DEVELOPING GENETICALLY ENGINEERED ONCOLYTIC VIRUSES FOR CANCER GENE THERAPY

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PART A

LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original publications, which are referred to in the text by their roman numerals

I Kangasniemi L., **PARVIAINEN S**., Pisto T., Koskinen M., Jokinen M., Kiviluoto T., Cerullo V., Jalonen H., Koski A., Kangasniemi A., Kanerva A., Pesonen S., Hemminki A. Effects of capsid-modified oncolytic adenoviruses and their combinations with gemcitabine or silica gel on pancreatic cancer. *Int J Cancer. 131:253-363, 2012*.

II PARVIAINEN S.,Ahonen M., Diaconu I.,Hirvinen M., Vähä-Koskela M., Karttunen Å., Hemminki A., Cerullo V. CD40-Ligand and tdTomato Armed Vaccinia Virus for Induction of Anti-Tumor Immune Response and tumor imaging. *Gene Ther. 21:195-204, 2014*.

III *PARVIAINEN S., *Ahonen M., Kipar A., Diaconu I., Vaha-Koskela M., Kanerva A., Cerullo V., Hemminki A. GMCSF –armed oncolytic vaccinia virus induces an anti-tumor immune response. *Int J Cancer. 135:1065-1072, 2014*.

IV PARVIAINEN S., Autio K., Guse K., Pesonen S., Karli E.,Vähä-Koskela M.,Rosol T.J., Zhao F., Hemminki A. Incomplete but infectious vaccinia virions are produced in the absence of oncolysis in feline SCCF1 cells. *PLoS One 10(3):e0120496,* 2015.

* Equal contribution

Publication I was included in the thesis of Lotta Kangasniemi (Improving oncolytic adenoviral therapies for gastrointestinal cancers and tumor initiating cells, University of Helsinki 2010).

ABBREVIATIONS

Ad	adenovirus
Ad3	adenovirus serotype 3
Ad5	adenovirus serotype 5
ADP	adenvirus death protein
APC	antigen-presenting cell
bp	base pair
BrdU	bromodeoxyuridine
CAR	coxackie-adenovirus receptor or chimeric antigen receptor
CEV	cell-associated enveloped virus
CPE	cytopathic effect
Cr	crescent
CTL	cytotoxic T-lymphocyte
CTLA-4	cytotoxic T-lymphocyte antigen 4
DC	dendritic cell
DMEM	Dulbecco´s Modified Eagle Medium
DNA	deoxyribonucleic acid
DSG-2	desmoglein-2
EGFR	epidermal growth factor receptor
FCS	fetal calf serum
EEV	extracellular enveloped virus
ELISA	enzyme-linked immunosorbent assay
GFP	green fluorescent protein
GMCSF	granulocyte-marcrophage colony-stimulating factor
FACS	fluorescence-activated cell sorting
HCC	hepatocellular carcinoma
HSPG	heparin sulfate proteoglycans
HSV	herpes-simplex virus
IFN	interferon

IL	interleukine
IMV	intracellular mature virus
LacZ	β-galactosidase
luc	luciferase
MHC	major histocompatibility complex
MOI	multiplicity of infection
MTD	maximum tolerated dose
MTS	[3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]
MV	mature virus
Nab	neutralizing antibody
NF-κB	nuclear factor κB
NK	natural killer cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethel glycol
pfu	plaque forming unit
pK	polylysine
qPCR	quantitative real-time polymerase chain reaction
Rb	retinoblastoma
RGD	arginine-lysine-aspartic acid
RNA	ribonucleic acid
SCID	severe combined immune deficiency
TCR	T-cell receptor
TIL	tumor-infiltrating lymphocyte
TLR	toll-like receptor
TK	thymidine kinase
VEGFR	vascular endothelial growth factor receptor
VGF	vaccinia growth factor
vp	virus particle

VSV	vesicular stomatitis virus
vv	vaccinia virus
wt	wild type

ABSTRACT

It has been estimated that up to half of people living in industrial societies will get cancer. Almost all cancer deaths are caused by metastatic disease and only few metastatic solid tumors can be cured with available therapies. Therefore, novel therapeutic modalities are needed. Genetically engineered oncolytic viruses such as adenoviruses and vaccinia viruses are a promising therapeutic approach given their capacity to specifically replicate in and kill tumor cells as well as to reach distant metastasis. Oncolytic viruses have demonstrated good safety, tolerability and promising signs of anti-tumor efficacy in several clinical trials, but the efficacy of the therapy needs improvement to reach its full clinical potential.

Adenoviruses are one of the most studied vectors in oncolytic virotherapy. Two major obstacles limiting the efficacy have been insufficient transduction of the tumor cells and the recognition of the virus by the immune system, which rapidly clears the virus from systemic circulation, hampering the overall efficacy. This study shows that novel capsid modifications can increase the transduction efficacy and oncolytic potency of the virus, and that embedding oncolytic virus in a silica implant might help to circumvent the problem of clearance by prompting only modest immune responses against the virus.

Recently, arming oncolytic viruses with immunomodulatory molecules has been a major focus in virotherapy. Immunotherapy means inducing the patient´s immune system to recognize and attack the tumor and has been recently linked with oncolytic virotherapy, as the replication of the virus in the tumor can be immunogenic *per se* and also release cancer epitopes available for antigen-presenting cells. Host immune responses have been shown to be a key player in oncolytic virotherapy as the balance between anti-viral and anti-tumoral immunity determines the efficacy of the therapy. Oncolytic vaccinia virus possesses unique immunogenicity and mechanisms of action that are distinct from other treatment modalities, and its self-perpetuating nature provides an ideal platform for therapeutic transgenic insertion. Therefore we engineered novel oncolytic vaccinia viruses with granulocyte-macrophage colony-stimulating factor (GMCSF) or CD40-ligand and studied their ability to increase the therapeutical outcome and anti-tumor immune responses in preclinical animal models. In the final part of the thesis the life cycle of oncolytic vaccinia virus was studied in more detail as we were able to show that the life cycle was compromised in a feline squamous cell carcinoma cell line. This finding might be important for a deeper understanding of the vaccinia virus-host cell interactions.

PART B

1. REVIEW OF THE LITERATURE

1.1 Introduction

Development of cancer is a multistep process causing cells to grow uncontrollably. In most cases, multiple cumulative genetic mutations and epigenetic changes are needed to cause cancer. One of the hallmarks of cancer is its invasive nature and these secondary cancer sites, called metastases, can cause local destruction and loss of normal tissue function (Hanahan et al. 2011). Despite the variety of successful therapies for different types of cancers, especially recurrent or metastatic cancers have low response rates to conventional treatments such as surgery, radiation therapy and chemotherapy (Chang et al. 2003, Kim et al. 2003) . Therefore, new alternative therapies for disseminated metastatic diseases are urgently needed.

Despite the remarkable improvements in cancer treatment modalities, diagnostic techniques and earlier access to cancer treatments, the global burden of cancer has more than doubled during the past 30 years. The corresponding estimates for total cancer deaths in 2012 were 8.2 million (about 22,000 cancer deaths per day) worldwide. It has been estimated that due to the continued growth and ageing of the world´s population the burden will further increase, and by 2030 there could be 75 million incident cases of cancer and 17 million cancer deaths worldwide (Ferlay et al. 2010, Ferlay et al. 2015). Cancer is a major public health concern and the leading cause of death in developed countries and the second most common in developing countries (Eaton 2003). In Finland, almost 30,000 new cases were diagnosed and about 11,800 people died due to cancer in 2012 (www.cancerregistry.fi, 2014).

