data).

4.3 Combining oncolytic immunotherapy with low-dose temozolomide and low-dose cyclophosphamide preclinically and in cancer patients

Adenoviruses induce autophagic cancer cell death, which has been associated with increased oncolysis and virus replication (Ito et al. 2006, Jiang et al. 2011, Rodriguez-Rocha et al. 2011), and preclinical data suggests that autophagy-inducing therapies might enhance the efficacy of oncolytic adenoviruses (Rajecki et al. 2009, Tyler et al. 2009). While baseline autophagy is essentially considered as a survival process, mortal autophagic flux, characterized by increased turnover of cellular organelles leading to cell death, can be exploited in cancer therapy (Chen and Karantza 2011). Moreover, autophagy has recently been suggested a prerequisite for immunogenic cell death (ICD), a phenomenon useful or even necessary for induction of antitumor immunity (Hannani et al. 2011, Michaud et al. 2011, Martins et al. 2012). ICD is characterized by exposure of calreticulin on cell surface, and release of adenosine triphosphate (ATP) and a nuclear protein high-mobility group box 1 (HMGB1) to the extracellular space. These danger signals are recognized by dendritic cells, which respond by secreting interleukin-1b for the activation of cytotoxic T-cells.

We studied therefore the combination of oncolytic adenovirus together with an autophagy-inducing, alkyting chemotherapeutic temozolomide (TMZ). As a chemotherapeutic drug, TMZ is used in the treatment of e.g. melanoma, pituitary cancer, and various gliomas, and its autophagy-inducing capacities have been regarded beneficial in the context of combination therapies (Gao et al. 2009, Palumbo et al. 2012). We hypothesized that combination of oncolytic adenovirus with low-dose TMZ could enhance efficacy *via* increased tumor autophagy and immunogenic cell death, leading to subsequent induction of antitumor immune responses. Since, tumors can evade antitumor T-cell responses at later stages by recruitment of immunosuppressive regulatory T-cells, we included low-dose cyclophosphamide in the treatments, which specifically decreases regulatory T-cells without compromising induction of antitumor immunity (Ghiringhelli et al. 2004, Ghiringhelli et al. 2007, Cerullo et al. 2011). We investigated the combinatorial effects of oncolytic adenovirus, low-dose temozolomide and low-dose cyclophosphamide preclinically, and the report, for the first time, safety, efficacy and immunological effects of the combination therapy in 17 patients with metastatic solid tumors refractory to conventional treatments.

4.3.1 Preclinical efficacy, autophagy induction and immunogenicity

We assessed cytotoxicity of the combination therapy in prostate PC3-MM2 and breast MDA-MB-436 cancer cells, by using an active metabolite of the prodrug cyclophosphamide (CP), i.e. 4-hydroperoxycyclophosphamide (4-HPCP), oncolytic adenovirus Ad5/3-Δ24-GMCSF, and TMZ *in vitro*: the triple combination as well as double combinations with either chemotherapeutic alone increased cell killing over oncolytic virus or chemotherapeutic agents alone (**Study III, Fig. 1A and Suppl. Fig. S2**). When drug combinations in PC3-MM2 cells were assessed for synergy using the median effect analysis developed by Chou and Talalay (Chou 2010), the triple combination showed synergism at the most relevant high fraction affected levels (**Study III, Suppl. Fig. S2**). Synergistic effect was the most pronounced with the combination of virus and TMZ, whereas virus with 4HPCP showed only slight synergism or an additive effect, as expected for its purpose as an

immunomodulating agent mediated only *in vivo* (Cerullo et al. 2011). Next, we studied the ability of the combination therapy to induce immunogenic cell death. Treatment of PC3-MM2 cells with virus, TMZ, and 4-HPCP resulted in significant increase of all mediators of ICD, namely calreticulin-positive cells, release of ATP, and secretion of HMGB1, when compared with control cells (**Study III, Fig. 1B**).

To assess the *in vivo* efficacy, subcutaneous prostate cancer PC3-MM2 xenograft bearing mice were treated with Ad5/3-Δ24-GMCSF or growth medium twice intratumorally, followed by concomitant treatment with TMZ (10 mg/kg) or saline, and CP (20 mg/kg) or saline intraperitoneally. Subtherapeutic doses of chemotherapeutic drugs were used as to mimic low-dose administration, which thus showed no efficacy alone (**Study III, Fig. 1C**). However, oncolytic virus combined to TMZ showed enhanced tumor growth inhibition ($P < 0.05$), and the effect was further enhanced when CP was added to the regimen ($P < 0.01$, both over control). We performed electron microscopy and immunohistochemistry on PC3-MM2 tumors to study autophagy induction, which has previously been reported as a key cell death mechanism in glioma virotherapy *in vivo* (Tyler et al. 2009). Electron microscopy revealed autophagic vacuoles in combination- and virus-treated tumor cells, together with evidence of progressive autophagic flux and virus progeny inside some of the combination treated dying tumor cells (**Study III, Fig. 1D**). To determine the extent of autophagy, we further performed immunohistochemical staining for LC3 protein isoform B (LC3B), a widely used marker of autophagy due to its involvement in the formation of autophagic vacuoles. Combination treated tumors presented a significantly higher frequency of LC3B punctate-positive cells as compared to control tumors (**Study III, Fig. 1D and Suppl. Fig. S3**).

4.3.2 Safety and biological virus activity in patients

We studied safety, efficacy and immunological data of 17 patients with metastatic solid tumors progressing after conventional therapies, who were treated with the combination therapy (1–6 treatment cycles) in the context of the ATAP. Patients had a WHO performance score ≤ 3 at baseline, and were heavily pre-treated with a median of 2 previous chemotherapy regimens and 1 surgery (**Study III, Suppl. Tables S1 and S2**). Combination treatments were sub-grouped according to administration of low-dose pulse of TMZ: Group 1 received TMZ (100 mg/day) for 5 days before virus treatment, group 2 for 5–7 days before and 2 weeks after the virus, and group 3 for 7–10 days following virus treatment. All but two patients received also low-dose CP, either concomitantly *per os* (50 mg/day) or as an intravenous infusion on the day of virus treatment (1,000 mg), as reported (Cerullo et al. 2011). Chemotherapeutic doses were adjusted for the two pediatric patients. Treatments appeared well-tolerated with mostly grade 1–2 adverse reactions (flu-like symptoms, fever, fatigue, nausea and pain), and no grade 4–5 clinical ARs. Laboratory ARs included grade 1–2 transient hemoglobin decreases, liver transaminase increases, and thrombocytopenia (**Study III, Table 1**). In addition, transient lymphopenia was commonly observed (88% of treatments), and accounted for majority of the grade 3, and the only grade 4 laboratory AR, that were observed (**Table 4**). In fact, accumulating evidence indicates that transient lymphocytopenia seen after virus treatment is likely to reflect redistribution of lymphocyte subsets from blood to the target tissues of infection, i.e. tumors, as suggested for potent immunotherapeutics and viruses (Reid et al. 2002b, Brahmer et al. 2010, Kanerva et al. 2013, Hemminki and Hemminki 2014). Moreover, observed transient lymphopenia did not appear to translate into clinical symptoms: First of all, none of the patients were reported to suffer from

clinical infections as AR. Second, as very expected, fever was observed after majority of the virus treatments (75,6%), but this did not correlate with lymphocytopenia, as both patient groups had fever: 75% (27/36) of patients with lymphopenia, and 80% (4/5) without lymphopenia. Transient lymphopenia after oncolytic immunotherapy may not therefore be an actual "adverse" reaction but rather a phenomenon contributing to efficacy, which was regarded later in study IV (see below).

When comparing to patient treatments given in the same therapy program but using oncolytic adenoviruses and low-dose CP (Cerullo et al. 2011), ARs did not appear increased by the addition of low-dose pulse of TMZ overall, although grade 2 nausea seemed slightly more common, as expected for TMZ therapy (22% *vs.* 11%, not significant). Interestingly, when compared between the TMZ administration subgroups, group 3 receiving TMZ for 7–10 days after the virus treatment seemed to exhibit less laboratory ARs, especially liver transaminase increases, than group 1 receiving TMZ for 5 days before the virus (grade 1 and 2 lab ARs recorded in 94 and 83% of treatments in group 1, and in 75 and 50% in group 3, respectively [$P < 0.05$]). One potentially treatment-related adverse reaction, grade 3 ileus in a cholangiocarcinoma patient, led to patient hospitalization and was therefore classified as a serious adverse event, while the other two clinical grade 3 ARs were alleviated by blood transfusions and antibiotics as outpatients.

To study biological viral activity in patients, we assessed inflammatory cytokines, neutralizing antibodies, and adenovirus genomes in blood (as a surrogate of virus replication). Patient serum titers for pro-inflammatory interleukin (IL)-6, and anti-inflammatory IL-10 showed transient increases at day 1 ($P < 0.05$ and $P < 0.0001$, respectively). In line with the differences seen in AR profiles between the TMZ administration subgroups, the most pronounced inflammatory cytokine changes were observed in patients receiving TMZ before the virus (group 1; **Study III, Suppl. Fig. S4**). Injected virus is rapidly cleared from the blood stream, and therefore extended presence of circulating specific adenovirus genomes is indicative of virus replication (Galanis et al. 2005). We observed prolonged (≥ day 3 up to day 74 post-treatment) circulating virus genomes in blood after 14 out of 30 evaluable treatments, and after 28 out of 38 treatments overall (when including earlier time-points; **Study III, Suppl. Table S3**). Circulating neutralizing antibody titers against specific adenovirus-capsid antigens showed gradual elevations during the first 1–5 weeks post-treatment (**Study III, Suppl. Table S4**). As expected, patients receiving their first oncolytic adenovirus treatment showed significantly lower titers at baseline and at 1 week post-treatment as compared to subsequent treatment cycles ($P < 0.05$, both time-points), followed by elevations to the overall median. In summary, treatments were well-tolerated and the addition of low-dose pulse of TMZ did not seem to essentially alter the safety profile. Since there were no differences between the TMZ administration subgroups in circulating adenovirus genomes or neutralizing antibody titers but the cytokine response and laboratory adverse reactions appeared more pronounced in patients receiving TMZ before the virus (group 1), our biological and safety data supports the administration of temozolomide after the virus treatment.

4.3.3 Evidence of autophagy, immunogenicity and immune responses in patients

We obtained pre- and post-treatment ascites tumor cell samples from two combination-treated patients and assessed them for LC3B-immunohistochemistry to study autophagy induction. In both cases, the assay revealed clear increase in LC3B punctate-positive tumor cells in one week post-treatment samples (**Study III, Fig. 2D**). To our knowledge, this is the first evidence of autophagy

induction in patients treated with oncolytic viruses. We also assessed the immunogenicity of patient treatments by measuring serum HMGB1 titers and antitumor T-cell responses in blood. Induction of tumor-specific T-cells in blood after combination treatment was seen in 8 out of 15 evaluable patients (**Study III, Fig. 3A**). Intriguingly, the immunogenic HMGB1 protein levels in serum showed corresponding changes, and trended for correlation with the antitumor T-cell responses even in this small patient analysis (*P* = 0.0833; **Study III, Fig. 3B**). Thus, our immunological results suggested activation of antitumor immunity in majority of the combination treated patients, and provided data to postulate that serum HMGB1 might be a candidate predictive marker for antitumor immune responses after oncolytic immunotherapy.

Of note, additional immune cell subtype analyses would have been required to address the overall immunological effects of the combination therapy. Effector T-cells can be inactivated by immunosuppressive subtypes, such as regulatory T-cells and myeloid-derived suppressor cells (MDSCs) that hamper antitumor T-cell functions also at later stages (Lindau et al. 2013). Low-dose CP was included in the treatments, because it has been shown to selectively inhibit regulatory T-cells (Ghiringhelli et al. 2004, Lutsiak et al. 2005), and further to mediate similar effects in combination treatments with oncolytic adenoviruses when administered either metronomically or intravenously (Cerullo et al. 2011). In addition, emerging evidence suggests that low-dose CP can also induce immunogenic type of cell death *in vivo*, very similar to low-dose TMZ and oncolytic adenoviruses (Sistigu et al. 2011). Despite these favourable effects, low-dose CP might on the other hand promote production of chronic inflammatory mediators, as reported in a model of *ret* transgenic mice (Sevko et al. 2013). The authors found that low-dose CP treatment lead to accumulation of immunosuppressive MDSCs that coincided with decreased effector cell activity in both tumor-bearing and chronic inflammation models. Interestingly, low-dose CP induced higher production of several inflammatory mediators of Th2 type response in tumors, including GMCSF, IL-1β, IL-5, IL-10, IFN-γ, and TNF-α. In contrast, cytokine profile involved in innate immune response triggered by oncolytic adenovirus is an acute response towards Th1 type (Tuve et al. 2009, Hendrickx et al. 2014), Indeed, the combination of low-dose CP and oncolytic adenovirus coding for CD40L shifted the inflammatory profile towards Th1-type response in majority of the cancer patients (Pesonen et al. 2012). Hence, current evidence still indicates beneficial effects using this combination in cancer patients. Nonetheless, evidence of MDSC induction and their immunosuppressive role requires attention. In fact, considerable progress has been done to design chemotherapeutic regimens and small-molecule inhibitors for their selective inhibition as well (Alizadeh and Larmonier 2014).

4.3.4 Clinical responses and survival

Patients treated in the context of the ATAP were radiologically imaged and assessed for tumor markers because, in addition to safety data, the local regulatory agency FIMEA requires reporting of all treatment responses and outcomes (for assessment of the risk-to-benefit ratio). As the patient treatments were not conducted in a context of a prospective clinical trial, especially the patient efficacy data described in this thesis and reported in studies III and IV must be regarded with caution and conclusions should await confirmation from ongoing and future clinical trials. Nevetheless, besides obliged to report data from all experimental therapies according to Declaration of Helsinki article 35, we also felt that the observed possible signs of treatment efficacy would be of interest to the scientific community, since some of the treatments in the ATAP represent novel empirically supported approaches and drug combinations.

In study III, radiological tumor responses (by CT, PET-CT or MRI imaging), and tumor markers were studied to evaluate signs of efficacy after the combination treatments. Since patients were progressing prior to oncolytic virus treatments, disease stabilization or better was regarded as treatment benefit (disease control). Overall, objective evidence of antitumor efficacy was observed in 67% of evaluable treatments in imaging, and in 4 out of 9 treatments by tumor markers (**Study III, Table 2**). Specifically with regards to imaging, one treatment response was classified as a minor response (**Study III, Fig. 2A-C**), twelve as stable diseases, and seven as progressive diseases. In addition, one pediatric patient with neuroblastoma responded in ultrasound imaging that could not be classified due to unconventional imaging method, but the same patient showed a partial response in tumor marker levels. Considering that patients were progressing after all conventional treatment modalities, several interesting cases of radiological disease control together with unusually long survival were reported in each TMZ administration subgroups: An endometrial sarcoma patient in group 1, with an overall survival of 951 days, showed clinical response to three rounds of combination treatment by experiencing a 15% decrease in the total tumor diameters at best, observed after the second treatment, and a minor response in tumor markers, seen after all three combination treatment rounds (**Study III, Fig. 2A-C and Table 2**). A mesothelioma patient in group 2 seemed to benefit from two rounds of combination treatment with stabilization of the disease and decrease in pleural effusion, together with an unexpectedly long survival of 779 days. A heavily pre-treated patient with malignant fibrous histiocytoma (a subtype of sarcoma) in group 3 showed disease stabilization that lasted for three rounds after initiation of the combination treatment and although progressing thereafter, had an overall survival of 553 days. Finally, at the end of follow-up (time of submitting the study III article: last updated in Jan 2013) one sarcoma patient receiving combination treatments first according to group 1 and then group 3 and showing sustained stabilization of the disease, was still alive with an ongoing survival of 1459 days. Interestingly, possible signs of clinical benefit (imaging/marker responses together with prolonged survival) were observed in all age groups as the aforementioned five patients were 6, 45, 65, 67, and 17 years old at baseline, respectively.

Overall survival is an endpoint affected by many prognostic and predictive factors, including tumor type, performance score, age, previous and following therapies, and comorbidities. While acknowledging this, however, survival is also the most relevant endpoint in cancer therapy research, and thus we wanted to examine the overall survival data of patients treated with oncolytic adenoviruses together with or without low-dose pulse of TMZ. Therefore, matched non-randomized control patients (n = 17) treated in the same therapy program, but without TMZ, were selected according to known prognostic factors. A Kaplan-Meier comparison between the treatment groups suggested a trend for improved survival in favour for combination-treated patients, with a median overall survival of 269 days in combination-treated *versus* 170 days in non-TMZ treated control patients (not significant; **Study III, Fig. 4**). It should be noted, however, that patients treated in the ATAP were thereafter allowed to receive other treatments, including other oncolytic adenovirus treatments. In fact, 3 out of 17 combination-treated patients and 4 out of 17 matched control patients were later treated with one or more additional cycles of virotherapy, which may have impacted the overall survival. To study whether immune activations would result in improved outcome, we studied correlations of immunological parameters to therapy responses. While T-cell responses or serum HMGB1 changes failed to correlate with disease control as assessed by imaging and/or tumor markers, a correlation between HMGB1 response (increase/ no increase post-treatment) and overall survival at median cutoff was observed even in this small patient series (P = 0.0119, Fisher's exact test). In other words, combination-treated patients who

experienced serum HMGB1 increase after treatment seemed to have a longer overall survival. We postulated that release of HMGB1 into circulation as a consequence of immunogenic cancer cell death triggered by combination therapy, could lead to activation of antitumor immunity as suggested by antitumor T-cell inductions observed in blood, which in turn would be reflected into long-term overall survival. However, these preliminary data were regarded as hypothesis forming, and lead us to investigate the role of serum HMGB1 in detail in study IV.

To conclude, our results demonstrate the safety of the combination treatment with oncolytic adenoviruses, low-dose TMZ and low-dose CP in cancer patients. Adverse reaction profile and liver enzyme elevations suggested that the low-dose TMZ pulse is optimally administered after the virus treatment. In addition, we provide important insights into mechanistic and immunological effects of the combination. Our findings are corroborated by earlier reports, suggesting that both agents, TMZ and oncolytic adenoviruses alone, can induce autophagy and immunogenic cell death preclinically (Kanzawa et al. 2004, Ulasov et al. 2009, Jiang et al. 2011, Diaconu et al. 2012). Interestingly, one study has also reported on combination of another adenoviral approach, called suicide gene therapy, together with TMZ and radiotherapy in the treatment of glioma *in vivo* (Curtin et al. 2009). The authors found that combination therapy as well as monotherapies resulted in HMGB1 release, and that HMGB1 binding to TLR-2 receptor on dendritic cells, as an endogenous danger signal, mediated effective antitumor immune responses. These results are compatible with our findings, and suggest further benefit by including also radiotherapy, which was under investigation in our study I. As an extension to the preclinical data, we report for the first time, evidence of the autophagy and immunogenic HMGB1 release after the combination therapy in patients, possibly in conjunction with the observed antitumor T-cell activations. In summary, autophagy induction and HMGB1 release together with signs of antitumor efficacy in both preclinical and clinical therapy setting indicates analogous effects at bench and patients' bedside. Adding the good safety and antitumor T-cell activity observed in patients, our translational findings encourage for clinical trials using oncolytic adenoviruses in combination with low-dose TMZ and CP.

4.4 Serum High-mobility group box 1 (HMGB1) protein is a predictive and prognostic biomarker for oncolytic immunotherapy in cancer patients

Emerging preclinical and clinical evidence by us and others suggest that extracellular HMGB1 protein plays a central role in both immunosuppression and immune activation, the former being relevant for tumor progression and the latter dominating in therapeutic setting. HMGB1 is a nuclear chromatin protein and a key cytokine in local inflammation. Under autophagic or necrotic cell death, HMGB1 is released from dying cells, acting as a damage-associated molecular pattern (DAMP) molecule that, in conjunction with other DAMP signals, stimulate dendritic cells and increase antigen-presentation (Apetoh et al. 2008, Martins et al. 2012, Guo et al. 2013). However, HMGB1 is also actively secreted by monocytes, macrophages and dendritic cells, and this chronic production has been linked to carcinogenesis, tumor progression and immunosuppression through chronic inflammation (Liu et al. 2011b, Kang et al. 2013, Li et al. 2013b). In study III, we observed a preliminary correlation between post-treatment HMGB1 surge in serum and survival, a tantalizing finding given that no specific prognostic or predictive biomarkers for oncolytic viruses exist. In fact, there is an urgent need for biomarkers for immunotherapy since it would facilitate planning of clinical trials and selecting the right patients for each therapy.

These aspects encouraged us to investigate the role of circulating HMGB1 in oncolytic immunotherapy in more detail. Given the two opposing roles of extracellular HMGB1, we hypothesized that serum HMGB1 level is an indicator of immunosurveillance status: High baseline values would indicate prior immunological detection of the tumor, which has subsequently resulted in local immunosuppression reflected by the chronic production of HMGB1. In contrast, low baseline levels coupled with HMGB1 surge after therapy could indicate lack of extensive baseline immunosuppression and therapeutic responses, representing immunologically naïve tumors that would be susceptible to immunogenic immunotherapy. We studied the role of serum HMGB1 in a large cohort of 202 cancer patients treated with oncolytic adenoviruses in the context of the ATAP. In order to rigorously assess the prognostic and predictive value of HMGB1, we considered possible confounding factors in multivariate analyses and performed additional correlative analyses to study the biological impact of serum HMGB1 status. Our clinical-epidemiological report sets the stage for, and helps in planning of, prospective biomarker studies and may eventually help in selecting the right patients for each therapy, thus sparing costs and human suffering.

4.4.1 Serum HMGB1 baseline levels, patient characteristics and treatments

The study population consisted of 202 patients with advanced solid tumors progressing after conventional therapies, who had available baseline serum samples for assessment of HMGB1 concentration by ELISA. Since normal physiological range of serum HMGB1 levels are unknown for advanced cancer patients, we used the overall median concentration at baseline (0.512 ng/mL) as a cutoff for dividing patients into low and high HMGB1-baseline groups. With regards to HMGB1 change, as assessed by subtracting individual baseline level from post-treatment values (total n = 172), patients experiencing HMGB1 increase, or surge, after treatment were considered as responders and otherwise deemed as non-responders. Our multivariate analyses focused on the HMGB1-baseline comparison where significant differences in therapy outcome were seen, and thus the HMGB1 change is discussed later and more shortly. Patient characteristics of the HMGB1 baseline groups were evenly distributed with regards to gender, age, tumor type and previous therapies (**Study IV, Table 1**). Patients were heavily pretreated with a median of 1 previous surgery and 3 chemotherapy regimens in both groups. The most common tumor types included colorectal, breast, pancreatic and ovarian cancer. Only WHO performance status at baseline showed mild discrepancy, with six more WHO performance score 3 (poor performance) patients in the high-baseline group (not significant), which was taken into account, similar to all other possible confounding factors, later in multivariate analyses. Of note, all five pediatric patients had low HMGB1 baselines (average of 0.382 ng/mL +/- 0.049 [SEM]). This is well in accord with our hypothesis, because pediatric tumors often develop more rapidly than adult tumors, and thus may feature less prior immunosurveillance, evasion and suppression (Vakkila et al. 2006).

Similar to patient demographics, the first oncolytic adenovirus treatments given to 202 cancer patients were well-balanced between the HMGB1-baseline groups (**Study IV, Table 2**). Most of the treatments featured adenoviruses armed with immunostimulatory transgenes, i.e. granulocyte-macrophage colony stimulating factor (GMCSF) or CD40-ligand (CD40L) (Koski et al. 2010, Pesonen et al. 2012). Low-dose CP, used for the selective reduction of immunosuppressive regulatory T-cells (Cerullo et al. 2011), and low-dose TMZ, intended to increase immunogenicity of the treatments (study III), were used in 66% and 13% of treatments overall, respectively. Virus types

were evenly distributed, while concomitant low-dose CP, serial treatment and intratumoral administration were slightly more frequent in the high-baseline group (none significantly), all of which were again later taken into account as possible confounding factors. It is important to note that HMGB1 baseline level was unknown at the time of treatment decision and follow-up, rendering clinical decision making and radiological response evaluation in our experimental setting unbiased. Furthermore, since none of the relevant characteristics or treatments correlated with the retrospectively analyzed HMGB1 status, we conclude that clinical aspects did not influence the biomarker findings in study IV.

4.4.2 Independent prognostic and predictive value of HMGB1 baseline status

Serum HMGB1 concentration at baseline showed an inverse correlation to overall survival (**Study IV, Fig. 1A**). When survival was examined using the median HMGB1 concentration as a cutoff, overall survival in the low HMGB1-baseline group was 151 days at median (95% CI 120 – 181 days), and in the high HMGB1-baseline group 102 days at median (95% CI 89 – 115 days), a significant difference as assessed by Log-Rank test (P = 0.008; **Study IV, Fig. 1B**). We next studied the radiological tumor responses of all 50 and 65 evaluable cases in the high and low HMGB1-baseline groups, respectively, and noticed a corresponding correlation: disease control (disease stabilization or better in previously progressing patients) was achieved in 49.2% of patient treatments in the low HMGB1-baseline group, whereas only 30.0% showed disease control in the high HMGB1-baseline group (P = 0.038, chi-squared test). Moreover, 18.5% of evaluable patients in the low-baseline group experienced tumor regression (minor response or better), including 6 complete responses, while none of the high-baseline patients had complete response and only 8.0% showed tumor regression (**Study IV, Table 2**).

Outcome variables, especially overall survival, are influenced by a number of prognostic and predictive factors that, if not adjusted for, can adversely affect the relation between the independent variable and the dependent outcome variable. Therefore we adjusted our findings for known confounding factors in two adequate multivariate models, Cox proportional hazards model for overall survival, and logistic regression model for radiological disease control. Importantly, both observations held in multivariate analyses (**Study IV, Table 3**): First, the prognostic value (overall survival) of the baseline HMGB1 status remained significant in the Cox model (P = 0.006), and second, the HMGB1 baseline status emerged as the only independent predictive factor for disease control in the logistic regression model (P = 0.049). With regards to prognostic impact of the HMGB1-baseline status, the hazard ratio (HR) was 0.638 (95% confidence interval at 0.462-0.881) and the adjusted survival and hazard functions appeared even more separated (**Study IV, Suppl. Fig. S1A-B**). Meanwhile in the logistic regression model, the odds ratio (OR) for predicting disease control by HMGB1-baseline status was 2.618 (95% confidence interval at 1.004-6.827). Overall, our logistic regression model showed fair to good predictive power, i.e. sensitivity and specificity, as suggested by the area under the receiver operating characteristic (ROC) curve value of 0.776 (+/- 0.043 SE; **Study IV, Suppl. Fig. S1C**).

With regard to prognostic factors, WHO performance status and tumor type were expectedly identified as other independent prognostic factors, whereas the third additional significant factor that emerged was less obvious: treatment with oncolytic adenovirus armed with GMCSF. This observation is in line with our previous reports suggesting that patients with GMCSF-susceptible tumor types may benefit from oncolytic immunotherapy with GMCSF-armed adenoviruses. In fact,

several clinical trials with GMCSF-armed oncolytic viruses are ongoing, and the first positive phase 3 trial results in the Western countries were recently reported with an oncolytic herpes simplex virus coding for GMCSF.

Considering predictive factors, the strong impact of the HMGB1-baseline status in predicting therapy outcome is underlined by the fact that even WHO performance status, which is considered as one of the most important parameters for clinical decision making in oncology (Ando et al. 2001), failed to reach statistical significance in this analysis (P = 0.055). Oncologists use the performance scores in planning anticancer treatments and selecting doses. Classically, physical condition/activity of the patient is considered to reflect the stamina to withstand heavy chemotherapeutic, surgical or radiotherapeutic regimens aimed at direct cell killing and reduction of tumor burden, thus predicting also responses to conventional therapies (Ando et al. 2001). In contrast, as the lack of clinically relevant predictive factors for cancer immunotherapy suggests, this is not directly applicable, or at least sufficient, in predicting responsiveness to immunotherapy, where patient's own immune system is ultimately responsible for antitumor responses. Hence, regarding the sole significance of the baseline HMGB1 as a predictive factor, these aspects may further increase the impact and potential clinical relevance of our finding. Considering that the WHO performance status did not correlate with HMGB1 status, these two parameters do not appear mutually exclusive and could in theory be utilized in parallel in planning of immunotherapeutic regimens for each patient. In summary, serum HMGB1 baseline status proved to be an independent prognostic and a tentative predictive biomarker for oncolytic immunotherapy, and it was the most strongly predictive factor of treatment efficacy.

4.4.3 Safety assessment and comparison between the patient cohorts

In study III, we specifically studied safety profile of the combination therapy using oncolytic adenoviruses, low-dose TMZ, and low-dose CP (see above), whereas in study IV, we studied overall safety of the first oncolytic adenovirus treatments given to a large cohort of cancer patients with or without concomitant therapies; Here we wanted to examine whether the safety profile differs between the HMGB1-low and HMGB1-high cancer patients, given their differential responsiveness to the therapy. In theory, poor therapy outcome could be due to premature therapy discontinuation after encountered adverse reactions. This was not the case, however, as oncolytic immunotherapy was equally well-tolerated in both groups (**Study IV, Suppl. Table S1**), and the adverse reaction profiles (most commonly grade 1–2 fever, fatigue, pain, transient anemia and electrolyte disturbances) were in line with previous reports (Koski et al. 2010, Cerullo et al. 2011, Pesonen et al. 2012). Most adverse reactions were self-limiting, however, 11 and 9 treatments in low and high HMGB1 groups, respectively, were associated with grade 2-4 serious adverse event (SAE) leading to patient hospitalization (**Study IV, Suppl. Table S1**). To conclude, the adverse reaction profiles did not differ between the HMGB1 groups, and therefore frequent therapy discontinuation in high baseline group seemed unlikely.

Safety of the oncolytic immunotherapy, with or without low-dose TMZ and CP, reported in studies III and IV was good throughout. In an attempt to compare between studies/groups, frequencies of ARs by grades in different patient cohorts are summarized in **Table 4**. Mild to moderate clinical grade 1 and 2 ARs were encountered both at a median of 2 per treatment in study IV, and all but one patient out of total 202 patients experienced one or more clinical AR. Meanwhile in study III, clinical grade 1 ARs were slightly more frequent (median of 3 per treatment), whereas the grade 2

ARs in turn were slightly less frequent (median of 1). Importantly, there were no treatment related deaths (grade 5), and only one grade 4 clinical AR emerged: pulmonary embolism was diagnosed in a colon carcinoma patient that led to patient hospitalization and was thus classified as a serious adverse reaction (SAE, see below). Thus, the frequency of symptomatic clinical ARs did not significantly differ between treatment regimens or any patient groups, and treatments appeared well-tolerated overall.

Table 4. Adverse reactions (AR) after oncolytic immunotherapy in studies III-IV.

	Study	***N***	**Cohort**	**Gr.1**	**Gr.2**	**Gr.3**	**Gr.4**	**Gr.5**
Clinical AR	III	41	TMZ-combined (*%*)	93	83	7	0	0
	IV	101	Low HMGB1 (*%*)	89	83	12	1	0
	IV	101	High HMGB1 (*%*)	93	89	14	0	0
	III-IV	238	Total (*%*)	91	86	11	0	0
Laboratory AR	III	41	TMZ-combined (*%*)	88	71	49	2	0
	III	**41**	**TMZ-comb. (*%*), no lymph.**[†]	**88**	**56**	**15**	**0**	**0**
	IV	101	Low HMGB1 (*%*)	48	30	11	4	0
	IV	101	High HMGB1 (*%*)	58	43	15	1	0
	III-IV	238	Total (*%*)[†]	59	39	13	2	0
Serious ARs[‡]	III	41	TMZ-combined (*n*)	0	0	1	0	0
	IV	101	Low HMGB1 (*n*)	0	0	10	1	0
	IV	101	High HMGB1 (*n*)	0	1	7	1	0
	III-IV	238	Total (*n*)	0	1	18	2	0

Of note, there were 5 patient treatments that were included both in study III and in study IV (4 in high and 1 in low HMGB1 group), which were thus counted only once in the total number of treatments, resulting in total *N* = 238. Adverse reactions (ARs) were reported according to Common Terminology Criteria for Adverse Events (CTCAE) v3.0 criteria, and the follow-up time for ARs in both studies was 28 days after the virus treatment. Pre-existing symptoms were recorded only if worsened, and in these cases were scored according to final severity.

[†] In order to reliably compare laboratory ARs between the groups, the TMZ-combined treatments of study III are also shown here without taking lymphocytopenia into account. Same applies to the Total % of the laboratory ARs.

[‡] ARs were classified as potentially treatment-related serious adverse reactions leading to patient hospitalization, malformation, life-threatening condition, or death (any of these constituting a "serious adverse event, SAE"), or not.

Interestingly with regards to laboratory ARs, when lymphocytopenia was retrospectively omitted from AR analysis of study III, as suggested by accumulating evidence (see above), the laboratory AR profile still showed more mild to moderate (grade 1 and 2) ARs in study III than in study IV ($P < 0.001$ and $P < 0.05$, respectively; see **Table 4**). In contrast, grade 3–4 laboratory ARs were explained by the transient lymphocytopenia. One explanation for the more frequent grade 1–2 laboratory ARs could be the liver transaminase increases, commonly seen in the study III subgroup that received TMZ before the virus. Liver enzyme elevations were indeed slightly more frequent in study III than in study IV overall (39% versus 32%, not significant), while even more so as compared to low HMGB1-baseline patients of study IV (39% versus 25%, $P = 0.096$; all chi-squared tests). The

laboratory ARs in study III were nevertheless asymptomatic or alleviated by outpatient care, as only one serious adverse reaction leading to hospitalization occurred (grade 3 ileus).

In clinical cancer research as well as in oncology practice, serious adverse reactions deserve special attention since these may constitute clinical or laboratory conditions potentially hazardous for patients. As a commonly used definition, serious adverse event (SAE) was defined as a potentially treatment-related serious adverse reaction leading to patient hospitalization, malformation, life-threatening condition, or death. Including safety data from studies III and IV, SAE was reported after 8,0% of all 238 treatments (two treatments resulted in two separate SAE reports), with altogether 21 SAE reports of grades 2–4 (see **Table 4**). One marginally more common SAE was grade 3 dyspnea. At least in 2 out of 3 occasions it was associated with pleural effusion, and might have been due to accumulation of effusion either caused by virus activity in lung/pleural lesions or because of tumor progression. Notably, close to a third (6 out of 21) of SAE reports were due to grade 3 ileus. These possibly treatment related SAEs might have been caused by virus-induced inflammation in cancer lesions residing on the gut lining, resulting in gut distension and paralysis, which is supported by the fact that 4/6 were ovarian carcinoma and 1/6 was a cholangiocarcinoma patient, both of which typically feature disseminated intraperitoneal diseases in advanced stage. One of these ovarian carcinoma patients was imaged by CT at 69 days post-treatment (over a month after the ileus), and had a minor response of 12% reduction in the total tumor diameters. She also experienced a 25% decrease in tumor markers (Ca12-5), also scored as a minor response. Moreover, circulating viral genomes (959 VP/ml) of the treatment virus Ad5/3-cox2L-Δ24 were detected in serum at day 4 post-treatment, suggestive of virus replication. These observations support the previous hypothesis that ileus may in fact relate to the replicative and inflammatory effects of the adenovirus, and should be taken into consideration when planning treatments and clinical trials for patients with peritoneally disseminated disease.

Of note, our analyses in study IV only included safety assessments done after the first oncolytic adenovirus treatment, and thus the impact of possible consecutive treatments on patient safety cannot be evaluated here. However, as previously reported and supported in study III, mounting of antiviral immune responses and induction of neutralizing antibodies is often observed soon after the first treatment, which are expected to protect the patient from inflammatory ARs of the consecutive treatments.

4.4.4 Correlative analyses of potential mechanistic factors

Another clinical variable besides ARs, which could account for the observed HMGB1 baseline status, was tumor burden at baseline, because tumor cells and the associated immune cells are regarded as the dominant source of HMGB1. In fact, we showed with available samples that HMGB1 levels in serum seemed to correlate with tumor-associated ascites/pleural effusion HMGB1 levels (**Study IV, Fig. 1C**). Thus, we speculated that the quantity of tumor cells releasing, and the amount of associated immune cells actively secreting HMGB1, might dictate the baseline HMGB1 level in serum. To address this, we studied the patient's tumor burden at baseline (pre-treatment whole-body imaging) and correlated the data with serum HMGB1-baseline levels. 55 patients from low and 40 from high baseline group were evaluable for assessment of tumor load correlation: The median tumor load score was 6 in both low and high HMGB1 groups (possible range: 0-21), with no statistical difference and lack of linear correlation (**Study IV, Suppl. Fig. S4**), indicating that serum HMGB1 levels are not directly dependent on tumor burden. Interestingly,

however, when only very high tumor loads (score >10) were studied, a significant linear correlation was observed (r^2=0.8662, P < 0.0001). Reason behind this correlation might be that very large, typically necrotic tumors are associated with rapid tumor cell turn-over. Alternatively, although not mutually exclusively, large bulky tumors often represent highly evolved immunosuppressive environments featuring lots of immune cells chronically producing HMGB1. Interestingly, when we further looked at the disease distribution pattern, presence of pleural/ascites effusion was observed significantly more frequently in low (27.3%) as compared to high HMGB1 baseline patients (10.0%) (P = 0.038, chi-squared test). When examined in more detail, none of the low HMGB1 patients with effusion (n = 15) had large tumor burden (median score of 6), while 3 out of 4 high HMGB1-baseline patients who had effusion, showed very high tumor loads (median score of 12.5). Hence, we postulated that large bulky tumor masses, as opposed to disseminated peritoneal/pleural disease, would be more prone to develop a highly evolved immunosuppressive tumor microenvironment, characterized by chronic HMGB1 production. Alternatively, these tumors might feature vast necrotic areas as a source of continuous HMGB1 shedding (Scaffidi et al. 2002). Finally, the amount of tumor-associated immunosuppressive cells, mediating also chronic production of HMGB1, has been identified as an independent prognostic factor (Tanchot et al. 2013), and may not directly depend on the tumor size. Tumor biopsies would have been required to assess this.

We further studied correlations of HMGB1-baseline status with relevant biological variables, such as classical virotherapy parameter virus replication: Circulating adenovirus genomes did not present greater virus replication in the low HMGB1-baseline patients, as could be expected for improved outcome. In fact, even a slight opposite trend towards higher replication in the high HMGB1-baseline group was noted. Nevertheless, a similar transient increase was observed in both groups after treatment, suggesting equal replication capacity in both patient groups (**Study IV, Suppl. Fig. S2**). Second, we studied inflammatory cytokines that may relate to antiviral immunity, antitumor immune activation, and immunosuppression as well. We used a panel of five relevant anti- and pro-inflammatory cytokines, IL-6, IL-8, IL-10, TNF-α, and GMCSF that was also used as a transgene in majority of the virus treatments: No significant differences between the HMGB1 groups emerged, but interestingly, HMGB1-high group showed a trend for higher total amount of IL-6 (**Study IV, Suppl. Fig. S3**). In addition to this, HMGB1-high patients presented a trend for higher increase in post-treatment GMCSF levels. Overall trends toward acute phase increase in IL-6 and a late increase in TNF-α were observed in both groups.

Our correlative analyses did not reveal mechanistic links to classical virotherapy parameters. There was, however, slight variation between the groups: high HMGB1-baseline patients showed a trend for higher virus replication, GMCSF production and acute phase IL-6 production, while featuring the highest tumor loads (score >10) that linearly correlated with increasing HMGB1 levels. These parameters may be connected because large bulky tumors that can be intratumorally injected, are optimal grounds for initial virus replication, coupled with efficient transgene expression (GMCSF), and antiviral cytokine response (IL-6). As discussed, however, these tumors also feature extensive immunosuppression, limiting their responsiveness. As a potential sign of this, the total amount of IL-6 was greater in the high-HMGB1 patients, which has been linked to advanced immunosuppressed tumors, and poor outcome after immunotherapy, mediated for example *via* chronic Stat3 activation (Burdelya et al. 2005, Zarogoulidis et al. 2013).

4.4.5 Evidence of immunological mechanisms underlying the HMGB1 status

We hypothesized that HMGB1 baseline level could distinguish between immunologically naïve responding patients and immunologically inert/suppressed non-responding individuals. Therefore, we analyzed antitumor T-cell activity in peripheral blood of 129 evaluable patients, and correlated the data with outcome variables and HMGB1 status. 49,6% of patients in total featured antitumor T-cell inductions, and the frequency was similar in both HMGB1-baseline groups, suggesting that the amount of T-cell inductions after oncolytic immunotherapy is not altered. However, when T-cell data was correlated to survival inside the HMGB1-groups, only the patients with low-HMGB1 baseline seemed to benefit from T-cell activations (**Study IV, Fig. 2**): The median survival of low-HMGB1 patients who featured T-cell inductions was 221 days (95% CI 113 – 329 days) *versus* 111 days (95% CI 101 – 121 days) in the high-HMGB1 group with T-cell inductions (not significant). Moreover, when also a decrease in antitumor T-cell counts, which is compatible with trafficking of T-cells from blood into tumors, was studied together with T-cell inductions, low-HMGB1 patients showed significantly improved survival over high-HMGB1 patients (P = 0.043), and importantly, over the remaining low-HMGB1 patients (P = 0.038).

Next, we assessed the more specific endpoint for therapy responsiveness, radiological responses. Again, we observed that in patients with T-cell induction, low-HMGB1 group showed a trend for further increased disease control rate over high-HMGB1 group (55.0% *versus* 27.3%; P = 0.068), and when trafficking was taken into account, a significantly improved response rate was observed (66.7% *versus* 20.0%; P = 0.040). Thus, we have identified the most immunologically active, best-responding subgroup, using HMGB1 and T-cell analyses: This subgroup features low-HMGB1 baseline levels, responds to oncolytic immunotherapy by showing induction and trafficking of antitumor T-cells in blood, and results in highest rate of objective signs of possible efficacy, both in tumor imaging and survival.

In addition to T-cell data, also the aforementioned trend for higher total IL-6 levels in the high-HMGB1 patients fits together with the proposed immunosuppression. Moreover, virus arming with the GMCSF cytokine, which has proven as a useful immunostimulatory adjuvant especially in the context of oncolytic viruses (Andtbacka et al. 2013), was observed to mediate the best signs of therapeutic efficacy in the low-HMGB1 patients (**Study IV, Fig. 3**): While the median survival was nearly doubled in the low-HMGB1 group over the high-HMGB1 patients (P = 0.004), the most notable improvement was seen in the long-term survival, which is well in line with the proposed immunological response profile, reported for other immunotherapeutic agents as well (Hodi et al. 2010, Hoos et al. 2010).

4.4.6 Post-treatment changes in serum HMGB1 and outcome parameters

Chronic production of HMGB1 by immune cells mediates several immunosuppressive actions (Liu et al. 2011b, Li et al. 2013a, Li et al. 2013b), whereas its acute release from immunogenically dying tumor cells is essential for immune activation (Scaffidi et al. 2002, Apetoh et al. 2007, Guo et al. 2014). Therefore, we hypothesized that these phenomena might have different circulation kinetics, and analyzed post-treatment serum HMGB1 levels, subtracted the individual baseline values, and correlated the data with outcome variables. Surprisingly, however, post-treatment HMGB1 increase in serum did not associate with improved outcome at any given time point after therapy (**Study IV, Suppl. Fig. S5**). On the contrary, late time point increase in HMGB1 (at 15 – 28

days post-treatment) trended towards shorter median survival (138 days *versus* 212 days in "no increase" group; not significant). Interestingly, this trend was due to low HMGB1-baseline patients, since HMGB1 elevation trended towards poor prognosis only in this baseline group (P = 0.098). Therefore, the low-baseline patients who maintained the low serum levels of HMGB1, especially at late time points after treatment, seemed to survive the longest. Accordingly with regards to treatment efficacy, the sustained low-HMGB1 patients showed an impressive disease control rate of 66.7% (2 complete responses, 1 partial response, 1 minor response, 6 stable diseases, and 5 progressive diseases), a much higher rate than in high-baseline patients (30.0%, P = 0.011) or in high-baseline patients with HMGB1 decrease post-treatment (26.1%, P = 0.013), but did not reach statistical significance as compared to low-baseline patients with HMGB1 increase post-treatment (43.9%, P = 0.131). These findings suggest that sustained low serum levels of HMGB1 associate with treatment benefits, but perhaps due to lack of multiple post-treatment samples and heterogeneity in treatments, predictive conclusions could not be drawn here.

These results are rather surprising given the preclinically well-established role of HMGB1 as an immunogenic DAMP molecule, also in the context of oncolytic viruses (Guo et al. 2014). Even though we observed an association between tumor-related ascites/pleural effusion and serum HMGB1, the absolute levels were constantly higher locally. Thus, it might be that a transient local release of HMGB1 is sufficient in activating dendritic cells – a spatiotemporal phenomenon that could not be recorded in serum, but only at the tumor site. The observed trend for poor outcome in patients with late time point HMGB1 elevation, on the other hand, could indicate harmful immunosuppression emerging. It should be noted that immunogenic cell death is mediated in conjunction with other DAMP signals as well (Guo et al. 2014), particularly calreticulin exposure and ATP release, that were not studied here. In contrast, our results in study III and previous data suggest that oncolytic adenoviruses, with or without low-dose chemotherapy, can induce immunogenic cell death (Diaconu et al. 2012). In study III, calreticulin exposure was observed at 24 h post-treatment, followed by ATP and HMGB1 release at 36 h post-treatment, while in study by Diaconu *et al.* using CD40-L coding oncolytic adenovirus, calreticulin, ATP, and HMGB1 were observed at 12 h, 18 h, and 24 h post-treatment, respectively. This suggests that dynamics of immunogenic cell death depend on the treatment agent and cell type, further complicating assessment *in vivo*, let alone in human biopsies. Nevertheless, the pivotal role of HMGB1 is important to take into account when designing novel treatments and biomarkers.

4.4.7 Potential clinical relevance of the biomarker

Effective responses seen after cancer immunotherapy, while often durable in nature due to immunological memory, can take time to mount and remain difficult to foresee, which complicates selection of appropriate immune-based treatments for each patient and slows down the progress in clinical trials. Therefore predictive biomarkers are urgently needed for cancer immunotherapy that would optimally, not only indicate biological activity of the drug, but also discriminate between immunologically responding and non-responding patients for each immunotherapeutic application. Good examples of the latter are immune checkpoint modulating antibodies that directly act on the immune system: programmed death-1 (PD-1) pathway, among others, mediates tumor-induced immunosuppression and monoclonal antibodies against PD-1 receptor or its ligands, such as nivolumab, are showing promise in the treatment of e.g. melanoma, lung, and renal cancer. Importantly, clinical data suggests that only patients with PD-L1-positive tumors seem to respond to antibody treatment, although warranting for confirmation in prospective

biomarker trials (McDermott and Atkins 2013). This would be logical since these patients feature strong PD-1 mediated immunosuppression that could be combated by pathway blocking antibodies releasing the specific breaks of the immune system.

For immunogenic immunotherapy, aimed at inducing antitumor immune responses rather than releasing the existing suppressive breaks, however, no such predictive biomarkers exist yet. Tumor lytic adenoviruses belong to this class, by featuring a strong immunostimulatory anticancer component *via* release of danger signals and tumor-associated antigens, which can be further boosted by immunogenic transgenes and/or adjuvant therapies. Interestingly, a very recent phase II randomized multicenter immunotherapy trial using chemotherapy together with or without oncolytic reovirus for the treatment of relapsed or metastatic pancreatic cancer failed meet its main endpoint of progression-free survival (PFS) in the total patient population, but instead showed a trend for improved PFS (39% increase in median PFS) in a sub-analysis where only KRAS-mutated patients were included (ClinicalTrials.gov Identifier: NCT01280058). Importantly, this subgroup analysis was based on previous preclinical and clinical reports suggesting that the oncolytic reovirus was mostly active in cancer cells with an activated RAS pathway (Thirukkumaran and Morris 2009). This predefined biomarker assessment was thus implied to have a major clinical significance, suggesting therapeutic benefit only in a subtype of pancreatic cancer, although the overall survival data remains to be seen. Unlike the optimal biomarker arsenal, however, the *KRAS*-mutation status as studied here only seems to predict biological activity of the drug (virus replication), thus ignoring the immunological aspects of the patient and tumor.

Closer to this end, a smaller pilot study focusing on oncolytic vaccinia virus treated melanoma patients recently investigated potential immunological biomarkers by gene expression analyses on peripheral blood T-cells. The authors identified immunoglobulin-like transcript 2 (ILT2) expression on immunosuppressive subset of CD8+ T-cells (CD8+FoxP3+ILT2+) as a candidate predictive biomarker of vaccinia virus immunotherapy outcome. Although not as straightforward to assess as serum HMGB1 and pending for further validation in large-scale studies, these findings are compatible with our results: In both studies, the overall frequency of (activated) CD8+ T-cells did not differ between responders and non-responders, but the antitumor T-cell activity reflected to improved outcome only in patients with low HMGB1 baseline, and the same was observed in vaccinia virus treated patients with low immunosuppressive CD8+FoxP3+ILT2+ T-cell population. It would be interesting to assess if these observations are connected by studying suppressive ILT2-positive T-cell populations in our patient series, and *vice versa*.

In a previous report by our group we studied if germline differences in the immunological mechanisms of patients, in particular, single nucleotide polymorphisms in Fc gamma receptors (FcgRs), could predict survival or disease control after oncolytic immunotherapy (Hirvinen et al. 2013). In that study we focused on FcgRs because of their central role in the immune defense against infections and potential impact on cancer therapy (van Sorge et al. 2003, Mellor et al. 2013). Blood samples of 235 patients treated with oncolytic viruses in the context of the ATAP were studied, with the Ethics Committee approval because receptor polymorphisms might also impact safety or efficacy of the treatments, and genotyped for two different Fc gamma receptor polymorphisms, FcgRIIa-H131R and FcgRIIIa-V158F. Comparisons between allotypes revealed that one particular genotype combination, FcgRIIIa-VV + FcgRIIa-HR, differed from others and was predictive of poor overall survival after virotherapy, but since only 10 patients carried this genotype, multivariate and further correlative analyses could not be performed. Nevertheless, our results suggested that genotypic differences in immunological charateristics may also have some

impact on the responsiveness to oncolytic immunotherapy, although predictive significance of FcgR polymorphisms remained to be evaluated.