1.2 Cancer gene therapy

Gene therapy is a field of research aiming to treat diseases caused by defective genes by altering the genomes of cells and tissues (Friedmann et al. 1972, Mulligan 1993). Disease entities for which gene therapy is being developed include for example cancer, cardiovascular disease, neurological diseases, hematological diseases, monogenic inherited diseases and infectious diseases. By June 2014, a total of 2076 clinical gene therapy trials had been initiated. The vast majority of these (64 %) were aimed at cancer gene therapy (Journal of Gene Medicine, http://www.abedia.com/wiley/index.html).

Cancer gene therapy approaches fall into four main strategies:

1) *Insertion of a normal gene into cancer cells to replace a mutated gene*

For example, mutation in p53 protein, which interferes with the ability of tumor cells to destruct themselves by apoptosis, is found in most of the cancers (Sherr et al. 2002). Restoring the functionality of the gene can direct cancer cell to apoptosis (Roth et al. 1996).

2) *Silence a mutated gene which is activated or overexpressed in cancer cells*

Such oncogenes can for example drive tumor growth, blood vessel formation, induce metastasis to other tissues, and allow for resistance to chemotherapy. Silencing can be accomplished by using e.g. small interfering RNA (siRNA) silencing technology, which has been used to specifically target for example tumor suppressor p53 molecules containing a single point mutation, leaving the wild-type suppressor intact (Martinez et al. 2002).

3) *Introducing genes that make cancer cells more sensitive to standard chemotherapy or for radiation treatments.*

Drug convertases ("suicide genes") which can turn an inactive pro-drug into an active drug can be introduced to tumor cells to cause cell-specific toxicity. For example, the herpes virus thymidine kinase can phosphorylate and convert non-toxic drug ganciclovir into toxic metabolites (Moolten et al. 1990). Additionally, the bystander effect can also affect the neighboring cells (Nicholas et al. 2003).

4) *Direct cell killing with targeted viruses*

After genetic engineering, oncolytic viruses selectively replicate in cancer cells leading to tumor cell destruction, oncolysis (Russell et al. 2012).

1.3. Oncolytic viruses

Different approaches utilizing viruses have been used for cancer treatment for several decades. Non-replicating or replicating viruses can be used as a gene transfer vector to introduce for example a therapeutic gene, co-stimulatory molecule or cytokine into cancer cells or to prime lymphocytes with tumor antigens in cancer vaccine approaches. By June 2014, viruses were being used as vector systems in approximately two thirds of all gene therapy trials. Out of different virus vectors, adenoviruses (23%) and retroviruses (19%) have

been reported as the most commonly used vectors (**Figure 1.**) (provided by Journal of Gene Medicine).

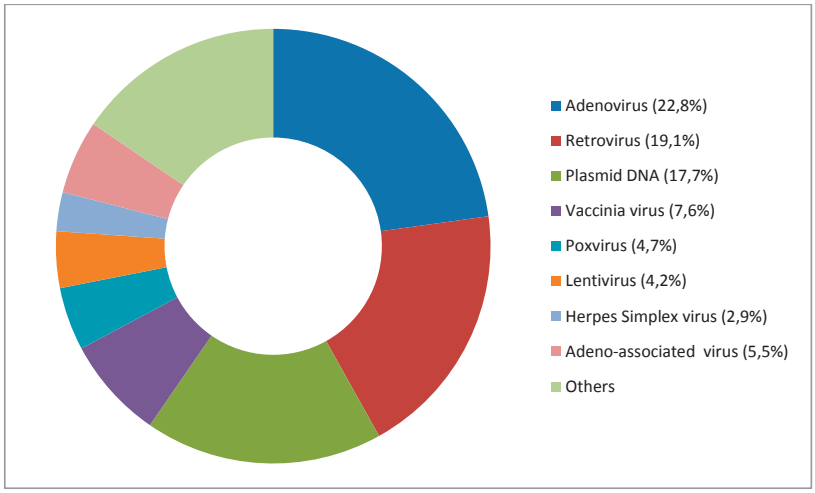

Figure 1. Cancer gene therapy trial reported until June 2014 in http://www.abedia.com/wiley/index.html database.

Oncolytic viruses are distinguished by their property to either inherently or after genetic modification replicates selectively in cancer cells. These viruses have multiple mechanisms to harm the host cells including direct lysis, induction of apoptosis and autophagy, expression of toxic proteins and shut-down of protein synthesis. At the end of the replication cycle, cells are destroyed and infective viral progeny is released into remaining tumor tissue. In addition to local amplifying antitumor effect, infective viral particles are able to enter systemic circulation and infect distant metastasis (**Figure 2.**) (Mullen et al. 2003, Russell et al. 2012). In addition to naturally occurring oncolytic viruses such as reovirus (Roberts et al. 2006), several human DNA and RNA viruses such as measles virus (MV), vesicular stomatitis virus (VSV), adenovirus, vaccinia virus (vv) and herpes simplex virus (HSV) have been genetically modified to selectively replicate in tumor cells, while their activity in normal cells is attenuated (Mullen et al. 2002, Kelly et al. 2007).

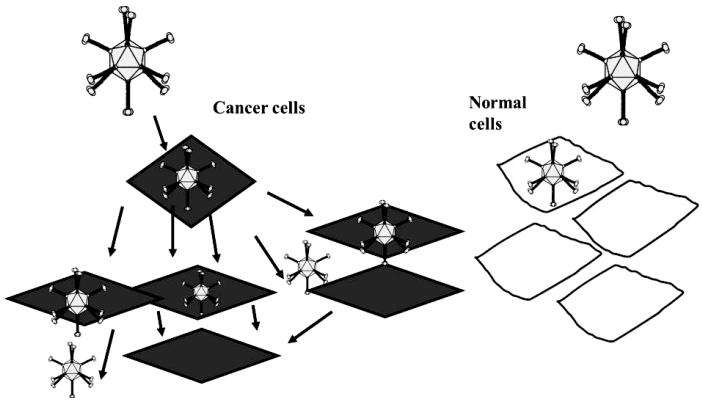

Figure 2. Oncolytic viruses can infect both normal and cancer cells but replication can only occur in cancer cells. New progeny of viruses is released from the lysed cancer cells, infecting other neighboring cancer cells.

1.3.1 Adenoviruses

Adenoviruses (Ad) are one of the most commonly used vectors for cancer gene therapy. Adenoviruses were first identified in the 1950s and ever since they have been intensively studied as gene therapy vectors (Rowe et al. 1953). Adenoviridae family can be divided into 4 genera and 6 species (Davison et al. 2003), and so far 59 serotypes of human adenoviruses have been identified (Chen et al. 2014). The various serotypes have been further classified in to subgroups A-G, depending on their ability to agglutinate erythrocytes (Rosen 1960). In this thesis the focus is on serotype 5 and 3 adenoviruses, which belong to the species C and B, respectively. Besides humans, they have a wide host-range but despite their ability to enter and infect different mammalian cells they tend to be species specific and replication in foreign host is quickly arrested. In general, adenoviruses are endemic in most parts of the world and have low pathogenicity in humans. Different serotypes have been shown to have different pathological effects but typically adenoviruses infect the epithelial cells in the respiratory and gastrointestinal track or the eyes causing mild flu, conjunctivitis and infantile gastroenteritis (Mautner et al. 1995, Berk 2007, Kunz et al. 2010).