In study IV, we have extended our attempt in identifying patients that possibly benefit from oncolytic immunotherapy. We have identified serum HMGB1 protein as an independent prognostic and predictive biomarker for oncolytic immunotherapy that may potentially discriminate between immunologically responding and non-responding patients. It would be interesting to study some of the aforementioned putative predictive factors together with our findings. In theory, this could further increase the sensitivity and specificity, similar to what we saw with regards to predictive impact of antitumor T-cell activations. Potentially, our findings could also apply to other immunogenic immunotherapy approaches including other oncolytic viruses, adoptive T-cell therapy, and cancer vaccines. Further mechanistic and prospective clinical studies using predefined levels of HMGB1 as a putative biomarker are warranted. Identifying novel predictive factors for emerging cancer immunotherapies is crucial not only for the individual patient benefit, but also to improve cost-effectiveness of these often expensive therapies (Geynisman et al. 2014). Ultimately, our results may help in selecting the right patients for each therapy, thus reducing costs and human suffering.

5. CONCLUSIONS AND FUTURE PROSPECTS

Cancer immunotherapy has recently provided several success stories, and is gradually meeting its expectations as a potent form of cancer therapy. CTLA-4 blocking antibody ipilimumab was shown to mediate durable antitumor responses and approved for treatment of metastatic melanoma (Hodi et al. 2010, Robert et al. 2011). A therapeutic cancer vaccine Sipuleucel-T was approved for treatment of castration-resistant prostate cancer (Kantoff et al. 2010). Anti-CD19 CAR T-cell therapy rendered pediatric leukemic patients into remission even after exhausting all other treatment options (Grupp et al. 2013). Most recently, oncolytic immunotherapy for treatment of metastatic melanoma, using an oncolytic herpes virus coding for GMCSF (T-VEC), met its primary endpoint in a phase III clinical trial, and is expected to receive marketing approval in the United States (Andtbacka et al. 2013). Owing to these advancements, the field was rightfully selected as the breakthrough therapy of the year by a leading scientific journal (Couzin-Frankel 2013).

Despite the evident progress, numerous immunotherapy trials have failed due to low efficacy. Cancer is the number one cause of death worldwide and incidence is rising. There are many tumor types still lacking curative treatments despite the advancements in conventional therapies. The aim of this thesis was to improve efficacy while maintaining favorable safety by combining oncolytic immunotherapy with conventional treatment modalities. We found that inherent potential of adenoviruses to sensitize infected cancer cells to radiotherapy is mediated by two proteins that inhibit DSB repair inhibition, and demonstrate the combination efficacy *in vivo*. Furthermore, we demonstrate that combination therapy of oncolytic adenovirus with low-dose TMZ and CP can mediate immunogenic cancer cell death and enhanced tumor regression in mice, and was well-tolerated with promising signs of possible efficacy in patients with advanced solid tumors, refractory to conventional therapies.

In addition, we aimed to identify acquired resistance mechanisms against oncolytic adenovirus, and novel predictive and prognostic biomarkers for oncolytic immunotherapy, which could ultimately lead to development of countermeasures, and better patient selection. Cancer entails enormous transforming capacity that renders each tumor individual and complicates planning of treatments. Nonetheless, also common growth promoting pathways and resistance mechanisms have been identified, which has allowed design of counteracting drugs and biomarkers, many of which are in clinical use. For immunotherapy in general, and oncolytic viruses in particular, predictive biomarkers are urgently needed, since it appears obvious that not all tumors respond to a given immunotherapeutic modality. To this end, we first identified a tentative immunohistochemical biomarker that correlated with the acquired antiviral phenotype preclinically, and provide here preliminary data on potential translation into cancer patients. Second, we propose a novel clinical prognostic and predictive biomarker for oncolytic adenoviral immunotherapy, which may distinguish between immunologically inert and reactive patients. Important for its utility, a simple serum sample could dictate between responsive and non-responsive cancer patient.

We used several methods in this thesis to study oncolytic immunotherapy, proceeding from bench to patients' bedside. In studies I and II we conducted basic research assessing molecular mechanisms behind treatment effects of oncolytic adenoviruses when combined with radiotherapy, and studied mouse models of acquired tumor resistance against oncolytic virotherapy. In this thesis we provide a translational extension of study II, by assessing the

potential antiviral resistance marker in available samples of advanced cancer patients treated with oncolytic adenoviruses. Study III was a translational study, addressing the underlying mechanisms and efficacy of oncolytic adenovirus with low-dose chemotherapy preclinically, and studying safety and possible signs of efficacy and immunological activity in cancer patients with solid tumors refractory to conventional treatments. Finally, in study IV, we evaluated the value of a novel prognostic and predictive biomarker, serum HMGB1, for oncolytic adenoviral immunotherapy in a large cohort of advanced cancer patients.

Both of our clinical-epidemiological reports (III, IV) serve as pilot research for future studies: First, they demonstrate safety and possible signs of efficacy of oncolytic adenovirus treatments in a heterogeneous group of cancer patients. Second, our reports suggest a feasible treatment combination, potential signs of immunological activity, and propose a novel prognostic and predictive biomarker. Hence, studies III and IV set the stage for clinical trials in specific groups of cancer patients. In particular, the promising combination of oncolytic adenovirus with low-dose TMZ and CP, must be evaluated in selected tumor types. On the contrary, serum HMGB1 would be expected to serve as a pan-tumoral biomarker, given the proposed immunological mechanism behind the predictive value. However, further mechanistic studies and prospective trials are warranted to confirm this.

Interestingly, when integrating the results between studies and reflecting them to research published by others, several aspects reappear in different contexts. For instance, besides the studied DSB repair inhibition (study I), synergy between oncolytic immunotherapy and radiotherapy is also mediated *via* autophagy and immunogenic cell death, which is analogous to the observed effects with low-dose temozolomide (study III). Moreover, HMGB1 protein, acting as a central cytokine for all lymphoid and innate immune cells, plays a key role in each of our studies: it is involved in autophagy and immunogenic cell death triggered by both combinatorial approaches (studies I and III), in innate antiviral immunity as an endogenous enhancer of danger-signaling (study II), and in tumor immunosuppression by activating regulatory T-cells and myeloid-derived suppressor cells (study IV). In fact, this congruence is beginning to emerge in all platforms of immunotherapy, which is an imminent indicator that the field has come of age: Developed far from the empirical times of Dr. William Coley, to the concept of immunosurveillance, and gradual understanding of the underlying tumor immunology and capacity of the human immune system, cancer immunotherapy may finally offer a remarkable addition to the standard care of many malignancies.

Nonetheless, many challenges lie ahead to achieve this. One major hurdle in the development of immunotherapeutics is the ever increasing costs. Considering an estimation that development of an approved drug will take approximately 20 years and cost over half a billion euros, it is not surprising that novel immunotherapeutics like ipilimumab and sipuleucel-T cost ninety-five and eighty-thousand euros per treatment, respectively (Couzin-Frankel 2013). Since academia is hardly to have the resources, collaboration with pharmaceutical companies at an early phase is imperative. Alternatively, fund raising or crowdfunding could provide means to proceed with drug development. One key element in improving the cost-effectiveness of the expensive cancer immunotherapies is to identify predictive biomarkers for better patient selection (Geynisman et al. 2014), which we have addressed in this thesis as well. The increasing costs raises also ethical and socio-economic questions of using expensive therapeutics that sometimes only transiently prolong patient survival. Another problem in drug development is the continuously increasing bureaucracy and the amount of regulations, which on one hand serve to ascertain scientific rigor, but on the

other hand slow down the progress and decrease availability of novel promising treatments. A balance between proper administration and urge to fill the unmet medical need should therefore be found. The United States Food and Drug Administration (FDA) Fast Track program is a refreshing attempt to this direction, which facilitates and prioritizes development of drugs that treat serious or life-threatening conditions.

In the near future, immunotherapy is likely to reach standard care in the treatment of many more malignancies. With non-overlapping safety profiles combination with conventional treatment modalities seems feasible. Oncolytic immunotherapy combined with standard or low-dose chemotherapy and radiotherapy holds promise, especially when moving to less immunosuppressed earlier patients. Specifically, oncolytic viruses could be utilized in earlier stage disease together with surgery, chemo- and radiotherapy, to boost the immune system to detect cancer and develop potent immunological memory, thus perhaps preventing many relapses. Along with improved diagnostics and personalized treatments, fewer tumors will advance and spread. However, occasionally also these highly resistant cases emerge, which could be treated with combination of different immunotherapeutics, such oncolytic immunotherapy and checkpoint blocking antibodies, that would increase immunogenicity, provide danger signals, and combat tumor immunosuppression. As an intriguing preliminary example of this, a very recent phase Ib trial using oncolytic T-VEC together with ipilimumab reported durable responses in 10 out of 18 (56%) evaluable patients, with 33% being complete responses, and no dose-limiting toxicities from the combination (Puzanov et al. 2014). Furthermore, along with longer life-spans, more immunocompromised patients will develop cancer. In these cases, an optimal combination of immunotherapy could be using oncolytic adenoviruses together with adoptive T-cell therapy given in specialized centers. Treatment with armed oncolytic adenoviruses would first provide danger-signals and tumor-antigens, distort the tumor microenvironment, and produce immunostimulatory molecules, followed by adoptive T-cell therapy, where patients' adaptive immune activation occurs *ex vivo*, thus surpassing patients' weak inherent immunity and creating enough potency to eradicate the remaining tumor cells.

The rapidly growing repertoire of targeted cancer treatments and improved methods to characterize individual tumors creates prospects for more personalized targeted medicine. Success of modern oncology, however, originates from well-designed clinical trials and evidence-based medicine, which ultimately either substantiate a given treatment as more beneficial than standard therapy, or not. Novel targeted strategies, in particular immunotherapy, must be evaluated with the same principles but with adapted criteria, that will take its specific features such as tumor swelling, long-term survival statistics, and specific side-effects into account when evaluating responses and adverse reactions. Also definitive judgements on utility should be held back until these specific characteristics are identified and tested for, which are based on comprehensive understanding of the underlying tumor-, virus- and immunobiology, together with human physiology and oncology. With regards to immunotherapy, time has come to encompass these features, as demonstrated by the vast number of ongoing clinical trials and formed clinical consortia. Ultimately, the emerging scientifically-proven immunotherapeutic approaches can change the way how we see and treat cancer: hopefully to a more optimistic direction.

6. ACKNOWLEDGEMENTS

This thesis work was carried out during the years 2007 – 2014 in the Cancer Gene Therapy Group (CGTG), which was formerly part of the Molecular Cancer Biology Program in Biomedicum Helsinki, University of Helsinki, and currently affiliated to the Department of Pathology and Transplantation Laboratory in Haartman Institute, University of Helsinki, Helsinki, Finland. I am deeply grateful to all the people working in these institutes for their valuable help and guidance during these years.

I want to thank the Dean of the Faculty of Medicine and Chairman of Transplantation Laboratory and former coordinator of the Rational Drug Design Program, Professor Risto Renkonen, for his continuous support and mentoring during my rotation. I also want to thank the former head of Transplantation Laboratory, Professor Pekka Häyry, the present and former directors of Molecular Cancer Biology Program, Professor Jorma Keski-Oja, Academy Professor Kari Alitalo and Professor Marikki Laiho, as well as the head of Biomedicum and Faculty Research Programs, Professor Olli A. Jänne, and the head of Haartman Institute, Professor Tom Böhling, for providing excellent research facilities and pleasant working environments. I want to express my sincere gratitude to the former head of Haartman Institute and the custos of the defense, Professor Seppo Meri, and the honored thesis opponent, Adjunct Professor Michal Besser for making my dissertation possible.

I wish to thank the Doctoral Programme in Biomedicine for the excellent graduate student education, continuous guidance and financial support. Thank you also for the valuable research experience I gained during my rotations in the laboratories of Professor Risto Renkonen, Docent Brendan Battersby, and Docent Eero Jokinen.

I want to express my deepest gratitude to my supervisor and the group leader of CGTG, Akseli Hemminki. Your optimism, initiative and pervasiveness have been truly inspiring to witness. Thank you for believing in me and giving me the opportunity to delve into this exciting and important research field. I am very grateful for your flexibility regarding my busy schedule as a medical student, a Speksi-actor and a graduate student who also wanted to see the world. In CGTG, I have never felt bored, nor doing things in vain. Sure enough, there have been times when PhD studies have stressed my nerves, but thankfully you have always had the time to discuss, answer my e-mails late at night, and encourage me to move forward. I admire your determination and courage, which has concretized in translation of our research “from bench to bedside” and potential benefit to cancer patients – something that very few cancer researchers are capable of achieving. You have also shown great ability to evolve along with science, which has reflected in shifting of our research focus towards immunotherapy; An area of research, which I find so intriguing and promising that I look forward to pursuing further in my postdoctoral studies – I wish to thank you especially for that!

While the research focus of CGTG has shifted from preclinical cancer gene therapy, via patient data experience, on to combination immunotherapy, I have simultaneously witnessed the three phases of our team, transforming from the original Akseli’s angels, via International Center of Action, on to the Adopting Immunotherapists (these nomenclatures may be impacted by the contemporary age and social status of the writer). These three phases and the many great colleagues I have had privilege to work with have really made my days at CGTG unforgettable.

I am sincerely grateful to my three postdoc supervisors, Laura, Sophie and Anniina – All of you have provided valuable advice for my projects and impacted my scientific thinking in different ways. I would also like to express my gratitude to the former postdoc room in Biomedicum, where it was always thrilling to pay a visit due to the great brain mass: thank you Kilian, Tanja, Sari, Anna, Merja, Mari R, Sari, Tuuli and Lotta! By the way, Kilian felt much more leveled already in Baja, in 2009. I also want to thank all the subsequent postdocs of CGTG, especially Markus and Vincenzo, with whom I have had truly inspiring scientific discussions. I would like to thank my hands-on mentors, at first Lotta who introduced me to the brave new world of pipetting and viruses, and then my "handsome" mentor João for introducing me to the mouse, helping me out with experiments and revisions, and for boosting my confidence in taking over the crown of the room.

Research is team-work and the team is what makes it so enjoyable. The CGTG would not have been the same without my dear graduate student friends. Iulia, thanks for being the true spirit of CGTG (and of Apollo with another crazy friend named Roxy). You have Iulia helped me in the lab with so many million things that I can help you out with the sugar piece, no problem. I want to thank Petri for his deep humor and caring mentality, Otto for his action-packed company during many conference trips, and Marko, Marta and Suvi for slack-lining along the PhD life. Thinking of which, there was a weird metamorphing symbiosis with the Aaltonen people and some other Biomedicum friends: thanks for crazy times, beach volley sessions and group-flossings outside the lab Alexandra, Heli, Erkka, Boris, Roxy, Lotta, Otto, and Marko! I am also grateful to all the other CGTG members and friends including Maria, Sergio, Anniina, Marta "U.", Theresia, Vale, Suvi, Topi, Noora, Cris, Matteo, Mari H. and Paula, as well as the current experts Siri, Mikko, Simona, Karo, Kristian and Riikka – thanks for your help in the lab, and for spicing up the PhD life! I also want to express my deep gratitude to Minna for her ever-helpful attitude and patience in endless practical matters, and to the talented technicians Aila, Kikka, Eerika, Saila, Suski, Päivi, Saija, and Maiju, whose laughter and expertise in the lab was highly appreciated.

I would like to thank all the collaborators participated in these studies. Thank you Dr. Marko Laakso, Professor Sampsa Hautaniemi and Dr. Vladia Monsurro for your great help in interpretation and visualization of microarray data, Dr. Fang Zhao for guiding me with the electron microscopy, and Dr. Tiina Hakonen for helping me set up the multivariate models. I would like to thank Professors Ari Ristimäki and Anja Kipar for their expertise in pathology, and Docents Kaarina Partanen and Leena Laasonen for their expertise in radiology. This thesis was enabled by fruitful collaborations with Professor Timo Joensuu, Docrates and Eira Hospitals, and the team at Oncos. And finally, I am deeply grateful to the patients in the ATAP, who made these studies possible.

My sincere gratitude goes to Docents Pirkko Mattila and Kirmo Wartiovaara for your time and effort in reviewing my (longish) thesis, as well as Docents Jaana Pihkala and Brendan Battersby for providing valuable advice and support as my Thesis Committee members.

These studies were financially supported by the Finnish Cultural Foundation, the Finnish Medical Foundation, Emil Aaltonen Foundation, Biomedicum Helsinki Foundation, Finnish-norwegian Medical Foundation, the Finnish Cancer Organizations, Finska Läkaresällskapet, K. Albin Johansson Foundation, Ida Montin Foundation, Finnish Oncology Association and Sigrid Juselius Foundation.

This time of my life has been about studying something that has never been studied before. When combining this with the thought of potential benefit for human kind, and a great team to work with, I could not have hoped for a more inspiring effort.

Exactly ten years ago I decided to apply for med school in University of Helsinki. I got to sleep at my friends' and relatives' places (thank you Heikki, Jaana, Anssi, Maarit and Ikka!), reading Galenos and attending cram school classes, just imagining what kind of adventures and encounters would lie ahead.

I want to thank my last name's initial, because on the first day of med school I got into the dream team of R10: I wish to thank my dear friends Jaakko, Mikko, Eva, Maija and Maria for their decade-long friendship and so many unforgettable memories and inside jokes not-appropriate mentioning here! I would also like to thank all my other med school friends including Stuba, Allu, Simo, Risto, Jenni, Sanna, Martta, Juha, Lauri, Anna, Alex, the late Petri, and many others – Whether it be a course trip in Harjavalta, surfing in Peniche, snurfing in Japan, or dancing the night away in Euphoria, you guys are always up for some peculiar recreational activity.

Life's a b.., but with Speksi it's a child's play. I want to thank all the fantastic people I got to know during my years in LKS-speksi. Acting and having meetings without borders even after finishing the med school is quite a brilliant idea – thank you for that, and let's keep it rolling my dear Aivastus friends Nunnu, Eetu, Matti, Iivo, Harri, Panu, Jouni, Jaakko, Anna O, Anna T, and Antti.

This journey actually started on a playground of Moisio school somewhere in deep Savo, when I met my trusted friends Viljami, Lauri, Perttu, and thereafter Juho and Tuukka. After many turns, thrown snow balls, and swetty sähly matches, these guys with their crazy ideas sparked my passion towards natural sciences. On the way, there have been countless of other friends and relatives who all mean very much to me – Thank you for everything!

My beloved Alexandra, you have truly opened my eyes for the soft side of life. Experiencing the world with you makes everything feel a notch more fascinating. It is quite a coincidence, or totally something else, that it was due to this research that we met and you became from a friend to min vän. I am eagerly looking forward to the many common adventures we have ahead of us.

Finally, I want to thank my beloved family. Lauri and Eero, my dear bros: growing up with you guys has been a privilege. Looking up to and competing with Lauri while still playing spider-man with Eero has probably done something permanent to my brain. Mom and dad, you have given me the hunger for knowledge and drive to wonder. Your love and support have boosted my energy and confidence when needed the most. I really admire both of your strength and wisdom. The few last years have provided such ups and downs that only a strong loving family such as ours could navigate through. Above all, I want to dedicate this thesis to you dad – the true rolling stone.

Helsinki 12.1.2015

Ilkka Liikanen

7. REFERENCES

Ahtiainen, L., C. Mirantes, T. Jahkola, S. Escutenaire, I. Diaconu, P. Osterlund, A. Kanerva, V. Cerullo and A. Hemminki (2010). Defects in innate immunity render breast cancer initiating cells permissive to oncolytic adenovirus. *PLoS One* 5(11): e13859.

Aiuti, A., L. Biasco, S. Scaramuzza, F. Ferrua, M. P. Cicalese, C. Baricordi, F. Dionisio, A. Calabria, S. Giannelli, M. C. Castiello, M. Bosticardo, C. Evangelio, A. Assanelli, M. Casiraghi, S. Di Nunzio, L. Callegaro, C. Benati, P. Rizzardi, D. Pellin, C. Di Serio, M. Schmidt, C. Von Kalle, J. Gardner, N. Mehta, V. Neduva, D. J. Dow, A. Galy, R. Miniero, A. Finocchi, A. Metin, P. P. Banerjee, J. S. Orange, S. Galimberti, M. G. Valsecchi, A. Biffi, E. Montini, A. Villa, F. Ciceri, M. G. Roncarolo and L. Naldini (2013). Lentiviral hematopoietic stem cell gene therapy in patients with wiskott-aldrich syndrome. *Science* 341(6148): 1233151.

Alba, R., A. Bosch and M. Chillon (2005). Gutless adenovirus: Last-generation adenovirus for gene therapy. *Gene Ther* 12 Suppl 1: S18-27.

Alemany, R. and M. Cascallo (2009). Oncolytic viruses from the perspective of the immune system. *Future Microbiol* 4(5): 527-36.

Alexandrov, L. B., S. Nik-Zainal, D. C. Wedge, S. A. Aparicio, S. Behjati, A. V. Biankin, G. R. Bignell, N. Bolli, A. Borg, A. L. Borresen-Dale, S. Boyault, B. Burkhardt, A. P. Butler, C. Caldas, H. R. Davies, C. Desmedt, R. Eils, J. E. Eyfjord, J. A. Foekens, M. Greaves, F. Hosoda, B. Hutter, T. Ilicic, S. Imbeaud, M. Imielinski, N. Jager, D. T. Jones, D. Jones, S. Knappskog, M. Kool, S. R. Lakhani, C. Lopez-Otin, S. Martin, N. C. Munshi, H. Nakamura, P. A. Northcott, M. Pajic, E. Papaemmanuil, A. Paradiso, J. V. Pearson, X. S. Puente, K. Raine, M. Ramakrishna, A. L. Richardson, J. Richter, P. Rosenstiel, M. Schlesner, T. N. Schumacher, P. N. Span, J. W. Teague, Y. Totoki, A. N. Tutt, R. Valdes-Mas, M. M. van Buuren, L. van 't Veer, A. Vincent-Salomon, N. Waddell, L. R. Yates, I. Australian Pancreatic Cancer Genome, I. B. C. Consortium, I. M.-S. Consortium, I. PedBrain, J. Zucman-Rossi, P. A. Futreal, U. McDermott, P. Lichter, M. Meyerson, S. M. Grimmond, R. Siebert, E. Campo, T. Shibata, S. M. Pfister, P. J. Campbell and M. R. Stratton (2013). Signatures of mutational processes in human cancer. *Nature* 500(7463): 415-21.

Alizadeh, D. and N. Larmonier (2014). Chemotherapeutic targeting of cancer-induced immunosuppressive cells. *Cancer Res* 74(10): 2663-8.

Anderson, K. P. and E. H. Fennie (1987). Adenovirus early region 1a modulation of interferon antiviral activity. *J Virol* 61(3): 787-95.

Anderson, S. L., T. Shen, J. Lou, L. Xing, N. E. Blachere, P. K. Srivastava and B. Y. Rubin (1994). The endoplasmic reticular heat shock protein gp96 is transcriptionally upregulated in interferon-treated cells. *J Exp Med* 180(4): 1565-9.

Ando, M., Y. Ando, Y. Hasegawa, K. Shimokata, H. Minami, K. Wakai, Y. Ohno and S. Sakai (2001). Prognostic value of performance status assessed by patients themselves, nurses, and oncologists in advanced non-small cell lung cancer. *Br J Cancer* 85(11): 1634-9.

Andtbacka, R. H. I., F. A. Collichio, T. Amatruda, N. N. Senzer, J. Chesney, K. A. Delman, L. E. Spitler, I. Puzanov, S. Doleman, Y. Ye, A. M. Vanderwalde, R. Coffin and H. Kaufman (2013). Optim: A randomized phase iii trial of talimogene laherparepvec (t-vec) versus subcutaneous (sc) granulocyte-macrophage colony-stimulating factor (gm-csf) for the treatment (tx) of unresected stage iiib/c and iv melanoma. *Proceedings of the ASCO Annual Meeting*, *Journal of Clinical Oncology*.

Apetoh, L., F. Ghiringhelli, A. Tesniere, A. Criollo, C. Ortiz, R. Lidereau, C. Mariette, N. Chaput, J. P. Mira, S. Delaloge, F. Andre, T. Tursz, G. Kroemer and L. Zitvogel (2007). The interaction between hmgb1 and tlr4 dictates the outcome of anticancer chemotherapy and radiotherapy. *Immunol Rev* 220: 47-59.

Apetoh, L., A. Tesniere, F. Ghiringhelli, G. Kroemer and L. Zitvogel (2008). Molecular interactions between dying tumor cells and the innate immune system determine the efficacy of conventional anticancer therapies. *Cancer Res* 68(11): 4026-30.