Species B and C human adenoviruses are good candidates for use as gene therapy vehicles since they have a natural, lytic replication cycle and they can infect both dividing and non-dividing cells. Adenoviruses replicate with high efficiency and therefore they are easy to produce in high titers (up to 10^{13} pfu/ml). It is relatively easy to engineer adenoviral capsid

and genome which can accommodate up to 105% of the wild type´s 36 kb genome and multiple tumor-targeting strategies have been identified (Choi et al. 2012).

1.3.1.1 Structure and life cycle of adenoviruses

Adenoviruses are non-enveloped, double-stranded DNA viruses of approximately 90 nm in diameter. The virus is protected by an icosahedral protein capsid consisting of penton and hexon proteins, knobbed fiber proteins extended from the twelve vertices. Each penton protein has flexible loops on its surface, featuring an arginine-glycine-aspartic acid (RGD) motif which is involved in cellular binding and internalization (Stewart et al. 1991).

Adenovirus enters the cells by binding to a high affinity cell surface receptor with its fiber knob. Most adenovirus species have been shown to bind to coxackie- and adenovirus receptor (CAR), which triggers secondary interaction with RGD motif and cellular αvβ-integrins leading to endocytosis via clathrin coated pits (Mathias et al. 1994, Roelvink et al. 1999). The adenovirus life cycle can be divided into two phases separated by the onset of viral DNA replication. The early phase, lasting 5 to 6 hours, includes adsorption and penetration of the virus, transportation of uncoated virions to the nucleus and initiation of early gene expression from early transcription cassettes E1A, E1B, E2, E3 and E4. Viral E1A is the first gene to be transcribed after the entry and a major modulator of early gene expression. E1A drives cells to enter the S phase of the cell cycle, which supports the replication of viral DNA and synthesis of gene products needed for viral replication. Proteins encoded form E2 region provide the machinery for viral DNA replication whereas genes in E3 region are responsible for inhibiting innate anti-viral responses by damping major histocompatibility complex I (MHC-I) expression and lysis of the host cell mediated by adenovirus death protein (ADP). During the second phase, late genes are expressed from late transcription cassettes L1-L5 and assembly of the virus progeny begins. Usually the entire life cycle of adenovirus is completed in 24 to 36 hours (Russell 2000, Berk 2007).

1.3.1.2. Transductional and transcriptional targeting of adenoviruses

Cancer gene therapy aims for tumor-restricted delivery of the vector and several approaches for transductional targeting of adenoviruses have been employed (Russell 2000). As many cancer cell types express low or even undetectable levels of the primary adenovirus receptor CAR, transductional targeting is necessary for improving the poor infectivity of adenovirus

(Cripe et al. 2001). Although CAR is ubiquitously expressed in epithelial cells, its expression is downregulated in many types of cancers due to the activation of the Raf-MAPK pathway (Anders et al. 2003). Thus, modifying the fiber knob domain to target other receptors could be beneficial (Glasgow et al. 2006) and it is the primarily exploited capsid locale for genetic engineering (**Figure 3.**). Several ligands have been studied as targeting tools by integrating these into the fiber. For example, a polylysine tail constituted of 7 lysine residues has been successfully shown to target the virus to cell surface heparan sulfate proteoglycans (HSPGs) (Wu et al. 2002, Kangasniemi et al. 2006, Ranki et al. 2007). Another approach involves modification of the fiber knob by incorporating Arg-Gly-Asp (RGD) containing peptide in the HI loop of the fiber knob, redirecting the virus to bind to cells which express αvβ-class integrins (Dmitriev et al. 1998, Kangasniemi et al. 2006). These integrins, responsible for binding an internalization of attached compounds, are highly expressed for example in pancreatic cancers (Grzesiak et al. 2007) and gastreatic cancers (Theocharis et al. 2003). Furthermore, double modification of adenovirus fiber with polylysine pK7 and RGD motifs has been shown to improve transduction in both CAR-positive and CAR-negative cells (Wu et al. 2002). Also other peptide candidates have been reported to be discovered by phage display library, featuring high affinity for vascular endothelial cells, cancer cells, transferrin receptor and vascular smooth muscle cells (Mizuguchi et al. 2004).

An alternative approach is pseudotyping the viruses by substituting the knob of most commonly used serotype Ad5 with its structural counterpart from another adenovirus serotype that bind a cellular receptor other than CAR (Mizuguchi et al. 2004). Some serotypes have inherently different cellular tropisms, for example the receptor for Ad3 is desmoglein 2 (DSG-2) (Wang et al. 2011), a receptor suggested to be highly expressed in many types of cancers (Tuve et al. 2006). By replacing the entire adenovirus serotype 5 knob with the knob from serotype 3 (Ad5/3) has shown increased gene transfer efficacy in the context of many tumor types (Kanerva et al. 2002, Kangasniemi et al. 2006, Guse et al. 2007, Bramante et al. 2014). Preliminary human data suggests that 5/3 chimerism may be a safe and effective approach also in cancer patients (Koski et al. 2010, Raki et al. 2011). Another problem related to serotypes is the induction of neutralizing antibodies (NAbs), which may prevent successful intravenous re-administration of the same agent. Most adults have developed adenovirus-specific cellular memory but seroprevalence rates of detected neutralizing antibodies are variable (Nayak et al. 2010). The neutralizing antibody response

can be partially overcome by modifying the adenoviral fiber knob, preferably to serotypes with lower natural prevalence (Petry et al. 2008).

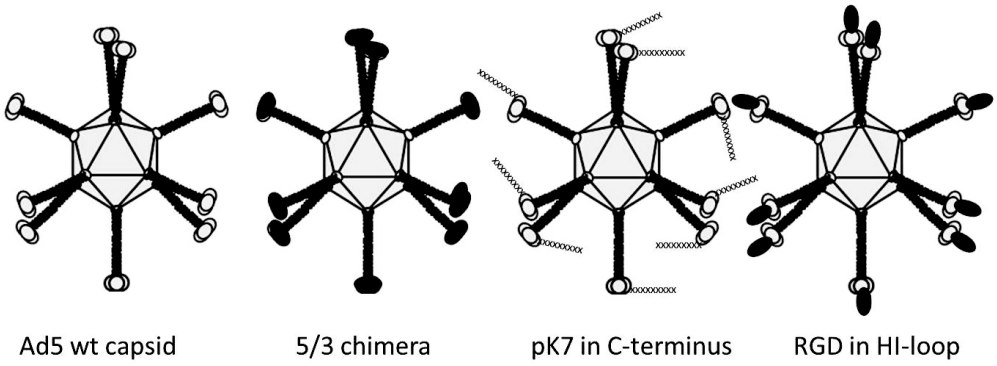

Figure 3. Ad5 wt capsid and capsid modified viruses to redirect adenoviral tropism: serotype 5/3 chimeric fiber with serotype 3 knob, pK7 modification in the C-terminus of the knob and RGD-modification in the HI-loop of the knob.

To further improve the safety and specificity of adenoviruses, transcriptional targeting has been extensively studied. This can be achieved by using cancer-specific promoters or by deleting adenoviral genes necessary for replication in normal cell but needless for replication in cancer cells (Doloff et al. 2008, Hsu et al. 2008). During the wild type adenovirus replication, E1A interacts with retinoblastoma protein (pRb) which is no longer able to repress E2F transcription factor. Release of E2F leads to loss of cell cycle control and pushes quiescent cells from G1 phase to S phase (Whyte et al. 1988). Therefore, adenoviruses featuring a 24 base pair deletion in constant region 2 of E1A, in which the pRb binding domain resides, have been generated (Δ24-mutated adenoviruses) (Fueyo et al. 2000). Most human tumors are deficient in the retinoblastoma/p16 pathway (Sherr et al. 2002), and thus in cancer cells Δ24 is complemented by inactivation of pRb by p16/Rb pathway defects, enabling virus replication (Heise et al. 2000). In normal cells, the interaction between E1A and pRb is lost and thus the virus replication is blocked.