Appledorn, D. M., S. Patial, A. McBride, S. Godbehere, N. Van Rooijen, N. Parameswaran and A. Amalfitano (2008). Adenovirus vector-induced innate inflammatory mediators, mapk signaling, as well as adaptive immune responses are dependent upon both tlr2 and tlr9 in vivo. *J Immunol* 181(3): 2134-44.

Aranda, F., E. Vacchelli, A. Eggermont, J. Galon, C. Sautes-Fridman, E. Tartour, L. Zitvogel, G. Kroemer and L. Galluzzi (2013). Trial watch: Peptide vaccines in cancer therapy. *Oncoimmunology* 2(12): e26621.

Arap, W., R. Pasqualini and E. Ruoslahti (1998). Cancer treatment by targeted drug delivery to tumor vasculature in a mouse model. *Science* 279(5349): 377-80.

Araujo, F. D., T. H. Stracker, C. T. Carson, D. V. Lee and M. D. Weitzman (2005). Adenovirus type 5 e4orf3 protein targets the mre11 complex to cytoplasmic aggresomes. *J Virol* 79(17): 11382-91.

Arellano, M. and S. Lonial (2008). Clinical uses of gm-csf, a critical appraisal and update. *Biologics* 2(1): 13-27.

Arnberg, N. (2012). Adenovirus receptors: Implications for targeting of viral vectors. *Trends Pharmacol Sci* 33(8): 442-8.

Ashburner, M., C. A. Ball, J. A. Blake, D. Botstein, H. Butler, J. M. Cherry, A. P. Davis, K. Dolinski, S. S. Dwight, J. T. Eppig, M. A. Harris, D. P. Hill, L. Issel-Tarver, A. Kasarskis, S. Lewis, J. C. Matese, J. E. Richardson, M. Ringwald, G. M. Rubin and G. Sherlock (2000). Gene ontology: Tool for the unification of biology. The gene ontology consortium. *Nat Genet* 25(1): 25-9.

Atkins, M. B., M. T. Lotze, J. P. Dutcher, R. I. Fisher, G. Weiss, K. Margolin, J. Abrams, M. Sznol, D. Parkinson, M. Hawkins, C. Paradise, L. Kunkel and S. A. Rosenberg (1999). High-dose recombinant interleukin 2 therapy for patients with metastatic melanoma: Analysis of 270 patients treated between 1985 and 1993. *J Clin Oncol* 17(7): 2105-16.

Bakacs, T., J. N. Mehrishi and R. W. Moss (2012). Ipilimumab (yervoy) and the tgn1412 catastrophe. *Immunobiology* 217(6): 583-9.

Banissi, C., F. Ghiringhelli, L. Chen and A. F. Carpentier (2009). Treg depletion with a low-dose metronomic temozolomide regimen in a rat glioma model. *Cancer Immunol Immunother* 58(10): 1627-34.

Bao, G., Q. Qiao, H. Zhao and X. He (2010). Prognostic value of hmgb1 overexpression in resectable gastric adenocarcinomas. *World J Surg Oncol* 8: 52.

Bauerschmitz, G. J., K. Guse, A. Kanerva, A. Menzel, I. Herrmann, R. A. Desmond, M. Yamamoto, D. M. Nettelbeck, T. Hakkarainen, P. Dall, D. T. Curiel and A. Hemminki (2006). Triple-targeted oncolytic adenoviruses featuring the cox2 promoter, e1a transcomplementation, and serotype chimerism for enhanced selectivity for ovarian cancer cells. *Mol Ther* 14(2): 164-74.

Bayo-Puxan, N., M. Gimenez-Alejandre, S. Lavilla-Alonso, A. Gros, M. Cascallo, A. Hemminki and R. Alemany (2009). Replacement of adenovirus type 5 fiber shaft heparan sulfate proteoglycan-binding domain with rgd for improved tumor infectivity and targeting. *Hum Gene Ther* 20(10): 1214-21.

Belousova, N., N. Korokhov, V. Krendelshchikova, V. Simonenko, G. Mikheeva, P. L. Triozzi, W. A. Aldrich, P. T. Banerjee, S. D. Gillies, D. T. Curiel and V. Krasnykh (2003). Genetically targeted adenovirus vector directed to cd40-expressing cells. *J Virol* 77(21): 11367-77.

Bennett, E. M., J. R. Bennink, J. W. Yewdell and F. M. Brodsky (1999). Cutting edge: Adenovirus e19 has two mechanisms for affecting class i mhc expression. *J Immunol* 162(9): 5049-52.

Bergelson, J. M., J. A. Cunningham, G. Droguett, E. A. Kurt-Jones, A. Krithivas, J. S. Hong, M. S. Horwitz, R. L. Crowell and R. W. Finberg (1997). Isolation of a common receptor for coxsackie b viruses and adenoviruses 2 and 5. *Science* 275(5304): 1320-3.

Berk, A. J. (2005). Recent lessons in gene expression, cell cycle control, and cell biology from adenovirus. *Oncogene* 24(52): 7673-85.

Biffi, A., E. Montini, L. Lorioli, M. Cesani, F. Fumagalli, T. Plati, C. Baldoli, S. Martino, A. Calabria, S. Canale, F. Benedicenti, G. Vallanti, L. Biasco, S. Leo, N. Kabbara, G. Zanetti, W. B. Rizzo, N. A. Mehta, M. P. Cicalese, M. Casiraghi, J. J. Boelens, U. Del Carro, D. J. Dow, M. Schmidt, A. Assanelli, V. Neduva, C. Di Serio, E. Stupka, J. Gardner, C. von Kalle, C. Bordignon, F. Ciceri, A. Rovelli, M. G. Roncarolo, A. Aiuti, M. Sessa and L. Naldini (2013). Lentiviral hematopoietic stem cell gene therapy benefits metachromatic leukodystrophy. *Science* 341(6148): 1233158.

Bilbao, G., J. L. Contreras, I. Dmitriev, C. A. Smyth, S. Jenkins, D. Eckhoff, F. Thomas, J. Thomas and D. T. Curiel (2002). Genetically modified adenovirus vector containing an rgd peptide in the hi loop of the fiber knob improves gene transfer to nonhuman primate isolated pancreatic islets. *Am J Transplant* 2(3): 237-43.

Bischoff, J. R., D. H. Kirn, A. Williams, C. Heise, S. Horn, M. Muna, L. Ng, J. A. Nye, A. Sampson-Johannes, A. Fattaey and F. McCormick (1996). An adenovirus mutant that replicates selectively in p53-deficient human tumor cells. *Science* 274(5286): 373-6.

Bortolanza, S., M. Bunuales, P. Alzuguren, O. Lamas, R. Aldabe, J. Prieto and R. Hernandez-Alcoceba (2009a). Deletion of the e3-6.7k/gp19k region reduces the persistence of wild-type adenovirus in a permissive tumor model in syrian hamsters. *Cancer Gene Ther* 16(9): 703-12.

Bortolanza, S., M. Bunuales, I. Otano, G. Gonzalez-Aseguinolaza, C. Ortiz-de-Solorzano, D. Perez, J. Prieto and R. Hernandez-Alcoceba (2009b). Treatment of pancreatic cancer with an oncolytic adenovirus expressing interleukin-12 in syrian hamsters. *Mol Ther* 17(4): 614-22.

Brahmer, J. R., C. G. Drake, I. Wollner, J. D. Powderly, J. Picus, W. H. Sharfman, E. Stankevich, A. Pons, T. M. Salay, T. L. McMiller, M. M. Gilson, C. Wang, M. Selby, J. M. Taube, R. Anders, L. Chen, A. J. Korman, D. M. Pardoll, I. Lowy and S. L. Topalian (2010). Phase i study of single-agent anti-programmed death-1 (mdx-1106) in refractory solid tumors: Safety, clinical activity, pharmacodynamics, and immunologic correlates. *J Clin Oncol* 28(19): 3167-75.

Bramante, S., A. Koski, A. Kipar, I. Diaconu, I. Liikanen, O. Hemminki, L. Vassilev, S. Parviainen, V. Cerullo, S. K. Pesonen, M. Oksanen, R. Heiskanen, N. Rouvinen-Lagerstrom, M. Merisalo-Soikkeli, T. Hakonen, T. Joensuu, A. Kanerva, S. Pesonen and A. Hemminki (2014). Serotype chimeric oncolytic adenovirus coding for gm-csf for treatment of sarcoma in rodents and humans. *Int J Cancer* 135(3): 720-30.

Brand, K., R. Klocke, A. Possling, D. Paul and M. Strauss (1999). Induction of apoptosis and g2/m arrest by infection with replication-deficient adenovirus at high multiplicity of infection. *Gene Ther* 6(6): 1054-63.

Brand, K., P. Loser, W. Arnold, T. Bartels and M. Strauss (1998). Tumor cell-specific transgene expression prevents liver toxicity of the adeno-hsvtk/gcv approach. *Gene Ther* 5(10): 1363-71.

Burdelya, L., M. Kujawski, G. Niu, B. Zhong, T. Wang, S. Zhang, M. Kortylewski, K. Shain, H. Kay, J. Djeu, W. Dalton, D. Pardoll, S. Wei and H. Yu (2005). Stat3 activity in melanoma cells affects migration of immune effector cells and nitric oxide-mediated antitumor effects. *J Immunol* 174(7): 3925-31.

Burnet, F. M. (1970). The concept of immunological surveillance. *Prog Exp Tumor Res* 13: 1-27.

Burroughs, K. D., D. B. Kayda, K. Sakhuja, Y. Hudson, J. Jakubczak, J. A. Bristol, D. Ennist, P. Hallenbeck, M. Kaleko and S. Connelly (2004). Potentiation of oncolytic adenoviral vector efficacy with gutless vectors encoding gmcsf or trail. *Cancer Gene Ther* 11(2): 92-102.

Campos, S. K. and M. A. Barry (2007). Current advances and future challenges in adenoviral vector biology and targeting. *Curr Gene Ther* 7(3): 189-204.

Carney, J. P., R. S. Maser, H. Olivares, E. M. Davis, M. Le Beau, J. R. Yates, 3rd, L. Hays, W. F. Morgan and J. H. Petrini (1998). The hmre11/hrad50 protein complex and nijmegen breakage syndrome: Linkage of double-strand break repair to the cellular DNA damage response. *Cell* 93(3): 477-86.

Carson, C. T., R. A. Schwartz, T. H. Stracker, C. E. Lilley, D. V. Lee and M. D. Weitzman (2003). The mre11 complex is required for atm activation and the g2/m checkpoint. *EMBO J* 22(24): 6610-20.

Cathcart, M. C., K. J. O'Byrne, J. V. Reynolds, J. O'Sullivan and G. P. Pidgeon (2012). Cox-derived prostanoid pathways in gastrointestinal cancer development and progression: Novel targets for prevention and intervention. *Biochim Biophys Acta* 1825(1): 49-63.

Cavazzana-Calvo, M., S. Hacein-Bey, G. de Saint Basile, F. Gross, E. Yvon, P. Nusbaum, F. Selz, C. Hue, S. Certain, J. L. Casanova, P. Bousso, F. L. Deist and A. Fischer (2000). Gene therapy of human severe combined immunodeficiency (scid)-x1 disease. *Science* 288(5466): 669-72.

Cerami, E. G., G. D. Bader, B. E. Gross and C. Sander (2006). Cpath: Open source software for collecting, storing, and querying biological pathways. *BMC Bioinformatics* 7: 497.

Cerullo, V., I. Diaconu, L. Kangasniemi, M. Rajecki, S. Escutenaire, A. Koski, V. Romano, N. Rouvinen, T. Tuuminen, L. Laasonen, K. Partanen, S. Kauppinen, T. Joensuu, M. Oksanen, S. L. Holm, E. Haavisto, A. Karioja-Kallio, A. Kanerva, S. Pesonen, P. T. Arstila and A. Hemminki (2011). Immunological effects of low-dose cyclophosphamide in cancer patients treated with oncolytic adenovirus. *Mol Ther* 19(9): 1737-46.

Cerullo, V., S. Pesonen, I. Diaconu, S. Escutenaire, P. T. Arstila, M. Ugolini, P. Nokisalmi, M. Raki, L. Laasonen, M. Sarkioja, M. Rajecki, L. Kangasniemi, K. Guse, A. Helminen, L. Ahtiainen, A. Ristimaki, A. Raisanen-Sokolowski, E. Haavisto, M. Oksanen, E. Karli, A. Karioja-Kallio, S. L. Holm, M. Kouri, T. Joensuu, A. Kanerva and A. Hemminki (2010). Oncolytic adenovirus coding for granulocyte macrophage colony-stimulating factor induces antitumoral immunity in cancer patients. *Cancer Res* 70(11): 4297-309.

Chaly, N. and X. Chen (1993). Assembly of adenovirus-specific nuclear inclusions in lytically infected hela cells: An ultrastructural and cytochemical study. *Biochem Cell Biol* 71(9-10): 475-87.

Chambers, C. A., M. S. Kuhns, J. G. Egen and J. P. Allison (2001). Ctla-4-mediated inhibition in regulation of t cell responses: Mechanisms and manipulation in tumor immunotherapy. *Annu Rev Immunol* 19: 565-94.

Chang, D. Z., W. Lomazow, C. Joy Somberg, R. Stan and M. A. Perales (2004a). Granulocyte-macrophage colony stimulating factor: An adjuvant for cancer vaccines. *Hematology* 9(3): 207-15.

Chang, J., X. Zhao, X. Wu, Y. Guo, H. Guo, J. Cao, Y. Guo, D. Lou, D. Yu and J. Li (2009). A phase i study of kh901, a conditionally replicating granulocyte-macrophage colony-stimulating factor: Armed oncolytic adenovirus for the treatment of head and neck cancers. *Cancer Biol Ther* 8(8): 676-82.

Chang, K. J., T. Reid, N. Senzer, S. Swisher, H. Pinto, N. Hanna, A. Chak and R. Soetikno (2012). Phase i evaluation of tnferade biologic plus chemoradiotherapy before esophagectomy for locally advanced resectable esophageal cancer. *Gastrointest Endosc* 75(6): 1139-46 e2.

Chang, S. M., K. R. Lamborn, M. Malec, D. Larson, W. Wara, P. Sneed, J. Rabbitt, M. Page, M. K. Nicholas and M. D. Prados (2004b). Phase ii study of temozolomide and thalidomide with radiation therapy for newly diagnosed glioblastoma multiforme. *Int J Radiat Oncol Biol Phys* 60(2): 353-7.

Chang, Y. W., M. C. Hung and J. L. Su (2014). The anti-tumor activity of e1a and its implications in cancer therapy. *Arch Immunol Ther Exp (Warsz)* 62(3): 195-204.

Chen, N. and V. Karantza (2011). Autophagy as a therapeutic target in cancer. *Cancer Biol Ther* 11(2): 157-68.

Chikamatsu, K., K. Sakakura, T. L. Whiteside and N. Furuya (2007). Relationships between regulatory t cells and cd8+ effector populations in patients with squamous cell carcinoma of the head and neck. *Head Neck* 29(2): 120-7.

Chiocca, E. A., K. M. Smith, B. McKinney, C. A. Palmer, S. Rosenfeld, K. Lillehei, A. Hamilton, B. K. DeMasters, K. Judy and D. Kirn (2008). A phase i trial of ad.Hifn-beta gene therapy for glioma. *Mol Ther* 16(3): 618-26.

Choi, K. J., J. H. Kim, Y. S. Lee, J. Kim, B. S. Suh, H. Kim, S. Cho, J. H. Sohn, G. E. Kim and C. O. Yun (2006). Concurrent delivery of gm-csf and b7-1 using an oncolytic adenovirus elicits potent antitumor effect. *Gene Ther* 13(13): 1010-20.

Chou, T. C. (2010). Drug combination studies and their synergy quantification using the chou-talalay method. *Cancer Res* 70(2): 440-6.

Chroboczek, J., F. Bieber and B. Jacrot (1992). The sequence of the genome of adenovirus type 5 and its comparison with the genome of adenovirus type 2. *Virology* 186(1): 280-5.

Chu, Y., D. Heistad, M. I. Cybulsky and B. L. Davidson (2001). Vascular cell adhesion molecule-1 augments adenovirus-mediated gene transfer. *Arterioscler Thromb Vasc Biol* 21(2): 238-42.

Cohen, C. J., J. T. Shieh, R. J. Pickles, T. Okegawa, J. T. Hsieh and J. M. Bergelson (2001). The coxsackievirus and adenovirus receptor is a transmembrane component of the tight junction. *Proc Natl Acad Sci U S A* 98(26): 15191-6.

Corthay, A. (2014). Does the immune system naturally protect against cancer? *Front Immunol* 5: 197.

Coussens, L. M., L. Zitvogel and A. K. Palucka (2013). Neutralizing tumor-promoting chronic inflammation: A magic bullet? *Science* 339(6117): 286-91.

Couzin-Frankel, J. (2013). Breakthrough of the year 2013. Cancer immunotherapy. *Science* 342(6165): 1432-3.

Cox, M. A., S. M. Kahan and A. J. Zajac (2013). Anti-viral cd8 t cells and the cytokines that they love. *Virology* 435(1): 157-69.

Cramarossa, G., E. K. Lee, L. Sivanathan, S. Georgsdottir, K. Lien, K. D. Santos, K. Chan and U. Emmenegger (2014). A systematic literature analysis of correlative studies in low-dose metronomic chemotherapy trials. *Biomark Med* 8(6): 893-911.

Crawford, E. D. (2004). Hormonal therapy in prostate cancer: Historical approaches. *Rev Urol* 6 Suppl 7: S3-S11.

Critchley-Thorne, R. J., D. L. Simons, N. Yan, A. K. Miyahira, F. M. Dirbas, D. L. Johnson, S. M. Swetter, R. W. Carlson, G. A. Fisher, A. Koong, S. Holmes and P. P. Lee (2009). Impaired interferon signaling is a common immune defect in human cancer. *Proc Natl Acad Sci U S A* 106(22): 9010-5.

Cuconati, A., C. Mukherjee, D. Perez and E. White (2003). DNA damage response and mcl-1 destruction initiate apoptosis in adenovirus-infected cells. *Genes Dev* 17(23): 2922-32.

Curran, M. A., M. K. Callahan, S. K. Subudhi and J. P. Allison (2012). Response to "ipilimumab (yervoy) and the tgn1412 catastrophe". *Immunobiology* 217(6): 590-2.

Curtin, J. F., N. Liu, M. Candolfi, W. Xiong, H. Assi, K. Yagiz, M. R. Edwards, K. S. Michelsen, K. M. Kroeger, C. Liu, A. K. Muhammad, M. C. Clark, M. Arditi, B. Comin-Anduix, A. Ribas, P. R. Lowenstein and M. G. Castro (2009). Hmgb1 mediates endogenous tlr2 activation and brain tumor regression. *PLoS Med* 6(1): e10.

Daniel, C., J. Nolting and H. von Boehmer (2009). Mechanisms of self-nonself discrimination and possible clinical relevance. *Immunotherapy* 1(4): 631-44.

Dechecchi, M. C., P. Melotti, A. Bonizzato, M. Santacatterina, M. Chilosi and G. Cabrini (2001). Heparan sulfate glycosaminoglycans are receptors sufficient to mediate the initial binding of adenovirus types 2 and 5. *J Virol* 75(18): 8772-80.

Demarest, S. J., K. Hariharan and J. Dong (2011). Emerging antibody combinations in oncology. *MAbs* 3(4): 338-51.

Dengjel, J., O. Schoor, R. Fischer, M. Reich, M. Kraus, M. Muller, K. Kreymborg, F. Altenberend, J. Brandenburg, H. Kalbacher, R. Brock, C. Driessen, H. G. Rammensee and S. Stevanovic (2005). Autophagy promotes mhc class ii presentation of peptides from intracellular source proteins. *Proc Natl Acad Sci U S A* 102(22): 7922-7.

DeWeese, T. L., H. van der Poel, S. Li, B. Mikhak, R. Drew, M. Goemann, U. Hamper, R. DeJong, N. Detorie, R. Rodriguez, T. Haulk, A. M. DeMarzo, S. Piantadosi, D. C. Yu, Y. Chen, D. R. Henderson, M. A. Carducci, W. G. Nelson and J. W. Simons (2001). A phase i trial of cv706, a replication-competent, psa selective oncolytic adenovirus, for the treatment of locally recurrent prostate cancer following radiation therapy. *Cancer Res* 61(20): 7464-72.

Diaconu, I., V. Cerullo, M. L. Hirvinen, S. Escutenaire, M. Ugolini, S. K. Pesonen, S. Bramante, S. Parviainen, A. Kanerva, A. S. Loskog, A. G. Eliopoulos, S. Pesonen and A. Hemminki (2012). Immune response is an important aspect of the antitumor effect produced by a cd40l-encoding oncolytic adenovirus. *Cancer Res* 72(9): 2327-38.

Diallo, J. S., F. Le Boeuf, F. Lai, J. Cox, M. Vaha-Koskela, H. Abdelbary, H. MacTavish, K. Waite, T. Falls, J. Wang, R. Brown, J. E. Blanchard, E. D. Brown, D. H. Kirn, J. Hiscott, H. Atkins, B. D. Lichty and J. C. Bell (2010). A high-throughput pharmacoviral approach identifies novel oncolytic virus sensitizers. *Mol Ther* 18(6): 1123-9.

Dias, J. D., K. Guse, P. Nokisalmi, M. Eriksson, D. T. Chen, I. Diaconu, M. Tenhunen, I. Liikanen, R. Grenman, M. Savontaus, S. Pesonen, V. Cerullo and A. Hemminki (2010). Multimodal approach using oncolytic adenovirus, cetuximab, chemotherapy and radiotherapy in hnscc low passage tumour cell cultures. *Eur J Cancer* 46(3): 625-35.

Dias, J. D., O. Hemminki, I. Diaconu, M. Hirvinen, A. Bonetti, K. Guse, S. Escutenaire, A. Kanerva, S. Pesonen, A. Loskog, V. Cerullo and A. Hemminki (2012). Targeted cancer immunotherapy with oncolytic adenovirus coding for a fully human monoclonal antibody specific for ctla-4. *Gene Ther* 19(10): 988-98.

Diaz, R. M., F. Galivo, T. Kottke, P. Wongthida, J. Qiao, J. Thompson, M. Valdes, G. Barber and R. G. Vile (2007). Oncolytic immunovirotherapy for melanoma using vesicular stomatitis virus. *Cancer Res* 67(6): 2840-8.

Dix, B. R., S. J. Edwards and A. W. Braithwaite (2001). Does the antitumor adenovirus onyx-015/dl1520 selectively target cells defective in the p53 pathway? *J Virol* 75(12): 5443-7.

Dmitriev, I., V. Krasnykh, C. R. Miller, M. Wang, E. Kashentseva, G. Mikheeva, N. Belousova and D. T. Curiel (1998). An adenovirus vector with genetically modified fibers demonstrates expanded tropism via utilization of a coxsackievirus and adenovirus receptor-independent cell entry mechanism. *J Virol* 72(12): 9706-13.

Dougan, M. and G. Dranoff (2009). Immune therapy for cancer. *Annu Rev Immunol* 27: 83-117.

Douglas, J. T., B. E. Rogers, M. E. Rosenfeld, S. I. Michael, M. Feng and D. T. Curiel (1996). Targeted gene delivery by tropism-modified adenoviral vectors. *Nat Biotechnol* 14(11): 1574-8.

Dunn, G. P., A. T. Bruce, H. Ikeda, L. J. Old and R. D. Schreiber (2002). Cancer immunoediting: From immunosurveillance to tumor escape. *Nat Immunol* 3(11): 991-8.

Dunn, G. P., L. J. Old and R. D. Schreiber (2004). The three es of cancer immunoediting. *Annu Rev Immunol* 22: 329-60.

Durinck, S. (2008). Pre-processing of microarray data and analysis of differential expression. *Methods Mol Biol* 452: 89-110.

Early Breast Cancer Trialists' Collaborative, G., R. Peto, C. Davies, J. Godwin, R. Gray, H. C. Pan, M. Clarke, D. Cutter, S. Darby, P. McGale, C. Taylor, Y. C. Wang, J. Bergh, A. Di Leo, K. Albain, S. Swain, M. Piccart and K. Pritchard (2012). Comparisons between different polychemotherapy regimens for early breast cancer: Meta-analyses of long-term outcome among 100,000 women in 123 randomised trials. *Lancet* 379(9814): 432-44.

Eisenhauer, E. A., P. Therasse, J. Bogaerts, L. H. Schwartz, D. Sargent, R. Ford, J. Dancey, S. Arbuck, S. Gwyther, M. Mooney, L. Rubinstein, L. Shankar, L. Dodd, R. Kaplan, D. Lacombe and J. Verweij (2009). New response evaluation criteria in solid tumours: Revised recist guideline (version 1.1). *Eur J Cancer* 45(2): 228-47.

Emens, L. A. (2012). Re-purposing cancer therapeutics for breast cancer immunotherapy. *Cancer Immunol Immunother* 61(8): 1299-305.

Escutenaire, S., V. Cerullo, I. Diaconu, L. Ahtiainen, P. Hannuksela, M. Oksanen, E. Haavisto, A. Karioja-Kallio, S. L. Holm, L. Kangasniemi, C. Ribacka, S. Kauppinen, T. Joensuu, T. P. Arstila, S. Pesonen, A.

Kanerva and A. Hemminki (2011). In vivo and in vitro distribution of type 5 and fiber-modified oncolytic adenoviruses in human blood compartments. *Ann Med* 43(2): 151-63.

Ezzeddine, Z. D., R. L. Martuza, D. Platika, M. P. Short, A. Malick, B. Choi and X. O. Breakefield (1991). Selective killing of glioma cells in culture and in vivo by retrovirus transfer of the herpes simplex virus thymidine kinase gene. *New Biol* 3(6): 608-14.

Fahmueller, Y. N., D. Nagel, R. T. Hoffmann, K. Tatsch, T. Jakobs, P. Stieber and S. Holdenrieder (2013). Immunogenic cell death biomarkers hmgb1, rage, and dnase indicate response to radioembolization therapy and prognosis in colorectal cancer patients. *Int J Cancer* 132(10): 2349-58.

Feig, C., J. O. Jones, M. Kraman, R. J. Wells, A. Deonarine, D. S. Chan, C. M. Connell, E. W. Roberts, Q. Zhao, O. L. Caballero, S. A. Teichmann, T. Janowitz, D. I. Jodrell, D. A. Tuveson and D. T. Fearon (2013). Targeting cxcl12 from fap-expressing carcinoma-associated fibroblasts synergizes with anti-pd-l1 immunotherapy in pancreatic cancer. *Proc Natl Acad Sci U S A* 110(50): 20212-7.

Ferlay, J., I. I. Soerjomataram, R. Dikshit, S. Eser, C. Mathers, M. Rebelo, D. M. Parkin, D. D. Forman and F. Bray (2014). Cancer incidence and mortality worldwide: Sources, methods and major patterns in globocan 2012. *Int J Cancer*.

Ferlazzo, G. and B. Morandi (2014). Cross-talks between natural killer cells and distinct subsets of dendritic cells. *Front Immunol* 5: 159.

Ferlazzo, G. and C. Munz (2004). Nk cell compartments and their activation by dendritic cells. *J Immunol* 172(3): 1333-9.

Fleischli, C., D. Sirena, G. Lesage, M. J. Havenga, R. Cattaneo, U. F. Greber and S. Hemmi (2007). Species b adenovirus serotypes 3, 7, 11 and 35 share similar binding sites on the membrane cofactor protein cd46 receptor. *J Gen Virol* 88(Pt 11): 2925-34.

Forbes, S. A., G. Tang, N. Bindal, S. Bamford, E. Dawson, C. Cole, C. Y. Kok, M. Jia, R. Ewing, A. Menzies, J. W. Teague, M. R. Stratton and P. A. Futreal (2010). Cosmic (the catalogue of somatic mutations in cancer): A resource to investigate acquired mutations in human cancer. *Nucleic Acids Res* 38(Database issue): D652-7.

Freytag, S. O., H. Stricker, J. Peabody, J. Pegg, D. Paielli, B. Movsas, K. N. Barton, S. L. Brown, M. Lu and J. H. Kim (2007). Five-year follow-up of trial of replication-competent adenovirus-mediated suicide gene therapy for treatment of prostate cancer. *Mol Ther* 15(3): 636-42.

Friggeri, A., Y. Yang, S. Banerjee, Y. J. Park, G. Liu and E. Abraham (2010). Hmgb1 inhibits macrophage activity in efferocytosis through binding to the alphavbeta3-integrin. *Am J Physiol Cell Physiol* 299(6): C1267-76.

Fueyo, J., C. Gomez-Manzano, R. Alemany, P. S. Lee, T. J. McDonnell, P. Mitlianga, Y. X. Shi, V. A. Levin, W. K. Yung and A. P. Kyritsis (2000). A mutant oncolytic adenovirus targeting the rb pathway produces anti-glioma effect in vivo. *Oncogene* 19(1): 2-12.

Fujiwara, T., Y. Urata and N. Tanaka (2008). Diagnostic and therapeutic application of telomerase-specific oncolytic adenoviral agents. *Front Biosci* 13: 1881-6.

Fukazawa, T., J. Matsuoka, T. Yamatsuji, Y. Maeda, M. L. Durbin and Y. Naomoto (2010). Adenovirus-mediated cancer gene therapy and virotherapy (review). *Int J Mol Med* 25(1): 3-10.

Fukuhara, H. and T. Todo (2007). Oncolytic herpes simplex virus type 1 and host immune responses. *Curr Cancer Drug Targets* 7(2): 149-55.

Gaggar, A., D. M. Shayakhmetov and A. Lieber (2003). Cd46 is a cellular receptor for group b adenoviruses. *Nat Med* 9(11): 1408-12.

Gajewski, T. F., H. Schreiber and Y. X. Fu (2013). Innate and adaptive immune cells in the tumor microenvironment. *Nat Immunol* 14(10): 1014-22.

Galanis, E., S. H. Okuno, A. G. Nascimento, B. D. Lewis, R. A. Lee, A. M. Oliveira, J. A. Sloan, P. Atherton, J. H. Edmonson, C. Erlichman, B. Randlev, Q. Wang, S. Freeman and J. Rubin (2005). Phase i-ii trial of

onyx-015 in combination with map chemotherapy in patients with advanced sarcomas. *Gene Ther* 12(5): 437-45.

Ganly, I., D. Kirn, G. Eckhardt, G. I. Rodriguez, D. S. Soutar, R. Otto, A. G. Robertson, O. Park, M. L. Gulley, C. Heise, D. D. Von Hoff and S. B. Kaye (2000). A phase i study of onyx-015, an e1b attenuated adenovirus, administered intratumorally to patients with recurrent head and neck cancer. *Clin Cancer Res* 6(3): 798-806.

Gao, S., X. J. Yang, W. G. Zhang, Y. W. Ji and Q. Pan (2009). Mechanism of thalidomide to enhance cytotoxicity of temozolomide in u251-mg glioma cells in vitro. *Chin Med J (Engl)* 122(11): 1260-6.

Garber, K. (2006). China approves world's first oncolytic virus therapy for cancer treatment. *J Natl Cancer Inst* 98(5): 298-300.

Gatei, M., A. W. Kijas, D. Biard, T. Dork and M. F. Lavin (2014). Rad50 phosphorylation promotes atr downstream signaling and DNA restart following replication stress. *Hum Mol Genet* 23(16): 4232-48.

Gaudet, D., J. Methot, S. Dery, D. Brisson, C. Essiembre, G. Tremblay, K. Tremblay, J. de Wal, J. Twisk, N. van den Bulk, V. Sier-Ferreira and S. van Deventer (2013). Efficacy and long-term safety of alipogene tiparvovec (aav1-lpls447x) gene therapy for lipoprotein lipase deficiency: An open-label trial. *Gene Ther* 20(4): 361-9.

Geynisman, D. M., C. R. Chien, F. Smieliauskas, C. Shen and Y. C. Tina Shih (2014). Economic evaluation of therapeutic cancer vaccines and immunotherapy: A systematic review. *Hum Vaccin Immunother* 10(11).

Ghiringhelli, F., N. Larmonier, E. Schmitt, A. Parcellier, D. Cathelin, C. Garrido, B. Chauffert, E. Solary, B. Bonnotte and F. Martin (2004). Cd4+cd25+ regulatory t cells suppress tumor immunity but are sensitive to cyclophosphamide which allows immunotherapy of established tumors to be curative. *Eur J Immunol* 34(2): 336-44.

Ghiringhelli, F., C. Menard, P. E. Puig, S. Ladoire, S. Roux, F. Martin, E. Solary, A. Le Cesne, L. Zitvogel and B. Chauffert (2007). Metronomic cyclophosphamide regimen selectively depletes cd4+cd25+ regulatory t cells and restores t and nk effector functions in end stage cancer patients. *Cancer Immunol Immunother* 56(5): 641-8.

Ginn, S. L., I. E. Alexander, M. L. Edelstein, M. R. Abedi and J. Wixon (2013). Gene therapy clinical trials worldwide to 2012 - an update. *J Gene Med* 15(2): 65-77.

Golden, E. B., I. Pellicciotta, S. Demaria, M. H. Barcellos-Hoff and S. C. Formenti (2012). The convergence of radiation and immunogenic cell death signaling pathways. *Front Oncol* 2: 88.

Good, J. S. and K. J. Harrington (2013). The hallmarks of cancer and the radiation oncologist: Updating the 5rs of radiobiology. *Clin Oncol (R Coll Radiol)* 25(10): 569-77.

Goodman, L. S., M. M. Wintrobe, W. Dameshek, M. J. Goodman, A. Gilman and M. T. McLennan (1984). Landmark article sept. 21, 1946: Nitrogen mustard therapy. Use of methyl-bis(beta-chloroethyl)amine hydrochloride and tris(beta-chloroethyl)amine hydrochloride for hodgkin's disease, lymphosarcoma, leukemia and certain allied and miscellaneous disorders. By louis s. Goodman, maxwell m. Wintrobe, william dameshek, morton j. Goodman, alfred gilman and margaret t. Mclennan. *JAMA* 251(17): 2255-61.

Greber, U. F., M. Suomalainen, R. P. Stidwill, K. Boucke, M. W. Ebersold and A. Helenius (1997). The role of the nuclear pore complex in adenovirus DNA entry. *EMBO J* 16(19): 5998-6007.

Green, D. R., T. Ferguson, L. Zitvogel and G. Kroemer (2009). Immunogenic and tolerogenic cell death. *Nat Rev Immunol* 9(5): 353-63.

Grewal, I. S. and R. A. Flavell (1998). Cd40 and cd154 in cell-mediated immunity. *Annu Rev Immunol* 16: 111-35.

Groves, M. D., V. K. Puduvalli, S. M. Chang, C. A. Conrad, M. R. Gilbert, I. W. Tremont-Lukats, T. J. Liu, P. Peterson, D. Schiff, T. F. Cloughesy, P. Y. Wen, H. Greenberg, L. E. Abrey, L. M. DeAngelis, K. R. Hess, K. R. Lamborn, M. D. Prados and W. K. Yung (2007). A north american brain tumor consortium (nabtc

99-04) phase ii trial of temozolomide plus thalidomide for recurrent glioblastoma multiforme. *J Neurooncol* 81(3): 271-7.

Grupp, S. A., M. Kalos, D. Barrett, R. Aplenc, D. L. Porter, S. R. Rheingold, D. T. Teachey, A. Chew, B. Hauck, J. F. Wright, M. C. Milone, B. L. Levine and C. H. June (2013). Chimeric antigen receptor-modified t cells for acute lymphoid leukemia. *N Engl J Med* 368(16): 1509-18.

Guo, J. and H. Xin (2006). Chinese gene therapy. Splicing out the west? *Science* 314(5803): 1232-5.

Guo, Z. S., Z. Liu and D. L. Bartlett (2014). Oncolytic immunotherapy: Dying the right way is a key to eliciting potent antitumor immunity. *Front Oncol* 4: 74.

Guo, Z. S., Z. Liu, D. L. Bartlett, D. Tang and M. T. Lotze (2013). Life after death: Targeting high mobility group box 1 in emergent cancer therapies. *Am J Cancer Res* 3(1): 1-20.

Hacein-Bey-Abina, S., J. Hauer, A. Lim, C. Picard, G. P. Wang, C. C. Berry, C. Martinache, F. Rieux-Laucat, S. Latour, B. H. Belohradsky, L. Leiva, R. Sorensen, M. Debre, J. L. Casanova, S. Blanche, A. Durandy, F. D. Bushman, A. Fischer and M. Cavazzana-Calvo (2010). Efficacy of gene therapy for x-linked severe combined immunodeficiency. *N Engl J Med* 363(4): 355-64.

Hagemann, T., F. Balkwill and T. Lawrence (2007). Inflammation and cancer: A double-edged sword. *Cancer Cell* 12(4): 300-1.

Haimovitz-Friedman, A., C. C. Kan, D. Ehleiter, R. S. Persaud, M. McLoughlin, Z. Fuks and R. N. Kolesnick (1994). Ionizing radiation acts on cellular membranes to generate ceramide and initiate apoptosis. *J Exp Med* 180(2): 525-35.

Haisma, H. J., M. Boesjes, A. M. Beerens, B. W. van der Strate, D. T. Curiel, A. Pluddemann, S. Gordon and A. R. Bellu (2009). Scavenger receptor a: A new route for adenovirus 5. *Mol Pharm* 6(2): 366-74.

Haisma, H. J., H. M. Pinedo, A. Rijswijk, I. der Meulen-Muileman, B. A. Sosnowski, W. Ying, V. W. Beusechem, B. W. Tillman, W. R. Gerritsen and D. T. Curiel (1999). Tumor-specific gene transfer via an adenoviral vector targeted to the pan-carcinoma antigen epcam. *Gene Ther* 6(8): 1469-74.

Hakkarainen, T., M. Rajecki, M. Sarparanta, M. Tenhunen, A. J. Airaksinen, R. A. Desmond, K. Kairemo and A. Hemminki (2009). Targeted radiotherapy for prostate cancer with an oncolytic adenovirus coding for human sodium iodide symporter. *Clin Cancer Res* 15(17): 5396-403.

Halbert, D. N., J. R. Cutt and T. Shenk (1985). Adenovirus early region 4 encodes functions required for efficient DNA replication, late gene expression, and host cell shutoff. *J Virol* 56(1): 250-7.

Halicka, H. D., X. Huang, F. Traganos, M. A. King, W. Dai and Z. Darzynkiewicz (2005). Histone h2ax phosphorylation after cell irradiation with uv-b: Relationship to cell cycle phase and induction of apoptosis. *Cell Cycle* 4(2): 339-45.

Hall, K., M. E. Blair Zajdel and G. E. Blair (2010). Unity and diversity in the human adenoviruses: Exploiting alternative entry pathways for gene therapy. *Biochem J* 431(3): 321-36.

Haller, O., P. Staeheli and G. Kochs (2007). Interferon-induced mx proteins in antiviral host defense. *Biochimie* 89(6-7): 812-8.

Hamid, O., M. L. Varterasian, S. Wadler, J. R. Hecht, A. Benson, 3rd, E. Galanis, M. Uprichard, C. Omer, P. Bycott, R. C. Hackman and A. F. Shields (2003). Phase ii trial of intravenous ci-1042 in patients with metastatic colorectal cancer. *J Clin Oncol* 21(8): 1498-504.

Hanahan, D. and R. A. Weinberg (2011). Hallmarks of cancer: The next generation. *Cell* 144(5): 646-74.

Hannani, D., A. Sistigu, O. Kepp, L. Galluzzi, G. Kroemer and L. Zitvogel (2011). Prerequisites for the antitumor vaccine-like effect of chemotherapy and radiotherapy. *Cancer J* 17(5): 351-8.

Hao, N. B., M. H. Lu, Y. H. Fan, Y. L. Cao, Z. R. Zhang and S. M. Yang (2012). Macrophages in tumor microenvironments and the progression of tumors. *Clin Dev Immunol* 2012: 948098.

Harrington, K., P. Jankowska and M. Hingorani (2007). Molecular biology for the radiation oncologist: The 5rs of radiobiology meet the hallmarks of cancer. *Clin Oncol (R Coll Radiol)* 19(8): 561-71.

Harrington, K. J., M. Hingorani, M. A. Tanay, J. Hickey, S. A. Bhide, P. M. Clarke, L. C. Renouf, K. Thway, A. Sibtain, I. A. McNeish, K. L. Newbold, H. Goldsweig, R. Coffin and C. M. Nutting (2010). Phase i/ii

study of oncolytic hsv gm-csf in combination with radiotherapy and cisplatin in untreated stage iii/iv squamous cell cancer of the head and neck. *Clin Cancer Res* 16(15): 4005-15.

Hart, L. S., D. Ornelles and C. Koumenis (2007). The adenoviral e4orf6 protein induces atypical apoptosis in response to DNA damage. *J Biol Chem* 282(9): 6061-7.

Hart, L. S., S. M. Yannone, C. Naczki, J. S. Orlando, S. B. Waters, S. A. Akman, D. J. Chen, D. Ornelles and C. Koumenis (2005). The adenovirus e4orf6 protein inhibits DNA double strand break repair and radiosensitizes human tumor cells in an e1b-55k-independent manner. *J Biol Chem* 280(2): 1474-81.

Hecht, J. R., R. Bedford, J. L. Abbruzzese, S. Lahoti, T. R. Reid, R. M. Soetikno, D. H. Kirn and S. M. Freeman (2003). A phase i/ii trial of intratumoral endoscopic ultrasound injection of onyx-015 with intravenous gemcitabine in unresectable pancreatic carcinoma. *Clin Cancer Res* 9(2): 555-61.

Hecht, J. R., J. J. Farrell, N. Senzer, J. Nemunaitis, A. Rosemurgy, T. Chung, N. Hanna, K. J. Chang, M. Javle, M. Posner, I. Waxman, A. Reid, R. Erickson, M. Canto, A. Chak, G. Blatner, M. Kovacevic and M. Thornton (2012). Eus or percutaneously guided intratumoral tnferade biologic with 5-fluorouracil and radiotherapy for first-line treatment of locally advanced pancreatic cancer: A phase i/ii study. *Gastrointest Endosc* 75(2): 332-8.

Hegenbarth, S., R. Gerolami, U. Protzer, P. L. Tran, C. Brechot, G. Gerken and P. A. Knolle (2000). Liver sinusoidal endothelial cells are not permissive for adenovirus type 5. *Hum Gene Ther* 11(3): 481-6.

Heise, C., I. Ganly, Y. T. Kim, A. Sampson-Johannes, R. Brown and D. Kirn (2000). Efficacy of a replication-selective adenovirus against ovarian carcinomatosis is dependent on tumor burden, viral replication and p53 status. *Gene Ther* 7(22): 1925-9.

Hemmi, H., O. Takeuchi, T. Kawai, T. Kaisho, S. Sato, H. Sanjo, M. Matsumoto, K. Hoshino, H. Wagner, K. Takeda and S. Akira (2000). A toll-like receptor recognizes bacterial DNA. *Nature* 408(6813): 740-5.

Hemminki, A., N. Belousova, K. R. Zinn, B. Liu, M. Wang, T. R. Chaudhuri, B. E. Rogers, D. J. Buchsbaum, G. P. Siegal, M. N. Barnes, J. Gomez-Navarro, D. T. Curiel and R. D. Alvarez (2001). An adenovirus with enhanced infectivity mediates molecular chemotherapy of ovarian cancer cells and allows imaging of gene expression. *Mol Ther* 4(3): 223-31.

Hemminki, O., G. Bauerschmitz, S. Hemmi, S. Lavilla-Alonso, I. Diaconu, K. Guse, A. Koski, R. A. Desmond, M. Lappalainen, A. Kanerva, V. Cerullo, S. Pesonen and A. Hemminki (2011). Oncolytic adenovirus based on serotype 3. *Cancer Gene Ther* 18(4): 288-96.

Hemminki, O., I. Diaconu, V. Cerullo, S. K. Pesonen, A. Kanerva, T. Joensuu, K. Kairemo, L. Laasonen, K. Partanen, L. Kangasniemi, A. Lieber, S. Pesonen and A. Hemminki (2012). Ad3-htert-e1a, a fully serotype 3 oncolytic adenovirus, in patients with chemotherapy refractory cancer. *Mol Ther* 20(9): 1821-30.

Hemminki, O. and A. Hemminki (2014). Patients treated with oncolytic adenovirus ad5/3-htert-e2f-gmcsf induce immunological responses against the tumor. *12th CIMT Annual Meeting (abstr 20)*, Mainz, Germany, *The Association for Cancer Immunotherapy*.

Hendrickx, R., N. Stichling, J. Koelen, L. Kuryk, A. Lipiec and U. F. Greber (2014). Innate immunity to adenovirus. *Hum Gene Ther* 25(4): 265-84.

Heo, J., T. Reid, L. Ruo, C. J. Breitbach, S. Rose, M. Bloomston, M. Cho, H. Y. Lim, H. C. Chung, C. W. Kim, J. Burke, R. Lencioni, T. Hickman, A. Moon, Y. S. Lee, M. K. Kim, M. Daneshmand, K. Dubois, L. Longpre, M. Ngo, C. Rooney, J. C. Bell, B. G. Rhee, R. Patt, T. H. Hwang and D. H. Kirn (2013). Randomized dose-finding clinical trial of oncolytic immunotherapeutic vaccinia jx-594 in liver cancer. *Nat Med* 19(3): 329-36.

Hermant, P. and T. Michiels (2014). Interferon-lambda in the context of viral infections: Production, response and therapeutic implications. *J Innate Immun* 6(5): 563-74.

Hernandez, J., J. Cooper, N. Babel, C. Morton and A. S. Rosemurgy (2010). Tnfalpha gene delivery therapy for solid tumors. *Expert Opin Biol Ther* 10(6): 993-9.

Hinrichs, C. S. and S. A. Rosenberg (2014). Exploiting the curative potential of adoptive t-cell therapy for cancer. *Immunol Rev* 257(1): 56-71.

Hirvinen, M., R. Heiskanen, M. Oksanen, S. Pesonen, I. Liikanen, T. Joensuu, A. Kanerva, V. Cerullo and A. Hemminki (2013). Fc-gamma receptor polymorphisms as predictive and prognostic factors in patients receiving oncolytic adenovirus treatment. *J Transl Med* 11: 193.

Hodi, F. S. and G. Dranoff (2010). The biologic importance of tumor-infiltrating lymphocytes. *J Cutan Pathol* 37 Suppl 1: 48-53.

Hodi, F. S., S. J. O'Day, D. F. McDermott, R. W. Weber, J. A. Sosman, J. B. Haanen, R. Gonzalez, C. Robert, D. Schadendorf, J. C. Hassel, W. Akerley, A. J. van den Eertwegh, J. Lutzky, P. Lorigan, J. M. Vaubel, G. P. Linette, D. Hogg, C. H. Ottensmeier, C. Lebbe, C. Peschel, I. Quirt, J. I. Clark, J. D. Wolchok, J. S. Weber, J. Tian, M. J. Yellin, G. M. Nichol, A. Hoos and W. J. Urba (2010). Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med* 363(8): 711-23.

Hojjat-Farsangi, M. (2014). Small-molecule inhibitors of the receptor tyrosine kinases: Promising tools for targeted cancer therapies. *Int J Mol Sci* 15(8): 13768-801.

Holsti, L. R. (1995). Development of clinical radiotherapy since 1896. *Acta Oncol* 34(8): 995-1003.

Hong, S. S., L. Karayan, J. Tournier, D. T. Curiel and P. A. Boulanger (1997). Adenovirus type 5 fiber knob binds to mhc class i alpha2 domain at the surface of human epithelial and b lymphoblastoid cells. *EMBO J* 16(9): 2294-306.

Hoos, A., A. M. Eggermont, S. Janetzki, F. S. Hodi, R. Ibrahim, A. Anderson, R. Humphrey, B. Blumenstein, L. Old and J. Wolchok (2010). Improved endpoints for cancer immunotherapy trials. *J Natl Cancer Inst* 102(18): 1388-97.

Houston, A., M. W. Bennett, G. C. O'Sullivan, F. Shanahan and J. O'Connell (2003). Fas ligand mediates immune privilege and not inflammation in human colon cancer, irrespective of tgf-beta expression. *Br J Cancer* 89(7): 1345-51.

Hubbard, T. J., B. L. Aken, S. Ayling, B. Ballester, K. Beal, E. Bragin, S. Brent, Y. Chen, P. Clapham, L. Clarke, G. Coates, S. Fairley, S. Fitzgerald, J. Fernandez-Banet, L. Gordon, S. Graf, S. Haider, M. Hammond, R. Holland, K. Howe, A. Jenkinson, N. Johnson, A. Kahari, D. Keefe, S. Keenan, R. Kinsella, F. Kokocinski, E. Kulesha, D. Lawson, I. Longden, K. Megy, P. Meidl, B. Overduin, A. Parker, B. Pritchard, D. Rios, M. Schuster, G. Slater, D. Smedley, W. Spooner, G. Spudich, S. Trevanion, A. Vilella, J. Vogel, S. White, S. Wilder, A. Zadissa, E. Birney, F. Cunningham, V. Curwen, R. Durbin, X. M. Fernandez-Suarez, J. Herrero, A. Kasprzyk, G. Proctor, J. Smith, S. Searle and P. Flicek (2009). Ensembl 2009. *Nucleic Acids Res* 37(Database issue): D690-7.

Hwang, T. H., A. Moon, J. Burke, A. Ribas, J. Stephenson, C. J. Breitbach, M. Daneshmand, N. De Silva, K. Parato, J. S. Diallo, Y. S. Lee, T. C. Liu, J. C. Bell and D. H. Kirn (2011). A mechanistic proof-of-concept clinical trial with jx-594, a targeted multi-mechanistic oncolytic poxvirus, in patients with metastatic melanoma. *Mol Ther* 19(10): 1913-22.