1.3.1.3. Immune response to adenoviruses

One of the obstacles for adenoviral gene therapy is host defense mechanisms which can lead to rapid clearance of the virus (Raper et al. 2003, Lenaerts et al. 2008). Innate immune responses mediated by pathogen-associated molecular patterns (PAMPs) such as toll-like receptors 2 and 9 (TLR-2 and TLR-9) evoke as a first line of defense immediately after

infection leading to release of cytokines and chemokines, activation of complement system and uptake of the virus by antigen-presenting cells such as macrophages an dendritic cells (Muruve et al. 1999, Guidotti et al. 2001). Adenovirus can suppress the MHC-I expression but for example natural killer (NK) cells can spontaneously kill MHC-I deficient tumor cells (Whiteside et al. 1995). The alarm signal provided by the innate immunity eventually activate adaptive immunity, which can target adenoviruses by secreting antibodies against adenovirus and priming T-cells to recognize cells infected by adenoviruses (Willcox et al. 1976, Russell 2000, Schagen et al. 2004).

In regard to the immune response against adenovirus, some concerns remain in the context of large virus doses and toxicity (Raper et al. 2003). Therefore systemic injection of large doses may not be an optimal approach. To overcome this, protracted release might be useful if anti-tumor efficacy can be retained.

1.3.1.4. Polymers and vehicles in adenoviral gene therapy

To evade immune-recognition, chemical engineering of the virus by coating it with biomaterials has been proposed as a way to hide the virus epitopes. Coating the virus into an implantable, biodegradable delivery matrix could lead to improved delivery to the tumor site, higher local concentrations of the virus, prolonged target exposure and reduced toxicity. The first polymer described for adenovirus coating was polyethel glycol (PEG), which was shown to reduce the clearance rate of the virus from blood but eventually reduced the infectivity of the virus (Alemany et al. 2000). As another representative polymers for chemical engineering, for example Poly-N-(2-hydroxypropyl) methacrylamide (pHPMA) (Fisher et al. 2001), Poly(ethylenimine) (PEI) (Baker et al. 1997) and Poly(L-lysine) (PLL) (Fasbender et al. 1997) have been used as a carriers for Ad vectors.

As another option, silica-based sol-polymers have been shown to successfully deliver oncolytic adenovirus *in vivo* without compromising the biological activity of the virus (Quintanar-Guerrero et al. 2009). Treatment of mice with silica gel-based delivery of adenovirus doubled their survival rate and slowed the development of anti-adenovirus antibodies (Kangasniemi et al. 2009). Silica-sol-gel implants have many desirable qualities as delivery devices. By changing the drug concentration, size of the implant or by adjusting the dissolution rate these implants can be applied in variable approaches (Viitala et al. 2007).

1.3.1.5. Clinical trials with oncolytic adenoviruses

The first clinical trials with naturally occurring oncolytic viruses were conducted as early as in the 1950s (Huebner et al. 1956, Southam et al. 1956) but there are no conclusive results from these early clinical trials. Eventually, it was not until 1996 when clinical trials with oncolytic adenoviruses were initiated again with ONYX-015, an oncolytic Ad2/Ad5 hybrid featuring deletions in its E1B 55K gene coding region. The E1B 55K protein is involved in p53 inhibition, viral mRNA transport and shutting off protein synthesis of the host cell, attenuating the replication in normal cells with intact p53 (Bischoff et al. 1996, Pearson et al. 2004). Due to the limited activity as a single agent, ONYX-015 has also been studied with chemotherapy and radiotherapy (Khuri et al. 2000). Eventually In 2005, a similar adenovirus with E1B 55K gene and E3B gene deletion H101 (Oncorine; Shanghai Sunway Biotech, Shanghai, China) was approved in China as the world's first oncolytic virus for head and neck cancer in 2005 (Garber 2006).

Despite encouraging results obtained *in vitro* and in animal models, these findings have not always been predictive of clinical trial results, probably due to the complex, multifactorial interactions between the tumor, its microenvironment, the virus and the host immunity (Wong et al. 2010). Currently a new generation of more effective adenoviral agents and combinations are entering clinical trials. In January 2015, official sources listed 14 open clinical trials that would evaluate the efficacy and safety of oncolytic adenoviruses in oncological indications (http://clinicaltrials.gov) (**Table 2.)**.

Table 2. Open adenoviral clinical gene therapy trials for cancer at January, 2015. Data adapted from www.ClinicalTrials.gov.

Virus	Type of cancer	Phase	Approach	Ref.
Ad-Delta24-RGD	Glioblastoma	I/II	As a single agent	NCT01582516
AdMA3	Solid tumours	I/II	Combined with Maraba virus MG1MA3	NCT02285816
CELYVIR	Solid tumors	I/II	CELYVIR consists in bone marrow-derived autologous mesenchymal stem cells infected with ICOVIR-5	NCT01844661
CG0070	Bladder cancer	II	As a single agent	NCT02143804
CG0070	Bladder cancer	II/III	As a single agent	NCT01438112
Colo-Ad1	Colon cancer, non-small cell lung cancer, bladder cancer, renal cell carcinoma	I	As a single agent	NCT02053220
DNX2401	Glioblastoma	I	Combined with Temozolomide	NCT01956734
DNX-2401	Glioblastoma or gliosarcoma	I	Combined with Interferon Gamma (IFN-γ)	NCT02197169
ICOVIR-5	Melanoma	I	As a single agent	NCT01864759
VCN-01	Solid tumors	I	Combined with Gemcitabine	NCT02045602

1.3.2. Vaccinia viruses

In 1798, Edward Jenner noticed that milkmaids exposed to cowpox developed protection against smallpox (Lakhani 1992). Smallpox was caused by variola, a member of the poxvirus family. This finding eventually lead to the development of a laboratory strain of poxvirus, vaccinia virus, used as a vaccine in the Smallpox Eradication Program led by the World Health Organization (Geddes 2006, Theves et al. 2014). Vaccinia is a member of the Orthopoxvirus genus and is its most extensively studied member. It was the first mammalian virus to be visualized microscopically, successfully grown in tissue culture, titrated accurately, purified physically and analyzed biochemically (Moss 2001). Due to this historical role, vaccinia virus has the longest and most extensive history of use in humans of any virus and has had a major impact on development of vaccines. Wild type vaccinia virus has been used in hundreds of millions of humans as a vaccine for the eradication of smallpox

and has shown a good safety profile as only rare serious side effects have been reported during the vaccination program (Halsell et al. 2003). Although smallpox has been completely eradicated from the 1980s onwards, vaccinia virus has been studied as a viral vector for the development of cancer virotherapies, immunotherapies, as well as development of next-generation smallpox vaccines due to its strong safety profile and high immunogenicity (Verardi et al. 2012).

1.3.2.1. Structure and life cycle of vaccinia

Vaccinia is a genetically complex double-stranded DNA virus, characterized as brick-shaped particles with a size of approximately 300 x 240 x 120 nm (Moss 2001). Infectious vaccinia virus particles have a lipoprotein envelope surrounding a complex core of linear double stranded DNA (191 636 bp, encodes for ~250 genes) (Upton et al. 2003). The composition of viral lipids and host cell membranes are similar. Vaccinia encodes all the proteins it needs for its replication in its genome, some of which have immune evading properties allowing the virus to establish infection (Moss 1990, Smith 1993).