Hwu, W. J., S. E. Krown, J. H. Menell, K. S. Panageas, J. Merrell, L. A. Lamb, L. J. Williams, C. J. Quinn, T. Foster, P. B. Chapman, P. O. Livingston, J. D. Wolchok and A. N. Houghton (2003). Phase ii study of temozolomide plus thalidomide for the treatment of metastatic melanoma. *J Clin Oncol* 21(17): 3351-6.

Ichim, C. V. (2005). Revisiting immunosurveillance and immunostimulation: Implications for cancer immunotherapy. *J Transl Med* 3(1): 8.

Im, S. A., J. S. Kim, C. Gomez-Manzano, J. Fueyo, T. J. Liu, M. S. Cho, C. M. Seong, S. N. Lee, Y. K. Hong and W. K. Yung (2001). Inhibition of breast cancer growth in vivo by antiangiogenesis gene therapy with adenovirus-mediated antisense-vegf. *Br J Cancer* 84(9): 1252-7.

Irizarry, R. A., B. M. Bolstad, F. Collin, L. M. Cope, B. Hobbs and T. P. Speed (2003). Summaries of affymetrix genechip probe level data. *Nucleic Acids Res* 31(4): e15.

Ito, H., H. Aoki, F. Kuhnel, Y. Kondo, S. Kubicka, T. Wirth, E. Iwado, A. Iwamaru, K. Fujiwara, K. R. Hess, F. F. Lang, R. Sawaya and S. Kondo (2006). Autophagic cell death of malignant glioma cells induced by a conditionally replicating adenovirus. *J Natl Cancer Inst* 98(9): 625-36.

Izumi, M., Y. Kawakami, J. N. Glasgow, N. Belousova, M. Everts, S. Kim-Park, S. Yamamoto, M. Wang, L. P. Le, P. N. Reynolds and D. T. Curiel (2005). In vivo analysis of a genetically modified adenoviral vector targeted to human cd40 using a novel transient transgenic model. *J Gene Med* 7(12): 1517-25.

Jayaram, S., T. Gilson, E. S. Ehrlich, X. F. Yu, G. Ketner and L. Hanakahi (2008). E1b 55k-independent dissociation of the DNA ligase iv/xrcc4 complex by e4 34k during adenovirus infection. *Virology* 382(2): 163-70.

Jiang, H., E. J. White, C. I. Rios-Vicil, J. Xu, C. Gomez-Manzano and J. Fueyo (2011). Human adenovirus type 5 induces cell lysis through autophagy and autophagy-triggered caspase activity. *J Virol* 85(10): 4720-9.

Jiang, M., W. Shi, Q. Zhang, X. Wang, M. Guo, Z. Cui, C. Su, Q. Yang, Y. Li, J. Sham, X. Liu, M. Wu and Q. Qian (2006). Gene therapy using adenovirus-mediated full-length anti-her-2 antibody for her-2 overexpression cancers. *Clin Cancer Res* 12(20 Pt 1): 6179-85.

Kalvakolanu, D. V., S. K. Bandyopadhyay, M. L. Harter and G. C. Sen (1991). Inhibition of interferon-inducible gene expression by adenovirus e1a proteins: Block in transcriptional complex formation. *Proc Natl Acad Sci U S A* 88(17): 7459-63.

Kanehisa, M., S. Goto, M. Furumichi, M. Tanabe and M. Hirakawa (2010). Kegg for representation and analysis of molecular networks involving diseases and drugs. *Nucleic Acids Res* 38(Database issue): D355-60.

Kanerva, A., S. Lavilla-Alonso, M. Raki, L. Kangasniemi, G. J. Bauerschmitz, K. Takayama, A. Ristimaki, R. A. Desmond and A. Hemminki (2008). Systemic therapy for cervical cancer with potentially regulatable oncolytic adenoviruses. *PLoS One* 3(8): e2917.

Kanerva, A., G. V. Mikheeva, V. Krasnykh, C. J. Coolidge, J. T. Lam, P. J. Mahasreshti, S. D. Barker, M. Straughn, M. N. Barnes, R. D. Alvarez, A. Hemminki and D. T. Curiel (2002a). Targeting adenovirus to the serotype 3 receptor increases gene transfer efficiency to ovarian cancer cells. *Clin Cancer Res* 8(1): 275-80.

Kanerva, A., P. Nokisalmi, I. Diaconu, A. Koski, V. Cerullo, I. Liikanen, S. Tahtinen, M. Oksanen, R. Heiskanen, S. Pesonen, T. Joensuu, T. Alanko, K. Partanen, L. Laasonen, K. Kairemo, S. Pesonen, L. Kangasniemi and A. Hemminki (2013). Antiviral and antitumor t-cell immunity in patients treated with gm-csf-coding oncolytic adenovirus. *Clin Cancer Res* 19(10): 2734-44.

Kanerva, A., M. Wang, G. J. Bauerschmitz, J. T. Lam, R. A. Desmond, S. M. Bhoola, M. N. Barnes, R. D. Alvarez, G. P. Siegal, D. T. Curiel and A. Hemminki (2002b). Gene transfer to ovarian cancer versus normal tissues with fiber-modified adenoviruses. *Mol Ther* 5(6): 695-704.

Kanerva, A., K. R. Zinn, T. R. Chaudhuri, J. T. Lam, K. Suzuki, T. G. Uil, T. Hakkarainen, G. J. Bauerschmitz, M. Wang, B. Liu, Z. Cao, R. D. Alvarez, D. T. Curiel and A. Hemminki (2003). Enhanced therapeutic efficacy for ovarian cancer with a serotype 3 receptor-targeted oncolytic adenovirus. *Mol Ther* 8(3): 449-58.

Kang, R., R. Chen, Q. Zhang, W. Hou, S. Wu, L. Cao, J. Huang, Y. Yu, X. G. Fan, Z. Yan, X. Sun, H. Wang, Q. Wang, A. Tsung, T. R. Billiar, H. J. Zeh, 3rd, M. T. Lotze and D. Tang (2014). Hmgb1 in health and disease. *Mol Aspects Med*.

Kang, R., Q. Zhang, H. J. Zeh, 3rd, M. T. Lotze and D. Tang (2013). Hmgb1 in cancer: Good, bad, or both? *Clin Cancer Res* 19(15): 4046-57.

Kantoff, P. W., C. S. Higano, N. D. Shore, E. R. Berger, E. J. Small, D. F. Penson, C. H. Redfern, A. C. Ferrari, R. Dreicer, R. B. Sims, Y. Xu, M. W. Frohlich, P. F. Schellhammer and I. S. Investigators (2010). Sipuleucel-t immunotherapy for castration-resistant prostate cancer. *N Engl J Med* 363(5): 411-22.

Kanzawa, T., I. M. Germano, T. Komata, H. Ito, Y. Kondo and S. Kondo (2004). Role of autophagy in temozolomide-induced cytotoxicity for malignant glioma cells. *Cell Death Differ* 11(4): 448-57.

Kawai, T. and S. Akira (2006). Tlr signaling. *Cell Death Differ* 13(5): 816-25.

Kavanagh, J. N., K. M. Redmond, G. Schettino and K. M. Prise (2013). DNA double strand break repair: A radiation perspective. *Antioxid Redox Signal* 18(18): 2458-72.

Kelly, E. and S. J. Russell (2007). History of oncolytic viruses: Genesis to genetic engineering. *Mol Ther* 15(4): 651-9.

Khare, R., C. Y. Chen, E. A. Weaver and M. A. Barry (2011). Advances and future challenges in adenoviral vector pharmacology and targeting. *Curr Gene Ther* 11(4): 241-58.

Khuri, F. R., J. Nemunaitis, I. Ganly, J. Arseneau, I. F. Tannock, L. Romel, M. Gore, J. Ironside, R. H. MacDougall, C. Heise, B. Randlev, A. M. Gillenwater, P. Bruso, S. B. Kaye, W. K. Hong and D. H. Kirn (2000). A controlled trial of intratumoral onyx-015, a selectively-replicating adenovirus, in combination with cisplatin and 5-fluorouracil in patients with recurrent head and neck cancer. *Nat Med* 6(8): 879-85.

Kim, J., P. H. Kim, J. Y. Yoo, A. R. Yoon, H. J. Choi, J. Seong, I. W. Kim, J. H. Kim and C. O. Yun (2009). Double e1b 19 kda- and e1b 55 kda-deleted oncolytic adenovirus in combination with radiotherapy elicits an enhanced anti-tumor effect. *Gene Ther* 16(9): 1111-21.

Kimball, K. J., M. A. Preuss, M. N. Barnes, M. Wang, G. P. Siegal, W. Wan, H. Kuo, S. Saddekni, C. R. Stockard, W. E. Grizzle, R. D. Harris, R. Aurigemma, D. T. Curiel and R. D. Alvarez (2010). A phase i study of a tropism-modified conditionally replicative adenovirus for recurrent malignant gynecologic diseases. *Clin Cancer Res* 16(21): 5277-87.

Kochs, G. and O. Haller (1999). Interferon-induced human mxa gtpase blocks nuclear import of thogoto virus nucleocapsids. *Proc Natl Acad Sci U S A* 96(5): 2082-6.

Koski, A., H. Ahtinen, H. Liljenback, A. Roivainen, A. Koskela, M. Oksanen, K. Partanen, L. Laasonen, K. Kairemo, T. Joensuu and A. Hemminki (2013a). [(18)f]-fluorodeoxyglucose positron emission tomography and computed tomography in response evaluation of oncolytic adenovirus treatments of patients with advanced cancer. *Hum Gene Ther* 24(12): 1029-41.

Koski, A., L. Kangasniemi, S. Escutenaire, S. Pesonen, V. Cerullo, I. Diaconu, P. Nokisalmi, M. Raki, M. Rajecki, K. Guse, T. Ranki, M. Oksanen, S. L. Holm, E. Haavisto, A. Karioja-Kallio, L. Laasonen, K. Partanen, M. Ugolini, A. Helminen, E. Karli, P. Hannuksela, S. Pesonen, T. Joensuu, A. Kanerva and A. Hemminki (2010). Treatment of cancer patients with a serotype 5/3 chimeric oncolytic adenovirus expressing gmcsf. *Mol Ther* 18(10): 1874-84.

Koski, A., E. Karli, A. Kipar, S. Escutenaire, A. Kanerva and A. Hemminki (2013b). Mutation of the fiber shaft heparan sulphate binding site of a 5/3 chimeric adenovirus reduces liver tropism. *PLoS One* 8(4): e60032.

Koski, A., M. Rajecki, K. Guse, A. Kanerva, A. Ristimaki, S. Pesonen, S. Escutenaire and A. Hemminki (2009). Systemic adenoviral gene delivery to orthotopic murine breast tumors with ablation of coagulation factors, thrombocytes and kupffer cells. *J Gene Med* 11(11): 966-77.

Krasnykh, V., N. Belousova, N. Korokhov, G. Mikheeva and D. T. Curiel (2001). Genetic targeting of an adenovirus vector via replacement of the fiber protein with the phage t4 fibritin. *J Virol* 75(9): 4176-83.

Krasnykh, V. N., G. V. Mikheeva, J. T. Douglas and D. T. Curiel (1996). Generation of recombinant adenovirus vectors with modified fibers for altering viral tropism. *J Virol* 70(10): 6839-46.

Krieg, A. M., T. Wu, R. Weeratna, S. M. Efler, L. Love-Homan, L. Yang, A. K. Yi, D. Short and H. L. Davis (1998). Sequence motifs in adenoviral DNA block immune activation by stimulatory cpg motifs. *Proc Natl Acad Sci U S A* 95(21): 12631-6.

Kunz, A. N. and M. Ottolini (2010). The role of adenovirus in respiratory tract infections. *Curr Infect Dis Rep* 12(2): 81-7.

Kurzen, H., S. Schmitt, H. Naher and T. Mohler (2003). Inhibition of angiogenesis by non-toxic doses of temozolomide. *Anticancer Drugs* 14(7): 515-22.

Kusmartsev, S. and D. I. Gabrilovich (2006). Role of immature myeloid cells in mechanisms of immune evasion in cancer. *Cancer Immunol Immunother* 55(3): 237-45.

Kyi, C. and M. A. Postow (2014). Checkpoint blocking antibodies in cancer immunotherapy. *FEBS Lett* 588(2): 368-76.

Kyula, J. N., V. Roulstone, E. M. Karapanagiotou, A. A. Melcher and K. J. Harrington (2012). Oncolytic reovirus type 3 (dearing) as a novel therapy in head and neck cancer. *Expert Opin Biol Ther* 12(12): 1669-78.

Laakso, M. and S. Hautaniemi (2010). Integrative platform to translate gene sets to networks. *Bioinformatics* 26(14): 1802-3.

Lauerova, L., L. Dusek, M. Simickova, I. Kocak, M. Vagundova, J. Zaloudik and J. Kovarik (2002). Malignant melanoma associates with th1/th2 imbalance that coincides with disease progression and immunotherapy response. *Neoplasma* 49(3): 159-66.

Lee, M. B., M. M. McMenamin, A. P. Byrnes, H. M. Charlton and M. J. Wood (2008). Th1 cytokines are upregulated by adenoviral vectors in the brains of primed mice. *Neuroreport* 19(12): 1187-92.

Lee, Y., S. L. Auh, Y. Wang, B. Burnette, Y. Wang, Y. Meng, M. Beckett, R. Sharma, R. Chin, T. Tu, R. R. Weichselbaum and Y. X. Fu (2009). Therapeutic effects of ablative radiation on local tumor require cd8+ t cells: Changing strategies for cancer treatment. *Blood* 114(3): 589-95.

Lefranc, F., V. Facchini and R. Kiss (2007). Proautophagic drugs: A novel means to combat apoptosis-resistant cancers, with a special emphasis on glioblastomas. *Oncologist* 12(12): 1395-403.

Leja, J., H. Dzojic, E. Gustafson, K. Oberg, V. Giandomenico and M. Essand (2007). A novel chromogranin-a promoter-driven oncolytic adenovirus for midgut carcinoid therapy. *Clin Cancer Res* 13(8): 2455-62.

Lejeune, F. J., C. Ruegg and D. Lienard (1998). Clinical applications of tnf-alpha in cancer. *Curr Opin Immunol* 10(5): 573-80.

Leone, P., E. C. Shin, F. Perosa, A. Vacca, F. Dammacco and V. Racanelli (2013). Mhc class i antigen processing and presenting machinery: Organization, function, and defects in tumor cells. *J Natl Cancer Inst* 105(16): 1172-87.

Leppard, K. N. and R. D. Everett (1999). The adenovirus type 5 e1b 55k and e4 orf3 proteins associate in infected cells and affect nd10 components. *J Gen Virol* 80 (Pt 4): 997-1008.

Li, C. Y., Q. Huang and H. F. Kung (2005). Cytokine and immuno-gene therapy for solid tumors. *Cell Mol Immunol* 2(2): 81-91.

Li, G., X. Liang and M. T. Lotze (2013a). Hmgb1: The central cytokine for all lymphoid cells. *Front Immunol* 4: 68.

Li, T., Y. Gui, T. Yuan, G. Liao, C. Bian, Q. Jiang, S. Huang, B. Liu and D. Wu (2012). Overexpression of high mobility group box 1 with poor prognosis in patients after radical prostatectomy. *BJU Int* 110(11 Pt C): E1125-30.

Li, W., K. Wu, E. Zhao, L. Shi, R. Li, P. Zhang, Y. Yin, X. Shuai, G. Wang and K. Tao (2013b). Hmgb1 recruits myeloid derived suppressor cells to promote peritoneal dissemination of colon cancer after resection. *Biochem Biophys Res Commun* 436(2): 156-61.

Lichty, B. D., C. J. Breitbach, D. F. Stojdl and J. C. Bell (2014). Going viral with cancer immunotherapy. *Nat Rev Cancer* 14(8): 559-67.

Lien, K., S. Georgsdottir, L. Sivanathan, K. Chan and U. Emmenegger (2013). Low-dose metronomic chemotherapy: A systematic literature analysis. *Eur J Cancer* 49(16): 3387-95.

Lilley, C. E., R. A. Schwartz and M. D. Weitzman (2007). Using or abusing: Viruses and the cellular DNA damage response. *Trends Microbiol* 15(3): 119-26.

Lind, M. J. (2008). Principles of cytotoxic chemotherapy. *Medicine* 36(1): 19-23.

Lindau, D., P. Gielen, M. Kroesen, P. Wesseling and G. J. Adema (2013). The immunosuppressive tumour network: Myeloid-derived suppressor cells, regulatory t cells and natural killer t cells. *Immunology* 138(2): 105-15.

Liu, E. B., D. A. Wadford, J. Seto, M. Vu, N. R. Hudson, L. Thrasher, S. Torres, D. W. Dyer, J. Chodosh, D. Seto and M. S. Jones (2012). Computational and serologic analysis of novel and known viruses in species human adenovirus d in which serology and genomics do not correlate. *PLoS One* 7(3): e33212.

Liu, G., J. Wang, Y. J. Park, Y. Tsuruta, E. F. Lorne, X. Zhao and E. Abraham (2008). High mobility group protein-1 inhibits phagocytosis of apoptotic neutrophils through binding to phosphatidylserine. *J Immunol* 181(6): 4240-6.

Liu, L., S. Wang, B. Shan, M. Sang, S. Liu and G. Wang (2010). Advances in viral-vector systemic cytokine gene therapy against cancer. *Vaccine* 28(23): 3883-7.

Liu, Y., S. Tuve, J. Persson, I. Beyer, R. Yumul, Z. Y. Li, K. Tragoolpua, K. E. Hellstrom, S. Roffler and A. Lieber (2011a). Adenovirus-mediated intratumoral expression of immunostimulatory proteins in combination with systemic treg inactivation induces tumor-destructive immune responses in mouse models. *Cancer Gene Ther* 18(6): 407-18.

Liu, Z., L. D. Falo, Jr. and Z. You (2011b). Knockdown of hmgb1 in tumor cells attenuates their ability to induce regulatory t cells and uncovers naturally acquired cd8 t cell-dependent antitumor immunity. *J Immunol* 187(1): 118-25.

Livak, K. J. and T. D. Schmittgen (2001). Analysis of relative gene expression data using real-time quantitative pcr and the 2(-delta delta c(t)) method. *Methods* 25(4): 402-8.

Loskog, A. S., M. E. Fransson and T. T. Totterman (2005). Adcd40l gene therapy counteracts t regulatory cells and cures aggressive tumors in an orthotopic bladder cancer model. *Clin Cancer Res* 11(24 Pt 1): 8816-21.

Loven, D., E. Hasnis, F. Bertolini and Y. Shaked (2013). Low-dose metronomic chemotherapy: From past experience to new paradigms in the treatment of cancer. *Drug Discov Today* 18(3-4): 193-201.

Lutsiak, M. E., R. T. Semnani, R. De Pascalis, S. V. Kashmiri, J. Schlom and H. Sabzevari (2005). Inhibition of cd4(+)25+ t regulatory cell function implicated in enhanced immune response by low-dose cyclophosphamide. *Blood* 105(7): 2862-8.

Lutz, P. and C. Kedinger (1996). Properties of the adenovirus iva2 gene product, an effector of late-phase-dependent activation of the major late promoter. *J Virol* 70(3): 1396-405.

Lutz, P., M. Rosa-Calatrava and C. Kedinger (1997). The product of the adenovirus intermediate gene ix is a transcriptional activator. *J Virol* 71(7): 5102-9.

MacGill, R. S., T. A. Davis, J. Macko, H. J. Mauceri, R. R. Weichselbaum and C. R. King (2007). Local gene delivery of tumor necrosis factor alpha can impact primary tumor growth and metastases through a host-mediated response. *Clin Exp Metastasis* 24(7): 521-31.

Malmstrom, P. U., A. S. Loskog, C. A. Lindqvist, S. M. Mangsbo, M. Fransson, A. Wanders, T. Gardmark and T. H. Totterman (2010). Adcd40l immunogene therapy for bladder carcinoma--the first phase i/iia trial. *Clin Cancer Res* 16(12): 3279-87.

Marcellus, R. C., J. G. Teodoro, R. Charbonneau, G. C. Shore and P. E. Branton (1996). Expression of p53 in saos-2 osteosarcoma cells induces apoptosis which can be inhibited by bcl-2 or the adenovirus e1b-55 kda protein. *Cell Growth Differ* 7(12): 1643-50.

Martins, I., M. Michaud, A. Q. Sukkurwala, S. Adjemian, Y. Ma, S. Shen, O. Kepp, L. Menger, E. Vacchelli, L. Galluzzi, L. Zitvogel and G. Kroemer (2012). Premortem autophagy determines the immunogenicity of chemotherapy-induced cancer cell death. *Autophagy* 8(3): 413-5.

Mastrangelo, M. J., H. C. Maguire, Jr., L. C. Eisenlohr, C. E. Laughlin, C. E. Monken, P. A. McCue, A. J. Kovatich and E. C. Lattime (1999). Intratumoral recombinant gm-csf-encoding virus as gene therapy in patients with cutaneous melanoma. *Cancer Gene Ther* 6(5): 409-22.

Mathias, P., M. Galleno and G. R. Nemerow (1998). Interactions of soluble recombinant integrin alphav beta5 with human adenoviruses. *J Virol* 72(11): 8669-75.

Matsumoto, K., S. F. Shariat, G. E. Ayala, K. A. Rauen and S. P. Lerner (2005). Loss of coxsackie and adenovirus receptor expression is associated with features of aggressive bladder cancer. *Urology* 66(2): 441-6.

Maude, S. L., N. Frey, P. A. Shaw, R. Aplenc, D. M. Barrett, N. J. Bunin, A. Chew, V. E. Gonzalez, Z. Zheng, S. F. Lacey, Y. D. Mahnke, J. J. Melenhorst, S. R. Rheingold, A. Shen, D. T. Teachey, B. L. Levine, C. H. June, D. L. Porter and S. A. Grupp (2014). Chimeric antigen receptor t cells for sustained remissions in leukemia. *N Engl J Med* 371(16): 1507-17.

McDermott, D. F. and M. B. Atkins (2013). Pd-1 as a potential target in cancer therapy. *Cancer Med* 2(5): 662-73.

McGovern, S. L. and A. Mahajan (2012). Progress in radiotherapy for pediatric sarcomas. *Curr Oncol Rep* 14(4): 320-6.

Mehlen, P. and A. Puisieux (2006). Metastasis: A question of life or death. *Nat Rev Cancer* 6(6): 449-58.

Melcher, A., K. Parato, C. M. Rooney and J. C. Bell (2011). Thunder and lightning: Immunotherapy and oncolytic viruses collide. *Mol Ther* 19(6): 1008-16.

Mellman, I., G. Coukos and G. Dranoff (2011). Cancer immunotherapy comes of age. *Nature* 480(7378): 480-9.

Mellor, J. D., M. P. Brown, H. R. Irving, J. R. Zalcberg and A. Dobrovic (2013). A critical review of the role of fc gamma receptor polymorphisms in the response to monoclonal antibodies in cancer. *J Hematol Oncol* 6: 1.

Michaud, M., I. Martins, A. Q. Sukkurwala, S. Adjemian, Y. Ma, P. Pellegatti, S. Shen, O. Kepp, M. Scoazec, G. Mignot, S. Rello-Varona, M. Tailler, L. Menger, E. Vacchelli, L. Galluzzi, F. Ghiringhelli, F. di Virgilio, L. Zitvogel and G. Kroemer (2011). Autophagy-dependent anticancer immune responses induced by chemotherapeutic agents in mice. *Science* 334(6062): 1573-7.

Miller, G., S. Lahrs, V. G. Pillarisetty, A. B. Shah and R. P. DeMatteo (2002). Adenovirus infection enhances dendritic cell immunostimulatory properties and induces natural killer and t-cell-mediated tumor protection. *Cancer Res* 62(18): 5260-6.

Mirzayans, R., B. Andrais, A. Scott, Y. W. Wang and D. Murray (2013). Ionizing radiation-induced responses in human cells with differing tp53 status. *Int J Mol Sci* 14(11): 22409-35.

Mizushima, N. and M. Komatsu (2011). Autophagy: Renovation of cells and tissues. *Cell* 147(4): 728-41.

Mocellin, S., C. R. Rossi, P. Pilati and D. Nitti (2005). Tumor necrosis factor, cancer and anticancer therapy. *Cytokine Growth Factor Rev* 16(1): 35-53.

Monsurro, V., S. Beghelli, R. Wang, S. Barbi, S. Coin, G. Di Pasquale, S. Bersani, M. Castellucci, C. Sorio, S. Eleuteri, A. Worschech, J. A. Chiorini, P. Pederzoli, H. Alter, F. M. Marincola and A. Scarpa (2010). Anti-viral state segregates two molecular phenotypes of pancreatic adenocarcinoma: Potential relevance for adenoviral gene therapy. *J Transl Med* 8: 10.

Morales, A., D. Eidinger and A. W. Bruce (1976). Intracavitary bacillus calmette-guerin in the treatment of superficial bladder tumors. *J Urol* 116(2): 180-3.

Morel, P. A. and T. B. Oriss (1998). Crossregulation between th1 and th2 cells. *Crit Rev Immunol* 18(4): 275-303.