Vaccinia virus enters the cell via fusion of viral and cellular membranes, which is mediated by entry-fusion complex (**Figure 4.**) (Carter et al. 2005, Senkevich et al. 2005). No specific receptor to facilitate entry of the virus into the cell has yet been discovered. After the entry, viral particles are uncoated, and transcription of early genes by the viral RNA polymerase starts followed by the expression of intermediate and late genes (Moss 2012). Vaccinia encodes all the enzymes and proteins needed for its replications in its genome along with viral genomic DNA including transcription factors, capping and methylation enzymes and a poly (A) polymerase (Moss 1990). Synthetization of translatable mRNA independently from host cells leads to assembly of several antigenic forms of new virus particles, which happens in the cytoplasmic "factories". The most numerous particle type is the intracellular mature virus, IMV, which is released during the cell lysis and it lacks the outer membrane (Sodeik et al. 1993). A small percentage of the IMVs are enwrapped with an additional Golgi-derived membrane and actively transported to the cell surface via actin tails (Cudmore et al. 1995). As long as they are attached to the cell these particles are called cell-associated enveloped viruses (CEV) and after release they become extracellular enveloped viruses (EEV) (Schmelz et al. 1994). These particles can exit the cell via direct budding through the plasma membrane without lysing the cell (Condit et al. 2006). As IMV particles usually infect neighboring cells,

enveloped viruses protected by a host-derived envelope can avoid recognition by the host immune system as only a few viral proteins are exposed. This can facilitate the systemic spread and re-infection of distant cancer cells (Payne 1980, Smith et al. 2002).

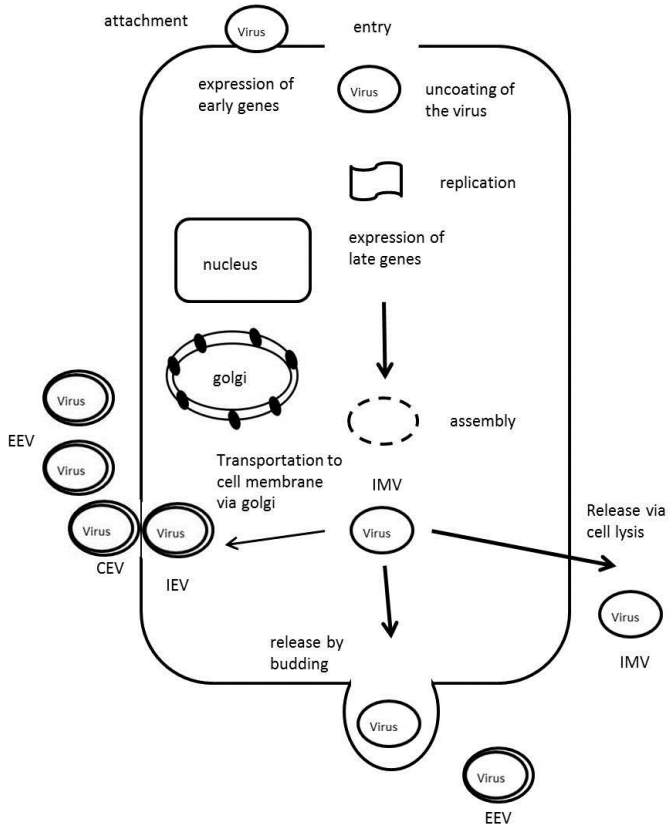

Figure 4. Vaccinia virus enters the cell via fusion of viral and cellular membranes. After the entry, transcription of early genes by the viral RNA polymerase starts. Viral particles are uncoated and the replication of viral DNA starts. The most numerous particle type is IMV, which is released during the cell lysis and it lacks the outer membrane. Some particles are packaged and released with an additional Golgi-derived membrane and are called IEV, CEV or EEV. EEV particles can also form via direct budding through the plasma membrane. IMV; intracellular mature virus, IEV; intracellular enveloped virus, CEV; cell-associated enveloped virus, EEV, extracellular enveloped virus.

Vaccinia infection results in profound changes in host cell function, morphology and metabolism, called cytopathic effect (CPE). *In vitro* these changes are visible and include cell rounding and detachment from neighboring cells (Bablanian et al. 1978). The virus induces cytopathic effects rapidly after infection, as early viral enzymes completely shut down host

cell functions. Already after 4-6 hours after viral entry, host protein synthesis is almost completely inhibited and actin cytoskeleton, microtubules and membrane permeability have been altered. The entire life cycle takes place in cytosol and is completed within 24 h releasing as many as 10,000 new virions (Salzman 1960).

1.3.2.2. Modified vaccinia viruses for cancer gene therapy

Vaccinia virus is appealing for biomedical research and gene therapy due to several characteristics. Genetic activity of the vaccinia virus occurs within the cytoplasm, providing physical separation from the nucleus. As vaccinia virus never enters the host cell nucleus, recombination between host and viral genomes is highly unlikely. The genome is fully sequenced and allows large inserts of foreign DNA up to 25 kb length to construct modified viruses carrying therapeutic transgenes (Smith et al. 1983). Using viral vectors to express therapeutic proteins in the target tissue leads to a high local concentration of the protein while systemic availability is limited to reduce side effects and toxicity (Gnant et al. 1999). Vv has a wide host range and is able to infect and replicate in almost all human and many other species´ cell types, allowing the use of syngeneic immune competent animal models in preclinical studies (McFadden 2005). Vaccinia virus is easy to produce in relatively high titers and the particles maintain their stability and infectivity even in prolonged storing as frozen solutions or dry power (Shen et al. 2005). Finally, antiviral agents are available to control possible toxicity and uncontrolled replication caused by virus administration. Such agents are for example vaccinia immune globulin (Wittek 2006), cidofovir (Andrei et al. 2010) and ST-246 (Yang et al. 2005).

Recombinant vvs are especially attractive as oncolytic cancer gene therapy vectors. Strong oncolytic effect paired with its high natural tropism for cancer tissue, efficient cell-to-cell spread, fast replication cycle and high infectivity has led to the design of novel cancer therapeutics based on vaccinia backbones (Zeh et al. 2002). Oncolysis seems to have features of both apoptosis and necrosis (Kirn et al. 2009), and additionally, vaccinia virus has been shown to cause vascular collapse in tumors (Breitbach et al. 2007).

Different strains of vaccinia virus have been used to create recombinant vaccinia viruses. Highly attenuated strains, such as Modified Vaccinia Ankara (MVA) and New York Vaccinia virus (NYVAC) exist, but they do not replicate in mammalian cells and therefore have a very little utility in gene therapy. Most commonly used oncolytic viruses are based on the Wyeth,

Lister, Western Reserve and Copenhagen strains. The Western Reserve strain seems to have the strongest oncolytic effect *in vitro* and *in vivo* (Naik et al. 2006).

The development of virotherapeutics for cancer therapy has led to the use of safety- and selectivity-enhanced viruses (Chiocca 2002). Vaccinia is shown to have a natural tropism for tumor since uncontrollably proliferating cancer cells have high concentrations of nucleotides needed for virus replication and the leaky vasculature of the tumor facilitates the access of relatively large virus to the tumor site (Thorne et al. 2007). In order to increase replication spesifically in cancerous tissue, different strategies based on genetic engineering of the vaccinia virus genome have been employed. Targeting can be achieved by engineering viral proteins which are needed for vaccinia virus to replicate in normal but not in cancer cells. Viral thymidine kinase (TK) is necessary for replication of the virus in normal cells since these cells have naturally low nucleotide concentrations and cellular thymidine kinase is only transiently expressed during the S phase of the cell cycle (Buller et al. 1985). TK is involved in the synthesis of deoxyribonucleotides in dividing cells and is expressed in large quantities in rapidly proliferating cancer cells (McKenna et al. 1988). Deletion of TK restricts virus replication to cells that overexpress E2F, the transcription factor that regulates cellular TK expression and have activated epithelial growth factor receptor pathways (Buller et al. 1985, Shen et al. 2005) and so far tumor selective replication of TK deleted vaccinia viruses have been shown *in vivo* including colon cancer, sarcoma, melanoma and liver metastasis models (Gnant et al. 1999, Puhlmann et al. 2000). Enhanced tumor selectivity has also been reported with anti-interferon (IFN) gene-deleted vaccinia virus. To counteract the cellular IFN anti-viral response, vaccinia virus produces many types of IFN –inhibiting proteins, such as B18R, whereas cancer cells frequently have inactivated IFN-pathway (Kirn et al. 2007).

Additionally, vaccinia growth factor (VGF) can be deleted to improve the safety and selectivity of the virus. VGF is a virulence factor of vaccinia virus and it is secreted early during the vaccinia virus infection. VGF is an epidermal growth factor (EGF) homologue and can drive the proliferation of neighboring cells by binding to the EGF receptor and stimulating the Raf/MEK/Erk pathway (Tzahar et al. 1998, Hanahan et al. 2000, de Magalhaes et al. 2001). As the EGFR–Ras pathway is activated in most human cancers (Hanahan et al. 2000), deletion of VGF restricts the replication and spread of the virus in normal cells. Together, deletion of TK and VGF genes (**Figure 5.**) have been shown to reduce the pathogenicity and to increase the selectivity of the virus compared to either of the single deletions alone. Good safety and preliminary evidence of efficacy has been seen with

double deleted oncolytic vaccinia virus in preclinical models (McCart et al. 2001, Haddad et al. 2012).

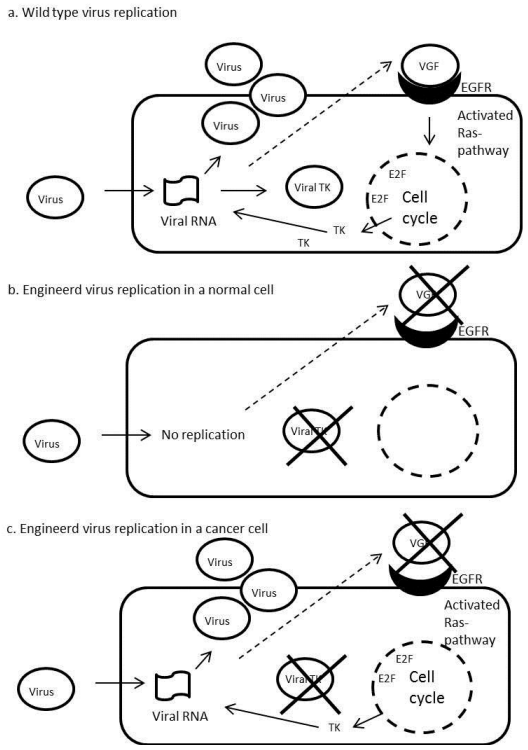

Figure 5. Tumor-restricted replication of double-deleted oncolytc vaccinia virus. a) Wild type virus replication in a normal cell. b) Engineered virus replication in a normal cell. c) Engineered virus replication in a cancer cell. *Modified from:* (Kirn et al. 2009).

Other approaches shown to reduce virulence in combination with TK deletions are for example deletions in the serpins SP-1 and SP-2 (Yang et al. 2007). Viral serpins block host response to vaccinia and inhibit apoptosis. By mutating these genes viral replication should only proceed normally in tumor cells harboring mutations in the apoptotic pathways, whereas in normal tissues infected cells would undergo apoptosis. A similar approach has been used by generating vaccinia and Fas ligand (FasL) protein (Taylor et al. 2006).

1.3.2.3 Immunological responses to vaccinia virus

1.3.2.3.1 Innate immunity responses

Innate immunity is the host´s first line of defense. As with other viruses, immediately after the entry of vaccinia virus rapid secretion of inflammatory cytokines such as type I interferons IFN-α and IFN-β is triggered by leukocytes and fibroblasts, which can induce an anti-viral state and upregulate adaptive immune functions (Samuel 1991, Perdiguero et al. 2009). The complement system is another crucial innate response that may destroy enveloped viruses or infected cells directly by lysis or indirectly by opsonizing pathogens for phagocytosis by macrophages and neutrophils. Natural killer cells are attracted to the site of infection as part of the inflammatory response and kill virus-infected cells, especially cells with reduced levels of MHC class I on their surface (See et al. 1997). Many different cells of the innate immune system have been shown to mediate the innate immunity against vaccinia virus. Macrophages have an important function as antigen presenting cells (APC) for priming and activation of specific immune response mediated by T-cells and it has been demonstrated that mice depleted with macrophages are unable to control vaccinia virus infections due to impaired virus clearance and antigen presentation (Karupiah et al. 1996). NK cells have a direct cytotoxic activity against vaccinia infected cells as depletion of NK cells *in vivo* was shown to enhance the virulence of vaccinia virus (Bukowski et al. 1983, Brutkiewicz et al. 1992).

1.3.2.3.2 Adaptive immunity responses

In addition to innate responses, also cellular responses are developed against vaccinia virus. Adaptive immunity is orchestrated by antigen-presenting cells such as dendritic cells (DCs) that present antigens to T cells. IFN-γ, a type II interferon secreted from macrophages, NK cells and T-cells, is important for the activation of immune and inflammatory responses and for cell mediated immunity (Boehm et al. 1997). Normally, peptides derived from endogenously expressed proteins, such as viral proteins produced as the virus replicates, activate antigen-presenting cells, which migrate to the lymph nodes and present virus antigens to T lymphocytes. Viral peptides are presented by dendritic cells via MHC class I (MHC I) molecules to cytotoxic CD8+ T lymphocytes (CTLs). After activation and proliferation, CTLs can directly kill virus infected cells. Vaccinia virus is a highly immunogenic virus, eliciting strong T-cell responses. Smallpox vaccines have been shown to generate a robust primary effector CD8 (+) T-cell response which was highly specific with

minimal bystander effects. Virus-specific CD8 (+) T-cells passed through an obligate effector phase and gradually differentiated into long-lived memory cells. These memory cells have been shown to be functional and undergo a memory differentiation program distinct from that described for human CD8(+) T-cells specific for persistent viruses (Miller et al. 2008). Alternatively, viruses can also be internalized and exogenously derived viral proteins can be loaded onto MHC class II (MHC II) for presentation to helper CD4+ T cells (Guidotti et al. 2001). The CD4+ T-helper cells activate B lymphocytes, leading to robust production of vaccinia-specific antibodies that can neutralize vaccinia virions. Unlike many other viruses, vaccinia virus is not endemic and smallpox immunizations were terminated in the 1970s. Therefore, only older patients will have pre-existing, circulating antibodies against vaccinia virus. Although neutralizing antibodies play a role in inhibiting the infection, T-cell responses to vaccinia virus seem to be more important as progressive vaccinia infection has been shown to correlate with T-cell deficiency (Putz et al. 2006). Successful re-infection in previously immunized patients has also been demonstrated in vaccine trials (Mastrangelo et al. 1999) and in a recent phase I clinical trial performed with oncolytic vaccinia virus where pre-existing antibody titers did not correlate with toxicity, systemic spread of the virus or antitumor activity (Zeh et al. 2015).