Muller, A. J. and G. C. Prendergast (2007). Indoleamine 2,3-dioxygenase in immune suppression and cancer. *Curr Cancer Drug Targets* 7(1): 31-40.

Mundt, A. J., S. Vijayakumar, J. Nemunaitis, A. Sandler, H. Schwartz, N. Hanna, T. Peabody, N. Senzer, K. Chu, C. S. Rasmussen, P. D. Kessler, H. S. Rasmussen, M. Warso, D. W. Kufe, T. D. Gupta and R. R. Weichselbaum (2004). A phase i trial of tnferade biologic in patients with soft tissue sarcoma in the extremities. *Clin Cancer Res* 10(17): 5747-53.

Murugesan, S. R., C. R. King, R. Osborn, W. R. Fairweather, E. M. O'Reilly, M. O. Thornton and L. L. Wei (2009). Combination of human tumor necrosis factor-alpha (htnf-alpha) gene delivery with gemcitabine is effective in models of pancreatic cancer. *Cancer Gene Ther* 16(11): 841-7.

Muruve, D. A. (2004). The innate immune response to adenovirus vectors. *Hum Gene Ther* 15(12): 1157-66.

Naik, S., R. Nace, M. J. Federspiel, G. N. Barber, K. W. Peng and S. J. Russell (2012). Curative one-shot systemic virotherapy in murine myeloma. *Leukemia* 26(8): 1870-8.

Nauts, H. C., W. E. Swift and B. L. Coley (1946). The treatment of malignant tumors by bacterial toxins as developed by the late william b. Coley, m.D., reviewed in the light of modern research. *Cancer Res* 6: 205-16.

Nayak, S. and R. W. Herzog (2010). Progress and prospects: Immune responses to viral vectors. *Gene Ther* 17(3): 295-304.

Nemunaitis, J., C. Cunningham, A. Buchanan, A. Blackburn, G. Edelman, P. Maples, G. Netto, A. Tong, B. Randlev, S. Olson and D. Kirn (2001). Intravenous infusion of a replication-selective adenovirus (onyx-015) in cancer patients: Safety, feasibility and biological activity. *Gene Ther* 8(10): 746-59.

Nemunaitis, J., I. Ganly, F. Khuri, J. Arseneau, J. Kuhn, T. McCarty, S. Landers, P. Maples, L. Romel, B. Randlev, T. Reid, S. Kaye and D. Kirn (2000). Selective replication and oncolysis in p53 mutant tumors with onyx-015, an e1b-55kd gene-deleted adenovirus, in patients with advanced head and neck cancer: A phase ii trial. *Cancer Res* 60(22): 6359-66.

Nemunaitis, J., A. W. Tong, M. Nemunaitis, N. Senzer, A. P. Phadke, C. Bedell, N. Adams, Y. A. Zhang, P. B. Maples, S. Chen, B. Pappen, J. Burke, D. Ichimaru, Y. Urata and T. Fujiwara (2010). A phase i study of telomerase-specific replication competent oncolytic adenovirus (telomelysin) for various solid tumors. *Mol Ther* 18(2): 429-34.

Nicklin, S. A., E. Wu, G. R. Nemerow and A. H. Baker (2005). The influence of adenovirus fiber structure and function on vector development for gene therapy. *Mol Ther* 12(3): 384-93.

Nielsen, L. L., M. Gurnani, J. Syed, J. Dell, B. Hartman, M. Cartwright and R. C. Johnson (1998). Recombinant e1-deleted adenovirus-mediated gene therapy for cancer: Efficacy studies with p53 tumor suppressor gene and liver histology in tumor xenograft models. *Hum Gene Ther* 9(5): 681-94.

Nijhawan, D., M. Fang, E. Traer, Q. Zhong, W. Gao, F. Du and X. Wang (2003). Elimination of mcl-1 is required for the initiation of apoptosis following ultraviolet irradiation. *Genes Dev* 17(12): 1475-86.

Nociari, M., O. Ocheretina, J. W. Schoggins and E. Falck-Pedersen (2007). Sensing infection by adenovirus: Toll-like receptor-independent viral DNA recognition signals activation of the interferon regulatory factor 3 master regulator. *J Virol* 81(8): 4145-57.

Nokisalmi, P., M. Rajecki, S. Pesonen, S. Escutenaire, R. Soliymani, M. Tenhunen, L. Ahtiainen and A. Hemminki (2012). Radiation-induced upregulation of gene expression from adenoviral vectors mediated by DNA damage repair and regulation. *Int J Radiat Oncol Biol Phys* 83(1): 376-84.

North, R. J. (1986). Radiation-induced, immunologically mediated regression of an established tumor as an example of successful therapeutic immunomanipulation. Preferential elimination of suppressor t cells allows sustained production of effector t cells. *J Exp Med* 164(5): 1652-66.

Nosho, K., Y. Baba, N. Tanaka, K. Shima, M. Hayashi, J. A. Meyerhardt, E. Giovannucci, G. Dranoff, C. S. Fuchs and S. Ogino (2010). Tumour-infiltrating t-cell subsets, molecular changes in colorectal cancer, and prognosis: Cohort study and literature review. *J Pathol* 222(4): 350-66.

O'Neal, W. K., H. Zhou, N. Morral, E. Aguilar-Cordova, J. Pestaner, C. Langston, B. Mull, Y. Wang, A. L. Beaudet and B. Lee (1998). Toxicological comparison of e2a-deleted and first-generation adenoviral vectors expressing alpha1-antitrypsin after systemic delivery. *Hum Gene Ther* 9(11): 1587-98.

O'Shea, C. C., L. Johnson, B. Bagus, S. Choi, C. Nicholas, A. Shen, L. Boyle, K. Pandey, C. Soria, J. Kunich, Y. Shen, G. Habets, D. Ginzinger and F. McCormick (2004). Late viral rna export, rather than p53 inactivation, determines onyx-015 tumor selectivity. *Cancer Cell* 6(6): 611-23.

Odaka, M., R. Wiewrodt, P. DeLong, T. Tanaka, Y. Zhang, L. Kaiser and S. Albelda (2002). Analysis of the immunologic response generated by ad.Ifn-beta during successful intraperitoneal tumor gene therapy. *Mol Ther* 6(2): 210-8.

Ohashi, M., F. Kanai, K. Tateishi, H. Taniguchi, P. A. Marignani, Y. Yoshida, Y. Shiratori, H. Hamada and M. Omata (2001). Target gene therapy for alpha-fetoprotein-producing hepatocellular carcinoma by e1b55k-attenuated adenovirus. *Biochem Biophys Res Commun* 282(2): 529-35.

Palumbo, S., L. Pirtoli, P. Tini, G. Cevenini, F. Calderaro, M. Toscano, C. Miracco and S. Comincini (2012). Different involvement of autophagy in human malignant glioma cell lines undergoing irradiation and temozolomide combined treatments. *J Cell Biochem* 113(7): 2308-18.

Pan, J. J., S. W. Zhang, C. B. Chen, S. W. Xiao, Y. Sun, C. Q. Liu, X. Su, D. M. Li, G. Xu, B. Xu and Y. Y. Lu (2009). Effect of recombinant adenovirus-p53 combined with radiotherapy on long-term prognosis of advanced nasopharyngeal carcinoma. *J Clin Oncol* 27(5): 799-804.

Park, B. H., T. Hwang, T. C. Liu, D. Y. Sze, J. S. Kim, H. C. Kwon, S. Y. Oh, S. Y. Han, J. H. Yoon, S. H. Hong, A. Moon, K. Speth, C. Park, Y. J. Ahn, M. Daneshmand, B. G. Rhee, H. M. Pinedo, J. C. Bell and D. H. Kirn (2008). Use of a targeted oncolytic poxvirus, jx-594, in patients with refractory primary or metastatic liver cancer: A phase i trial. *Lancet Oncol* 9(6): 533-42.

Parviainen, S., M. Ahonen, I. Diaconu, M. Hirvinen, A. Karttunen, M. Vaha-Koskela, A. Hemminki and V. Cerullo (2014). Cd40 ligand and tdtomato-armed vaccinia virus for induction of antitumor immune response and tumor imaging. *Gene Ther* 21(2): 195-204.

Pearson, S., H. Jia and K. Kandachi (2004). China approves first gene therapy. *Nat Biotechnol* 22(1): 3-4.

Pegg, A. E. (1990). Mammalian o6-alkylguanine-DNA alkyltransferase: Regulation and importance in response to alkylating carcinogenic and therapeutic agents. *Cancer Res* 50(19): 6119-29.

Pellegrini, M., T. W. Mak and P. S. Ohashi (2010). Fighting cancers from within: Augmenting tumor immunity with cytokine therapy. *Trends Pharmacol Sci* 31(8): 356-63.

Peng, Z. (2005). Current status of gendicine in china: Recombinant human ad-p53 agent for treatment of cancers. *Hum Gene Ther* 16(9): 1016-27.

Perrot, I., D. Blanchard, N. Freymond, S. Isaac, B. Guibert, Y. Pacheco and S. Lebecque (2007). Dendritic cells infiltrating human non-small cell lung cancer are blocked at immature stage. *J Immunol* 178(5): 2763-9.

Perry, J. R., K. Belanger, W. P. Mason, D. Fulton, P. Kavan, J. Easaw, C. Shields, S. Kirby, D. R. Macdonald, D. D. Eisenstat, B. Thiessen, P. Forsyth and J. F. Pouliot (2010). Phase ii trial of continuous dose-intense temozolomide in recurrent malignant glioma: Rescue study. *J Clin Oncol* 28(12): 2051-7.

Pesonen, S., I. Diaconu, L. Kangasniemi, T. Ranki, A. Kanerva, S. K. Pesonen, U. Gerdemann, A. M. Leen, K. Kairemo, M. Oksanen, E. Haavisto, S. L. Holm, A. Karioja-Kallio, S. Kauppinen, K. P. Partanen, L. Laasonen, T. Joensuu, T. Alanko, V. Cerullo and A. Hemminki (2012). Oncolytic immunotherapy of advanced solid tumors with a cd40l-expressing replicating adenovirus: Assessment of safety and immunologic responses in patients. *Cancer Res* 72(7): 1621-31.

Pesonen, S., P. Nokisalmi, S. Escutenaire, M. Sarkioja, M. Raki, V. Cerullo, L. Kangasniemi, L. Laasonen, C. Ribacka, K. Guse, E. Haavisto, M. Oksanen, M. Rajecki, A. Helminen, A. Ristimaki, A. Karioja-Kallio, E. Karli, T. Kantola, G. Bauerschmitz, A. Kanerva, T. Joensuu and A. Hemminki (2010). Prolonged systemic circulation of chimeric oncolytic adenovirus ad5/3-cox2l-d24 in patients with metastatic and refractory solid tumors. *Gene Ther* 17(7): 892-904.

Platzer, B., M. Stout and E. Fiebiger (2014). Antigen cross-presentation of immune complexes. *Front Immunol* 5: 140.

Pol, J., N. Bloy, F. Obrist, A. Eggermont, J. Galon, I. Cremer, P. Erbs, J. M. Limacher, X. Preville, L. Zitvogel, G. Kroemer and L. Galluzzi (2014). Trial watch:: Oncolytic viruses for cancer therapy. *Oncoimmunology* 3: e28694.

Prestwich, R. J., E. J. Ilett, F. Errington, R. M. Diaz, L. P. Steele, T. Kottke, J. Thompson, F. Galivo, K. J. Harrington, H. S. Pandha, P. J. Selby, R. G. Vile and A. A. Melcher (2009). Immune-mediated antitumor activity of reovirus is required for therapy and is independent of direct viral oncolysis and replication. *Clin Cancer Res* 15(13): 4374-81.

Pukkala, E., R. Sankila and M. Rautalahti (2011). Syöpä suomessa 2011.

Puzanov, I., M. M. Milhem, R. H. I. Andtbacka, M. D. R., O. Hamid, A. Li, M. Chastain, K. Gorski, A. Anderson, A. M. Vanderwalde, J. Chou and H. Kaufman (2014). Primary analysis of a phase 1b multicenter trial to evaluate safety and efficacy of talimogene laherparepvec (t-vec) and ipilimumab (ipi) in previously untreated, unresected stage iiib-iv melanoma. *Proceedings of the ASCO Annual Meeting*, *Journal of Clinical Oncology*.

Querido, E., R. C. Marcellus, A. Lai, R. Charbonneau, J. G. Teodoro, G. Ketner and P. E. Branton (1997). Regulation of p53 levels by the e1b 55-kilodalton protein and e4orf6 in adenovirus-infected cells. *J Virol* 71(5): 3788-98.

Quesada, J. R., E. M. Hersh, J. Manning, J. Reuben, M. Keating, E. Schnipper, L. Itri and J. U. Gutterman (1986). Treatment of hairy cell leukemia with recombinant alpha-interferon. *Blood* 68(2): 493-7.

Rainov, N. G. (2000). A phase iii clinical evaluation of herpes simplex virus type 1 thymidine kinase and ganciclovir gene therapy as an adjuvant to surgical resection and radiation in adults with previously untreated glioblastoma multiforme. *Hum Gene Ther* 11(17): 2389-401.

Rajecki, M., T. af Hallstrom, T. Hakkarainen, P. Nokisalmi, S. Hautaniemi, A. I. Nieminen, M. Tenhunen, V. Rantanen, R. A. Desmond, D. T. Chen, K. Guse, U. H. Stenman, R. Gargini, M. Kapanen, J. Klefstrom, A. Kanerva, S. Pesonen, L. Ahtiainen and A. Hemminki (2009). Mre11 inhibition by oncolytic adenovirus associates with autophagy and underlies synergy with ionizing radiation. *Int J Cancer* 125(10): 2441-9.

Randall, R. E. and S. Goodbourn (2008). Interferons and viruses: An interplay between induction, signalling, antiviral responses and virus countermeasures. *J Gen Virol* 89(Pt 1): 1-47.

Rauen, K. A., D. Sudilovsky, J. L. Le, K. L. Chew, B. Hann, V. Weinberg, L. D. Schmitt and F. McCormick (2002). Expression of the coxsackie adenovirus receptor in normal prostate and in primary and metastatic prostate carcinoma: Potential relevance to gene therapy. *Cancer Res* 62(13): 3812-8.

Raverot, G., F. Castinetti, E. Jouanneau, I. Morange, D. Figarella-Branger, H. Dufour, J. Trouillas and T. Brue (2012). Pituitary carcinomas and aggressive pituitary tumours: Merits and pitfalls of temozolomide treatment. *Clin Endocrinol (Oxf)* 76(6): 769-75.

Reid, T., E. Galanis, J. Abbruzzese, D. Sze, L. M. Wein, J. Andrews, B. Randlev, C. Heise, M. Uprichard, M. Hatfield, L. Rome, J. Rubin and D. Kirn (2002a). Hepatic arterial infusion of a replication-selective oncolytic adenovirus (dl1520): Phase ii viral, immunologic, and clinical endpoints. *Cancer Res* 62(21): 6070-9.

Reid, T., R. Warren and D. Kirn (2002b). Intravascular adenoviral agents in cancer patients: Lessons from clinical trials. *Cancer Gene Ther* 9(12): 979-86.

Rein, D. T., M. Breidenbach and D. T. Curiel (2006). Current developments in adenovirus-based cancer gene therapy. *Future Oncol* 2(1): 137-43.

Rekosh, D. M., W. C. Russell, A. J. Bellet and A. J. Robinson (1977). Identification of a protein linked to the ends of adenovirus DNA. *Cell* 11(2): 283-95.

Restifo, N. P., M. E. Dudley and S. A. Rosenberg (2012). Adoptive immunotherapy for cancer: Harnessing the t cell response. *Nat Rev Immunol* 12(4): 269-81.

Robert, C., L. Thomas, I. Bondarenko, S. O'Day, D. J. M, C. Garbe, C. Lebbe, J. F. Baurain, A. Testori, J. J. Grob, N. Davidson, J. Richards, M. Maio, A. Hauschild, W. H. Miller, Jr., P. Gascon, M. Lotem, K. Harmankaya, R. Ibrahim, S. Francis, T. T. Chen, R. Humphrey, A. Hoos and J. D. Wolchok (2011). Ipilimumab plus dacarbazine for previously untreated metastatic melanoma. *N Engl J Med* 364(26): 2517-26.

Rodriguez-Rocha, H., J. G. Gomez-Gutierrez, A. Garcia-Garcia, X. M. Rao, L. Chen, K. M. McMasters and H. S. Zhou (2011). Adenoviruses induce autophagy to promote virus replication and oncolysis. *Virology* 416(1-2): 9-15.

Rodriguez, R., E. R. Schuur, H. Y. Lim, G. A. Henderson, J. W. Simons and D. R. Henderson (1997). Prostate attenuated replication competent adenovirus (arca) cn706: A selective cytotoxic for prostate-specific antigen-positive prostate cancer cells. *Cancer Res* 57(13): 2559-63.

Roelvink, P. W., A. Lizonova, J. G. Lee, Y. Li, J. M. Bergelson, R. W. Finberg, D. E. Brough, I. Kovesdi and T. J. Wickham (1998). The coxsackievirus-adenovirus receptor protein can function as a cellular attachment protein for adenovirus serotypes from subgroups a, c, d, e, and f. *J Virol* 72(10): 7909-15.

Rojas, J. J., M. Cascallo, S. Guedan, A. Gros, J. Martinez-Quintanilla, A. Hemminki and R. Alemany (2009). A modified e2f-1 promoter improves the efficacy to toxicity ratio of oncolytic adenoviruses. *Gene Ther* 16(12): 1441-51.

Rosenberg, S. A., P. Aebersold, K. Cornetta, A. Kasid, R. A. Morgan, R. Moen, E. M. Karson, M. T. Lotze, J. C. Yang, S. L. Topalian and et al. (1990). Gene transfer into humans--immunotherapy of patients with advanced melanoma, using tumor-infiltrating lymphocytes modified by retroviral gene transduction. *N Engl J Med* 323(9): 570-8.

Ross, J. S., E. A. Slodkowska, W. F. Symmans, L. Pusztai, P. M. Ravdin and G. N. Hortobagyi (2009). The her-2 receptor and breast cancer: Ten years of targeted anti-her-2 therapy and personalized medicine. *Oncologist* 14(4): 320-68.

Rowe, W. P., R. J. Huebner, L. K. Gilmore, R. H. Parrott and T. G. Ward (1953). Isolation of a cytopathogenic agent from human adenoids undergoing spontaneous degeneration in tissue culture. *Proc Soc Exp Biol Med* 84(3): 570-3.

Russell, S. J., K. W. Peng and J. C. Bell (2012). Oncolytic virotherapy. *Nat Biotechnol* 30(7): 658-70.

Russell, W. C. (2000). Update on adenovirus and its vectors. *J Gen Virol* 81(Pt 11): 2573-604.

Russell, W. C. (2009). Adenoviruses: Update on structure and function. *J Gen Virol* 90(Pt 1): 1-20.

Russi, E. G., J. E. Raber-Durlacher and S. T. Sonis (2014). Local and systemic pathogenesis and consequences of regimen-induced inflammatory responses in patients with head and neck cancer receiving chemoradiation. *Mediators Inflamm* 2014: 518261.

Saban, S. D., M. Silvestry, G. R. Nemerow and P. L. Stewart (2006). Visualization of alpha-helices in a 6-angstrom resolution cryoelectron microscopy structure of adenovirus allows refinement of capsid protein assignments. *J Virol* 80(24): 12049-59.

Sangro, B., G. Mazzolini, J. Ruiz, M. Herraiz, J. Quiroga, I. Herrero, A. Benito, J. Larrache, J. Pueyo, J. C. Subtil, C. Olague, J. Sola, B. Sadaba, C. Lacasa, I. Melero, C. Qian and J. Prieto (2004). Phase i trial of intratumoral injection of an adenovirus encoding interleukin-12 for advanced digestive tumors. *J Clin Oncol* 22(8): 1389-97.

Sanoff, H. K., W. R. Carpenter, J. Freburger, L. Li, K. Chen, L. L. Zullig, R. M. Goldberg, M. J. Schymura and D. Schrag (2012). Comparison of adverse events during 5-fluorouracil versus 5-fluorouracil/oxaliplatin adjuvant chemotherapy for stage iii colon cancer: A population-based analysis. *Cancer* 118(17): 4309-20.

Sarnow, P., Y. S. Ho, J. Williams and A. J. Levine (1982). Adenovirus e1b-58kd tumor antigen and sv40 large tumor antigen are physically associated with the same 54 kd cellular protein in transformed cells. *Cell* 28(2): 387-94.

Sato, M., S. Goto, R. Kaneko, M. Ito, S. Sato and S. Takeuchi (1998). Impaired production of th1 cytokines and increased frequency of th2 subsets in pbmc from advanced cancer patients. *Anticancer Res* 18(5D): 3951-5.

Scaffidi, P., T. Misteli and M. E. Bianchi (2002). Release of chromatin protein hmgb1 by necrotic cells triggers inflammation. *Nature* 418(6894): 191-5.

Schiavoni, G., A. Sistigu, M. Valentini, F. Mattei, P. Sestili, F. Spadaro, M. Sanchez, S. Lorenzi, M. T. D'Urso, F. Belardelli, L. Gabriele, E. Proietti and L. Bracci (2011). Cyclophosphamide synergizes with type i interferons through systemic dendritic cell reactivation and induction of immunogenic tumor apoptosis. *Cancer Res* 71(3): 768-78.

Schwartz, R. A., S. S. Lakdawala, H. D. Eshleman, M. R. Russell, C. T. Carson and M. D. Weitzman (2008). Distinct requirements of adenovirus e1b55k protein for degradation of cellular substrates. *J Virol* 82(18): 9043-55.

Seliger, B., F. M. Marincola, S. Ferrone and H. Abken (2008). The complex role of b7 molecules in tumor immunology. *Trends Mol Med* 14(12): 550-9.

Semino, C., G. Angelini, A. Poggi and A. Rubartelli (2005). Nk/idc interaction results in il-18 secretion by dcs at the synaptic cleft followed by nk cell activation and release of the dc maturation factor hmgb1. *Blood* 106(2): 609-16.

Senzer, N., S. Mani, A. Rosemurgy, J. Nemunaitis, C. Cunningham, C. Guha, N. Bayol, M. Gillen, K. Chu, C. Rasmussen, H. Rasmussen, D. Kufe, R. Weichselbaum and N. Hanna (2004). Tnferade biologic, an adenovector with a radiation-inducible promoter, carrying the human tumor necrosis factor alpha gene: A phase i study in patients with solid tumors. *J Clin Oncol* 22(4): 592-601.

Senzer, N. N., H. L. Kaufman, T. Amatruda, M. Nemunaitis, T. Reid, G. Daniels, R. Gonzalez, J. Glaspy, E. Whitman, K. Harrington, H. Goldsweig, T. Marshall, C. Love, R. Coffin and J. J. Nemunaitis (2009). Phase ii clinical trial of a granulocyte-macrophage colony-stimulating factor-encoding, second-generation oncolytic herpesvirus in patients with unresectable metastatic melanoma. *J Clin Oncol* 27(34): 5763-71.

Serafini, P., R. Carbley, K. A. Noonan, G. Tan, V. Bronte and I. Borrello (2004). High-dose granulocyte-macrophage colony-stimulating factor-producing vaccines impair the immune response through the recruitment of myeloid suppressor cells. *Cancer Res* 64(17): 6337-43.

Sevko, A., M. Sade-Feldman, J. Kanterman, T. Michels, C. S. Falk, L. Umansky, M. Ramacher, M. Kato, D. Schadendorf, M. Baniyash and V. Umansky (2013). Cyclophosphamide promotes chronic inflammation-dependent immunosuppression and prevents antitumor response in melanoma. *J Invest Dermatol* 133(6): 1610-9.

Sherr, C. J. (1996). Cancer cell cycles. *Science* 274(5293): 1672-7.

Shevchenko, I., S. Karakhanova, S. Soltek, J. Link, J. Bayry, J. Werner, V. Umansky and A. V. Bazhin (2013). Low-dose gemcitabine depletes regulatory t cells and improves survival in the orthotopic panc02 model of pancreatic cancer. *Int J Cancer* 133(1): 98-107.

Shi, L., M. Ramaswamy, L. J. Manzel and D. C. Look (2007). Inhibition of jak1-dependent signal transduction in airway epithelial cells infected with adenovirus. *Am J Respir Cell Mol Biol* 37(6): 720-8.

Short, J. J., A. V. Pereboev, Y. Kawakami, C. Vasu, M. J. Holterman and D. T. Curiel (2004). Adenovirus serotype 3 utilizes cd80 (b7.1) and cd86 (b7.2) as cellular attachment receptors. *Virology* 322(2): 349-59.

Sims, G. P., D. C. Rowe, S. T. Rietdijk, R. Herbst and A. J. Coyle (2010). Hmgb1 and rage in inflammation and cancer. *Annu Rev Immunol* 28: 367-88.

Sistigu, A., S. Viaud, N. Chaput, L. Bracci, E. Proietti and L. Zitvogel (2011). Immunomodulatory effects of cyclophosphamide and implementations for vaccine design. *Semin Immunopathol* 33(4): 369-83.