1.3.2.3.3 Immune evasion

Vaccinia virus has evolved many mechanisms for evading the immune system (Haga et al. 2005) by encoding proteins that counteract the activity of interferons IFN-α, IFN-β, IFN-γ and soluble cytokines such as IL1β and TNF-α. These viral proteins have sequence similarity to the extracellular binding domains of host cytokine receptors and can bind cytokines with high affinity and to neutralize their activity (Smith et al. 2000). To counteract the complement, vaccinia virus expresses and secretes virus complement protein (VCP) which binds to complement components C3b and C4b and functions as a co-factor blocking activation of the complement cascade by either the classical or alternative pathway (Kotwal et al. 1988). In addition, extracellular envelope of the virus is known to be almost completely resistant to neutralization (Smith et al. 2002). Additionally, vaccinia encodes several anti-apoptotic proteins, such as serpins and an inhibitor of apoptosis-related cytochrome c release (Kettle et al. 1997, Taylor et al. 2006).

1.3.2.4. Clinical trials with vaccinia viruses

Over the past decade, hundreds of cancer patients have been treated with vaccinia virus in clinical trials, evaluating several different genetically engineered vaccinia viruses. The first trials were performed by repeatedly injecting wild type vaccinia virus directly in the melanoma lesions. Altogether, 44 patients were treated in these early trials and the overall objective tumor response rate was estimated to be approximately 50% with complete regression in 25% of the cases. These studies demonstrated that repeated injections are feasible and can lead to further responses (Burdick et al. 1964, Hunter-Craig et al. 1970, Thorne et al. 2005).

Currently the leading vaccinia-based clinical candidate is Pexa-Vec (JX-594), an oncolytic vaccinia engineered by insertion of human GM-CSF on the disrupted TK gene region. This vector has three main mechanisms of action; selective infection of cancer cells, induction of an antitumor immune response and disruption of tumor-associated vasculature (Parato et al. 2012, Breitbach et al. 2013). So far over 250 patients with advanced cancers have received Pexa-Vec treatments.

In two phase I trials, intratumoral Pexa-Vec was well tolerated, with only mild systemic toxicity reported. In the first phase I trial, seven patients with melanoma were treated and one partial response and one complete response after surgery were observed. In addition, inflammation of cutaneous lesions was observed and eosinophilic and lymphocytic infiltrations were detected in tumors (Mastrangelo et al. 1999). In another phase I trial in patients with hepatic carcinoma, three out of ten evaluable patients had a partial response and six had stable disease. Responses were seen in both injected and non-injected lesions and the maximum tolerated dose (MTD) was also established at $1x10^9$ plaque forming units (pfu) (Park et al. 2008, Merrick et al. 2009).

In order to explore the relationship between the dose and the desired activity, a mechanistic proof-of-concept trial with Pexa-Vec was conducted. Ten patients with advanced metastatic melanoma were treated with a low dose of Pexa-Vec, equivalent to 10% of the maximum tolerated dose in the previous trial. Delayed re-emergence of circulating Pexa-Vec was detected in 5 patients, which is suggestive of replication and progeny shedding into the blood. Antibodies against vaccinia were induced in all patients. Pexa-Vec replication, perivascular lymphocytic infiltration and diffuse tumor necrosis were observed in tumor biopsies (Hwang et al. 2011).

A Phase II dose-finding trial of Pexa-Vec as a single agent was performed in patients with advanced hepatocellular carcinoma (HCC). In this study, significant difference in median overall survival rate between the group of patients receiving a high dose of Pexa-Vec versus those receiving a low dose (14.1 months for the high-dose group versus 6.7 months for the low-dose group) (Heo et al. 2013) was observed.

To further improve the efficacy, Pexa-Vec was studied in combination with sorafenib in a pilot study with three patients. Sorafenib (Nexavar; Bayer), a small molecule inhibitor of B-raf and vascular endothelial growth factor receptor, is currently considered the global standard of care and is the only product approved for the first-line treatment of advanced hepatocellular carcinoma (HCC) (Llovet et al. 2008). After the sequential treatment, all three patients exhibited rapid necrosis and responses on sorafenib. These results might indicate that Pexa-Vec can sensitize HCC tumors to sorafenib and potentially also other vascular endothelial growth factor receptor (VEGFR) inhibitors (Heo et al. 2011). In September 2013, Transgene announced that a Phase IIb trial evaluating Pexa-Vec in patients with second-line HCC did not meet its primary endpoint of overall survival. However, a phase III trial is slated to begin next year in partnership with the biotech company Transgene, Sillajen and Lee's Pharmaceutical (Scudellari 2014).

Recently, the first results of a phase 1 study of double-deleted (TK-/VGF-), Western Reserve strain oncolytic vaccinia virus were published. In addition, the virus has been modified to encode cytosine deaminase (CD) gene for controlling the viral infection and somatostatin receptor (SR) gene allowing imaging of the virus (vvDD-CDSR). Dose escalation proceeded without dose-limiting toxicities to a maximum feasible dose of 3×10^9 pfu, and viral genomes and/or infectious particles were recovered from injected (n = 5 patients) and noninjected (n = 2 patients) tumors. (Zeh et al. 2015). In January 2015, official sources listed 3 open clinical trials that would evaluate the efficacy and safety of oncolytic vaccinia viruses. All three Phase I/II trials were designed for GL-ONC1, an attenuated vaccinia virus as a single agent in peritoneal carcinomatosis, solid tumors and head & neck cancer (http://clinicaltrials.gov).

1.3.2.5. Safety concerns

Vaccinia virus has been used clinically as a vaccine for smallpox for over 150 years, and thus is associated with a good safety profile and extensive clinical experience (Mastrangelo et al. 2000). In the United States, during the vaccination program only 0.003 % of the vaccinated

population was reported to suffer from vaccinia necrosum, encephalitis, myopericarditis, eczema vaccinatum, or death (Poland et al. 2005). However, one possible safety concern is the contagious transmission of vaccinia virus, which has been reported to occur between recently vaccinated subjects and individuals naïve to vaccinia (Wertheimer et al. 2012). Also the unlikely but theoretical possibility of bioterrorism utilizing smallpox has raised some safety concerns (Artenstein et al. 2008). In clinical cancer trials, wild-type and engineered vvs have generally shown only mild toxicity, mostly consisting of transient fever, malaise, skin reactions and pain at the injection site. However, oncolytic vvs have not yet been tested in large populations to reliably determine the occurrence of adverse events. In case of uncontrolled replication, vaccinia immunoglobulin and cidofovir are recommended as first and second line therapy (Cono et al. 2003).

1.4 Cancer immunotherapy

Numerous innate and adaptive immune effector cells and molecules participate in the recognition and destruction of cancer cells, a process that is known as cancer immunosurveillance (Burnet 1970). In brief, the immune system can react against cancer cells in two ways: by responding to molecules that are unique to cancer cells (tumor-specific antigens) or by recognizing molecules that are expressed differently by cancer cells and normal cells (tumor-associated antigens) (Graziano et al. 2005). In healthy individuals, the immune system can recognize and kill cell featuring antigenic variations presented by malignant cells, but many cancers have multiple mechanisms to escape from the immune system (Cheever et al. 2009). Such mechanisms include for example reduced immunogenicity, resistance to immune cell killing and selection of non-immunogenic tumor-cell variants. This process is also known as immunoediting, characterized by changes in the immunogenicity of tumors due to the anti-tumor response of the immune system, resulting in the emergence of immune-resistant variants. It is made up of three phases: elimination, equilibrium, and escape (Dunn et al. 2002, Zitvogel et al. 2006)..