Skoetz, N., S. Trelle, M. Rancea, H. Haverkamp, V. Diehl, A. Engert and P. Borchmann (2013). Effect of initial treatment strategy on survival of patients with advanced-stage hodgkin's lymphoma: A systematic review and network meta-analysis. *Lancet Oncol* 14(10): 943-52.

Small, E. J., M. A. Carducci, J. M. Burke, R. Rodriguez, L. Fong, L. van Ummersen, D. C. Yu, J. Aimi, D. Ando, P. Working, D. Kirn and G. Wilding (2006). A phase i trial of intravenous cg7870, a replication-

selective, prostate-specific antigen-targeted oncolytic adenovirus, for the treatment of hormone-refractory, metastatic prostate cancer. *Mol Ther* 14(1): 107-17.

Smith, E., J. Breznik and B. D. Lichty (2011). Strategies to enhance viral penetration of solid tumors. *Hum Gene Ther* 22(9): 1053-60.

Smith, J. G., C. M. Wiethoff, P. L. Stewart and G. R. Nemerow (2010). Adenovirus. *Curr Top Microbiol Immunol* 343: 195-224.

Smith, K. A. (2014). Toward a molecular understanding of adaptive immunity: A chronology, part iii. *Front Immunol* 5: 29.

Smith, T. A., N. Idamakanti, M. L. Rollence, J. Marshall-Neff, J. Kim, K. Mulgrew, G. R. Nemerow, M. Kaleko and S. C. Stevenson (2003). Adenovirus serotype 5 fiber shaft influences in vivo gene transfer in mice. *Hum Gene Ther* 14(8): 777-87.

Staeheli, P. and J. Pavlovic (1991). Inhibition of vesicular stomatitis virus mrna synthesis by human mxa protein. *J Virol* 65(8): 4498-501.

Sterman, D. H., A. Recio, A. R. Haas, A. Vachani, S. I. Katz, C. T. Gillespie, G. Cheng, J. Sun, E. Moon, L. Pereira, X. Wang, D. F. Heitjan, L. Litzky, C. H. June, R. H. Vonderheide, R. G. Carroll and S. M. Albelda (2010). A phase i trial of repeated intrapleural adenoviral-mediated interferon-beta gene transfer for mesothelioma and metastatic pleural effusions. *Mol Ther* 18(4): 852-60.

Stillman, B. (1986). Functions of the adenovirus e1b tumour antigens. *Cancer Surv* 5(2): 389-404.

Stoetzer, O. J., D. M. Fersching, C. Salat, O. Steinkohl, C. J. Gabka, U. Hamann, M. Braun, A. M. Feller, V. Heinemann, B. Siegele, D. Nagel and S. Holdenrieder (2013). Circulating immunogenic cell death biomarkers hmgb1 and rage in breast cancer patients during neoadjuvant chemotherapy. *Tumour Biol* 34(1): 81-90.

Stracker, T. H., C. T. Carson and M. D. Weitzman (2002). Adenovirus oncoproteins inactivate the mre11-rad50-nbs1 DNA repair complex. *Nature* 418(6895): 348-52.

Strauss, R., P. Sova, Y. Liu, Z. Y. Li, S. Tuve, D. Pritchard, P. Brinkkoetter, T. Moller, O. Wildner, S. Pesonen, A. Hemminki, N. Urban, C. Drescher and A. Lieber (2009). Epithelial phenotype confers resistance of ovarian cancer cells to oncolytic adenoviruses. *Cancer Res* 69(12): 5115-25.

Strunze, S., L. C. Trotman, K. Boucke and U. F. Greber (2005). Nuclear targeting of adenovirus type 2 requires crm1-mediated nuclear export. *Mol Biol Cell* 16(6): 2999-3009.

Stupp, R., W. P. Mason, M. J. van den Bent, M. Weller, B. Fisher, M. J. Taphoorn, K. Belanger, A. A. Brandes, C. Marosi, U. Bogdahn, J. Curschmann, R. C. Janzer, S. K. Ludwin, T. Gorlia, A. Allgeier, D. Lacombe, J. G. Cairncross, E. Eisenhauer, R. O. Mirimanoff, R. European Organisation for, T. Treatment of Cancer Brain, G. Radiotherapy and G. National Cancer Institute of Canada Clinical Trials (2005). Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *N Engl J Med* 352(10): 987-96.

Sumida, S. M., D. M. Truitt, A. A. Lemckert, R. Vogels, J. H. Custers, M. M. Addo, S. Lockman, T. Peter, F. W. Peyerl, M. G. Kishko, S. S. Jackson, D. A. Gorgone, M. A. Lifton, M. Essex, B. D. Walker, J. Goudsmit, M. J. Havenga and D. H. Barouch (2005). Neutralizing antibodies to adenovirus serotype 5 vaccine vectors are directed primarily against the adenovirus hexon protein. *J Immunol* 174(11): 7179-85.

Sung, M. W., S. H. Chen, S. N. Thung, D. Y. Zhang, T. G. Huang, J. P. Mandeli and S. L. Woo (2002). Intratumoral delivery of adenovirus-mediated interleukin-12 gene in mice with metastatic cancer in the liver. *Hum Gene Ther* 13(6): 731-43.

Sung, R. S., L. Qin and J. S. Bromberg (2001). Tnfalpha and ifngamma induced by innate anti-adenoviral immune responses inhibit adenovirus-mediated transgene expression. *Mol Ther* 3(5 Pt 1): 757-67.

Suzuki, K., R. Alemany, M. Yamamoto and D. T. Curiel (2002). The presence of the adenovirus e3 region improves the oncolytic potency of conditionally replicative adenoviruses. *Clin Cancer Res* 8(11): 3348-59.

Tadmor, T., R. Fell, A. Polliack and D. Attias (2013). Absolute monocytosis at diagnosis correlates with survival in diffuse large b-cell lymphoma-possible link with monocytic myeloid-derived suppressor cells. *Hematol Oncol* 31(2): 65-71.

Takeda, T., K. Nakajima, H. Kojima and T. Hirano (1994). E1a repression of il-6-induced gene activation by blocking the assembly of il-6 response element binding complexes. *J Immunol* 153(10): 4573-82.

Takeshima, T., K. Chamoto, D. Wakita, T. Ohkuri, Y. Togashi, H. Shirato, H. Kitamura and T. Nishimura (2010). Local radiation therapy inhibits tumor growth through the generation of tumor-specific ctl: Its potentiation by combination with th1 cell therapy. *Cancer Res* 70(7): 2697-706.

Tamanini, A., E. Nicolis, A. Bonizzato, V. Bezzerri, P. Melotti, B. M. Assael and G. Cabrini (2006). Interaction of adenovirus type 5 fiber with the coxsackievirus and adenovirus receptor activates inflammatory response in human respiratory cells. *J Virol* 80(22): 11241-54.

Tanchot, C., M. Terme, H. Pere, T. Tran, N. Benhamouda, M. Strioga, C. Banissi, L. Galluzzi, G. Kroemer and E. Tartour (2013). Tumor-infiltrating regulatory t cells: Phenotype, role, mechanism of expansion in situ and clinical significance. *Cancer Microenviron* 6(2): 147-57.

Taniguchi, M., K. Seino and T. Nakayama (2003). The nkt cell system: Bridging innate and acquired immunity. *Nat Immunol* 4(12): 1164-5.

Tarca, A. L., S. Draghici, P. Khatri, S. S. Hassan, P. Mittal, J. S. Kim, C. J. Kim, J. P. Kusanovic and R. Romero (2009). A novel signaling pathway impact analysis. *Bioinformatics* 25(1): 75-82.

Tatar, Z., E. Thivat, E. Planchat, P. Gimbergues, E. Gadea, C. Abrial and X. Durando (2013). Temozolomide and unusual indications: Review of literature. *Cancer Treat Rev* 39(2): 125-35.

Tazawa, H., S. Kagawa and T. Fujiwara (2013). Oncolytic adenovirus-induced autophagy: Tumor-suppressive effect and molecular basis. *Acta Med Okayama* 67(6): 333-42.

Tesniere, A., F. Schlemmer, V. Boige, O. Kepp, I. Martins, F. Ghiringhelli, L. Aymeric, M. Michaud, L. Apetoh, L. Barault, J. Mendiboure, J. P. Pignon, V. Jooste, P. van Endert, M. Ducreux, L. Zitvogel, F. Piard and G. Kroemer (2010). Immunogenic death of colon cancer cells treated with oxaliplatin. *Oncogene* 29(4): 482-91.

Thaci, B., I. V. Ulasov, D. A. Wainwright and M. S. Lesniak (2011). The challenge for gene therapy: Innate immune response to adenoviruses. *Oncotarget* 2(3): 113-21.

Thirukkumaran, C. and D. G. Morris (2009). Oncolytic viral therapy using reovirus. *Methods Mol Biol* 542: 607-34.

Thomas, M. A., J. F. Spencer, M. C. La Regina, D. Dhar, A. E. Tollefson, K. Toth and W. S. Wold (2006). Syrian hamster as a permissive immunocompetent animal model for the study of oncolytic adenovirus vectors. *Cancer Res* 66(3): 1270-6.

Tian, M., J. R. Neil and W. P. Schiemann (2011). Transforming growth factor-beta and the hallmarks of cancer. *Cell Signal* 23(6): 951-62.

Tollefson, A. E., J. S. Ryerse, A. Scaria, T. W. Hermiston and W. S. Wold (1996a). The e3-11.6-kda adenovirus death protein (adp) is required for efficient cell death: Characterization of cells infected with adp mutants. *Virology* 220(1): 152-62.

Tollefson, A. E., A. Scaria, T. W. Hermiston, J. S. Ryerse, L. J. Wold and W. S. Wold (1996b). The adenovirus death protein (e3-11.6k) is required at very late stages of infection for efficient cell lysis and release of adenovirus from infected cells. *J Virol* 70(4): 2296-306.

Tollefson, A. E., A. Scaria, S. K. Saha and W. S. Wold (1992). The 11,600-mw protein encoded by region e3 of adenovirus is expressed early but is greatly amplified at late stages of infection. *J Virol* 66(6): 3633-42.

Tong, A. W., J. Nemunaitis, D. Su, Y. Zhang, C. Cunningham, N. Senzer, G. Netto, D. Rich, A. Mhashilkar, K. Parker, K. Coffee, R. Ramesh, S. Ekmekcioglu, E. A. Grimm, J. van Wart Hood, J. Merritt and S. Chada (2005). Intratumoral injection of ingn 241, a nonreplicating adenovector expressing the melanoma-

differentiation associated gene-7 (mda-7/il24): Biologic outcome in advanced cancer patients. *Mol Ther* 11(1): 160-72.

Topalian, S. L., F. S. Hodi, J. R. Brahmer, S. N. Gettinger, D. C. Smith, D. F. McDermott, J. D. Powderly, R. D. Carvajal, J. A. Sosman, M. B. Atkins, P. D. Leming, D. R. Spigel, S. J. Antonia, L. Horn, C. G. Drake, D. M. Pardoll, L. Chen, W. H. Sharfman, R. A. Anders, J. M. Taube, T. L. McMiller, H. Xu, A. J. Korman, M. Jure-Kunkel, S. Agrawal, D. McDonald, G. D. Kollia, A. Gupta, J. M. Wigginton and M. Sznol (2012). Safety, activity, and immune correlates of anti-pd-1 antibody in cancer. *N Engl J Med* 366(26): 2443-54.

Tuve, S., Y. Liu, K. Tragoolpua, J. D. Jacobs, R. C. Yumul, Z. Y. Li, R. Strauss, K. E. Hellstrom, M. L. Disis, S. Roffler and A. Lieber (2009). In situ adenovirus vaccination engages t effector cells against cancer. *Vaccine* 27(31): 4225-39.

Tyler, M. A., I. V. Ulasov, A. Borovjagin, A. M. Sonabend, A. Khramtsov, Y. Han, P. Dent, P. B. Fisher, D. T. Curiel and M. S. Lesniak (2006). Enhanced transduction of malignant glioma with a double targeted ad5/3-rgd fiber-modified adenovirus. *Mol Cancer Ther* 5(9): 2408-16.

Tyler, M. A., I. V. Ulasov and M. S. Lesniak (2009). Cancer cell death by design: Apoptosis, autophagy and glioma virotherapy. *Autophagy* 5(6): 856-7.

Tyynela, K., A. M. Sandmair, M. Turunen, R. Vanninen, P. Vainio, R. Kauppinen, R. Johansson, M. Vapalahti and S. Yla-Herttuala (2002). Adenovirus-mediated herpes simplex virus thymidine kinase gene therapy in bt4c rat glioma model. *Cancer Gene Ther* 9(11): 917-24.

Ulasov, I. V., A. M. Sonabend, S. Nandi, A. Khramtsov, Y. Han and M. S. Lesniak (2009). Combination of adenoviral virotherapy and temozolomide chemotherapy eradicates malignant glioma through autophagic and apoptotic cell death in vivo. *Br J Cancer* 100(7): 1154-64.

Wahl, R. L., H. Jacene, Y. Kasamon and M. A. Lodge (2009). From recist to percist: Evolving considerations for pet response criteria in solid tumors. *J Nucl Med* 50 Suppl 1: 122S-50S.

Vakkila, J., R. Jaffe, M. Michelow and M. T. Lotze (2006). Pediatric cancers are infiltrated predominantly by macrophages and contain a paucity of dendritic cells: A major nosologic difference with adult tumors. *Clin Cancer Res* 12(7 Pt 1): 2049-54.

Walters, R. W., P. Freimuth, T. O. Moninger, I. Ganske, J. Zabner and M. J. Welsh (2002). Adenovirus fiber disrupts car-mediated intercellular adhesion allowing virus escape. *Cell* 110(6): 789-99.

van der Most, R. G., A. Currie, B. W. Robinson and R. A. Lake (2006). Cranking the immunologic engine with chemotherapy: Using context to drive tumor antigen cross-presentation towards useful antitumor immunity. *Cancer Res* 66(2): 601-4.

van Kooten, C. (2000). Immune regulation by cd40-cd40-l interactions - 2; y2k update. *Front Biosci* 5: D880-693.

van Kooten, C. and J. Banchereau (2000). Cd40-cd40 ligand. *J Leukoc Biol* 67(1): 2-17.

van Sorge, N. M., W. L. van der Pol and J. G. van de Winkel (2003). Fcgammar polymorphisms: Implications for function, disease susceptibility and immunotherapy. *Tissue Antigens* 61(3): 189-202.

Wang, D. and R. N. Dubois (2006). Prostaglandins and cancer. *Gut* 55(1): 115-22.

Wang, H., O. Bloom, M. Zhang, J. M. Vishnubhakat, M. Ombrellino, J. Che, A. Frazier, H. Yang, S. Ivanova, L. Borovikova, K. R. Manogue, E. Faist, E. Abraham, J. Andersson, U. Andersson, P. E. Molina, N. N. Abumrad, A. Sama and K. J. Tracey (1999). Hmg-1 as a late mediator of endotoxin lethality in mice. *Science* 285(5425): 248-51.

Wang, H., Z. Y. Li, Y. Liu, J. Persson, I. Beyer, T. Moller, D. Koyuncu, M. R. Drescher, R. Strauss, X. B. Zhang, J. K. Wahl, 3rd, N. Urban, C. Drescher, A. Hemminki, P. Fender and A. Lieber (2011). Desmoglein 2 is a receptor for adenovirus serotypes 3, 7, 11 and 14. *Nat Med* 17(1): 96-104.

Wang, K., S. Huang, A. Kapoor-Munshi and G. Nemerow (1998). Adenovirus internalization and infection require dynamin. *J Virol* 72(4): 3455-8.

VanOosten, R. L. and T. S. Griffith (2007). Activation of tumor-specific cd8+ t cells after intratumoral ad5-trail/cpg oligodeoxynucleotide combination therapy. *Cancer Res* 67(24): 11980-90.

Vasey, P. A., L. N. Shulman, S. Campos, J. Davis, M. Gore, S. Johnston, D. H. Kirn, V. O'Neill, N. Siddiqui, M. V. Seiden and S. B. Kaye (2002). Phase i trial of intraperitoneal injection of the e1b-55-kd-gene-deleted adenovirus onyx-015 (dl1520) given on days 1 through 5 every 3 weeks in patients with recurrent/refractory epithelial ovarian cancer. *J Clin Oncol* 20(6): 1562-9.

Weiden, P. L., N. Flournoy, E. D. Thomas, R. Prentice, A. Fefer, C. D. Buckner and R. Storb (1979). Antileukemic effect of graft-versus-host disease in human recipients of allogeneic-marrow grafts. *N Engl J Med* 300(19): 1068-73.

Weigel, S. and M. Dobbelstein (2000). The nuclear export signal within the e4orf6 protein of adenovirus type 5 supports virus replication and cytoplasmic accumulation of viral mrna. *J Virol* 74(2): 764-72.

Velho, T. R. (2012). Metastatic melanoma - a review of current and future drugs. *Drugs Context* 2012: 212242.

Verheije, M. H. and P. J. Rottier (2012). Retargeting of viruses to generate oncolytic agents. *Adv Virol* 2012: 798526.

Westphal, M., S. Yla-Herttuala, J. Martin, P. Warnke, P. Menei, D. Eckland, J. Kinley, R. Kay, Z. Ram and A. S. Group (2013). Adenovirus-mediated gene therapy with sitimagene ceradenovec followed by intravenous ganciclovir for patients with operable high-grade glioma (aspect): A randomised, open-label, phase 3 trial. *Lancet Oncol* 14(9): 823-33.

Wheeler, M. D., S. Yamashina, M. Froh, I. Rusyn and R. G. Thurman (2001). Adenoviral gene delivery can inactivate kupffer cells: Role of oxidants in nf-kappab activation and cytokine production. *J Leukoc Biol* 69(4): 622-30.

Whyte, P., K. J. Buchkovich, J. M. Horowitz, S. H. Friend, M. Raybuck, R. A. Weinberg and E. Harlow (1988). Association between an oncogene and an anti-oncogene: The adenovirus e1a proteins bind to the retinoblastoma gene product. *Nature* 334(6178): 124-9.

Whyte, P., N. M. Williamson and E. Harlow (1989). Cellular targets for transformation by the adenovirus e1a proteins. *Cell* 56(1): 67-75.

Wierda, W. G., J. E. Castro, R. Aguillon, D. Sampath, A. Jalayer, J. McMannis, C. E. Prussak, M. Keating and T. J. Kipps (2010). A phase i study of immune gene therapy for patients with cll using a membrane-stable, humanized cd154. *Leukemia* 24(11): 1893-900.

Wiethoff, C. M., H. Wodrich, L. Gerace and G. R. Nemerow (2005). Adenovirus protein vi mediates membrane disruption following capsid disassembly. *J Virol* 79(4): 1992-2000.

Williams, R. S., J. S. Williams and J. A. Tainer (2007). Mre11-rad50-nbs1 is a keystone complex connecting DNA repair machinery, double-strand break signaling, and the chromatin template. *Biochem Cell Biol* 85(4): 509-20.

Wisskirchen, C., T. H. Ludersdorfer, D. A. Muller, E. Moritz and J. Pavlovic (2011). Interferon-induced antiviral protein mxa interacts with the cellular rna helicases uap56 and urh49. *J Biol Chem* 286(40): 34743-51.

Wittwer, C., S. Boeck, V. Heinemann, M. Haas, P. Stieber, D. Nagel and S. Holdenrieder (2013). Circulating nucleosomes and immunogenic cell death markers hmgb1, srage and dnase in patients with advanced pancreatic cancer undergoing chemotherapy. *Int J Cancer* 133(11): 2619-30.

Vogel, G. (2014). Assisted reproduction. Fda considers trials of 'three-parent embryos'. *Science* 343(6173): 827-8.

Wolchok, J. D., A. Hoos, S. O'Day, J. S. Weber, O. Hamid, C. Lebbe, M. Maio, M. Binder, O. Bohnsack, G. Nichol, R. Humphrey and F. S. Hodi (2009). Guidelines for the evaluation of immune therapy activity in solid tumors: Immune-related response criteria. *Clin Cancer Res* 15(23): 7412-20.

Vonderheide, R. H., J. P. Dutcher, J. E. Anderson, S. G. Eckhardt, K. F. Stephans, B. Razvillas, S. Garl, M. D. Butine, V. P. Perry, R. J. Armitage, R. Ghalie, D. A. Caron and J. G. Gribben (2001). Phase i study of recombinant human cd40 ligand in cancer patients. *J Clin Oncol* 19(13): 3280-7.

Worgall, S., G. Wolff, E. Falck-Pedersen and R. G. Crystal (1997). Innate immune mechanisms dominate elimination of adenoviral vectors following in vivo administration. *Hum Gene Ther* 8(1): 37-44.

Wu, H., T. Seki, I. Dmitriev, T. Uil, E. Kashentseva, T. Han and D. T. Curiel (2002). Double modification of adenovirus fiber with rgd and polylysine motifs improves coxsackievirus-adenovirus receptor-independent gene transfer efficiency. *Hum Gene Ther* 13(13): 1647-53.

Wu, J., T. Vallenius, K. Ovaska, J. Westermarck, T. P. Makela and S. Hautaniemi (2009). Integrated network analysis platform for protein-protein interactions. *Nat Methods* 6(1): 75-7.

Xia, Z. J., J. H. Chang, L. Zhang, W. Q. Jiang, Z. Z. Guan, J. W. Liu, Y. Zhang, X. H. Hu, G. H. Wu, H. Q. Wang, Z. C. Chen, J. C. Chen, Q. H. Zhou, J. W. Lu, Q. X. Fan, J. J. Huang and X. Zheng (2004). [phase iii randomized clinical trial of intratumoral injection of e1b gene-deleted adenovirus (h101) combined with cisplatin-based chemotherapy in treating squamous cell cancer of head and neck or esophagus]. *Ai Zheng* 23(12): 1666-70.

Yamazaki, T., D. Hannani, V. Poirier-Colame, S. Ladoire, C. Locher, A. Sistigu, N. Prada, S. Adjemian, J. P. Catani, M. Freudenberg, C. Galanos, F. Andre, G. Kroemer and L. Zitvogel (2014). Defective immunogenic cell death of hmgb1-deficient tumors: Compensatory therapy with tlr4 agonists. *Cell Death Differ* 21(1): 69-78.

Yang, J. C. and S. A. Rosenberg (1997). An ongoing prospective randomized comparison of interleukin-2 regimens for the treatment of metastatic renal cell cancer. *Cancer J Sci Am* 3 Suppl 1: S79-84.

Yang, Z. X., D. Wang, G. Wang, Q. H. Zhang, J. M. Liu, P. Peng and X. H. Liu (2010). Clinical study of recombinant adenovirus-p53 combined with fractionated stereotactic radiotherapy for hepatocellular carcinoma. *J Cancer Res Clin Oncol* 136(4): 625-30.

Young, H., R. Baum, U. Cremerius, K. Herholz, O. Hoekstra, A. A. Lammertsma, J. Pruim and P. Price (1999). Measurement of clinical and subclinical tumour response using [18f]-fluorodeoxyglucose and positron emission tomography: Review and 1999 eortc recommendations. European organization for research and treatment of cancer (eortc) pet study group. *Eur J Cancer* 35(13): 1773-82.

Yu, W. and H. Fang (2007). Clinical trials with oncolytic adenovirus in china. *Curr Cancer Drug Targets* 7(2): 141-8.

Yue, P., E. Melamud and J. Moult (2006). Snps3d: Candidate gene and snp selection for association studies. *BMC Bioinformatics* 7: 166.

Zaiss, A. K., H. B. Machado and H. R. Herschman (2009). The influence of innate and pre-existing immunity on adenovirus therapy. *J Cell Biochem* 108(4): 778-90.

Zanussi, S., E. Vaccher, C. Caffau, C. Pratesi, C. Crepaldi, M. T. Bortolin, R. Tedeschi, D. Politi, L. Barzan, U. Tirelli and P. De Paoli (2003). Interferon-gamma secretion and perforin expression are impaired in cd8+ t lymphocytes from patients with undifferentiated carcinoma of nasopharyngeal type. *Cancer Immunol Immunother* 52(1): 28-32.

Zarogoulidis, P., L. Yarmus, K. Darwiche, R. Walter, H. Huang, Z. Li, B. Zaric, K. Tsakiridis and K. Zarogoulidis (2013). Interleukin-6 cytokine: A multifunctional glycoprotein for cancer. *Immunome Res* 9(62): 16535.

Zheng, S., I. V. Ulasov, Y. Han, M. A. Tyler, Z. B. Zhu and M. S. Lesniak (2007). Fiber-knob modifications enhance adenoviral tropism and gene transfer in malignant glioma. *J Gene Med* 9(3): 151-60.

Zhu, J., X. Huang and Y. Yang (2007). Innate immune response to adenoviral vectors is mediated by both toll-like receptor-dependent and -independent pathways. *J Virol* 81(7): 3170-80.

Zhu, W., L. Zhou, J. Q. Qian, T. Z. Qiu, Y. Q. Shu and P. Liu (2014). Temozolomide for treatment of brain metastases: A review of 21 clinical trials. *World J Clin Oncol* 5(1): 19-27.

PART C – ORIGINAL PUBLICATIONS