Immunotherapy aims to fight diseases such as cancer by inducing, enhancing or suppressing immune response. In most human solid tumors, there is wide variation in which degree they are infiltrated by immune effector cells. Solid tumors are infiltrated by cells of the immune system and some correlation between increased number of cytotoxic CD8+ cells and prolonged survival has been seen for example in epithelial ovarian carcinoma, endometrial cancer and breast cancer (Menard et al. 1997, Tomsova et al. 2008, Yamagami et al. 2011). However, one of the major hurdles in cancer immunotherapy is the limited trafficking of

tumor-specific T-cells into the tumor and the low activity of these cells due to the immunosuppressive nature of the tumor microenvironment. In tumor-draining lymph nodes both cross-priming and cross-toleration have been reported and tumor antigen–specific T-cell proliferation has been detected, but the numbers of proliferating T cells are often too low. Therefore the overall effect of CD8+ T-cell activation does not always result in inhibition of tumor growth and the tumors remain unaffected, refractory and continue progressing (Mellman et al. 2011, Vesely et al. 2011).

1.4.1. Cancer immunotherapy with oncolytic viruses

Oncolytic viruses are naturally immunogenic and therefore a promising platform for immunotherapy. Classically, the immune system is thought to limit the efficacy of therapy, leading to viral clearance. However, preclinical and clinical data suggest that in some cases virotherapy may in fact act as cancer immunotherapy. (Diaz et al. 2007, Alemany et al. 2009). The replication of oncolytic virus in the tumor is an immunogenic phenomenon, releasing tumor-specific antigens that can be taken up by infiltrating antigen-presenting cells for cross-presentation to cytotoxic T-cells (Toda et al. 1998, Diaz et al. 2007). The power of combining viral oncolysis and tumor-specific immunity has been demonstrated for example in a study by Chuang et al., where tumor-bearing mice were first primed with highly foreign antigen ovalbumin (OVA). Priming was followed by intratumoral injection of vaccinia virus encoding the same antigen resulting in increased infiltration of OVA-specific CTLs and significantly enhanced therapeutic effects (Chuang et al. 2009).

Activation of the immune system can be further improved by inserting genes encoding immunomodulatory proteins such as cytokines, interferons or chemokines in the viral genome (**Figure 6**.). Outbalancing the tumor immunosuppression mechanisms and breaking the immune tolerance of tumors could lead to a significant anti-tumor effect (Melcher et al. 2011).

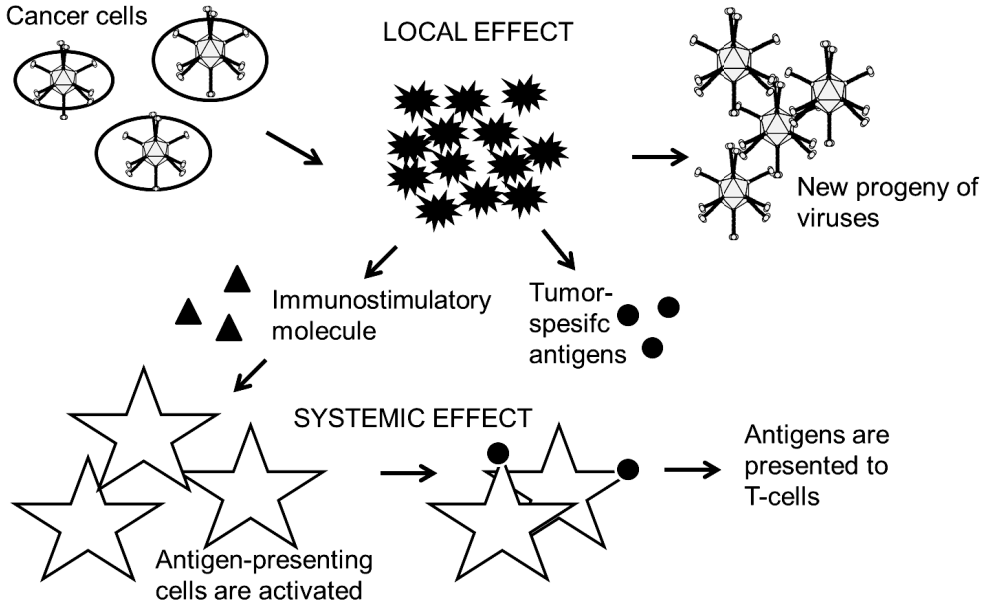

Figure 6. Dual-mechanism of oncolytic virotherapy consists of the local lytic effect and systemic effect when the immune system is activated against the tumor.

Cytokines are signaling molecules secreted by numerous cells of the immune system. They are key players in immune reactions and modulate many cell processes including cell growth, proliferation, migration and activation. However, systemic administration of cytokines might lead to severe adverse reactions and even systemic toxicity (Li et al. 2005). In addition, local concentrations often remain inefficiently low. Virus vectors are a suitable platform for cytokines, since by expressing cytokines locally their anticancer activities can be safely taken advantage of without evoking systemic toxicity. Cytokines that can activate dendritic cells and natural killer cells and mediate induction of tumor-specific CD8+ cytotoxic T-lymphocytes are especially interesting for anticancer therapies for promoting anti-tumoral effects. In addition to cytokines, for example chemokines, T-cell engagers and co-factor molecules can be paired with oncolytic viruses (Chen et al. 2013). Multiple oncolytic virus vectors have been armed with various immunomodulatory and some examples of such viruses have been collected to Table 1.

Table 1. Immunostimulatory transgenes encoded by oncolytic viruses.

Transgene	Vectors	Action
CD40 ligand	• Adenovirus (Diaconu et al. 2012) • HSV-1 (Terada et al. 2006) • VSV (Galivo et al. 2010)	Co-stimulates T-cells
CD80 T lymphocyte activation antigen/ B7-1	• Adenovirus (Lee et al. 2006) • HSV-1 (Todo et al. 2001)	Co-stimulates T-cells
Chemokine ligand 3 (CCL3)	• Adenovirus (Edukulla et al. 2009)	Attracts leukocytes
Chemokine ligand 5 (CCL5)/ RANTES	• Adenovirus (Lapteva et al. 2009)	Recruits T-cells
EphA2 T-cell engager	• Vaccinia virus (Yu et al. 2014)	Binds to CD3 and tumor cell surface antigen EphA2
FMS related tyrosine kinase 3 ligand (FLT3L)	• Adenovirus (Bernt et al. 2005) • VSV (Leveille et al. 2011)	Activates DCs and NK cells
Granulocyte-macrophage colony-stimulating factor (GMCSF)	• HSV-1 (Liu et al. 2013) • Vaccinia (Breitbach et al. 2011) • Adenovirus (Koski et al. 2010) • Measles virus (Grote et al. 2003) • VSV (Bergman et al. 2007)	Stimulates granulocytes and monocytes, promotes maturation of dendritic cells
Interleukin 2 (IL-2)	• HSV-1 (Carew et al. 2001) • Vaccinia (Perera et al. 2001)	Activates T-cells
Interleukin 4 (IL-4)	• Adenovirus (Post et al. 2007) • HSV-1 (Terada et al. 2006)	Activates T-cells and B-cells
Interleukin 12 (IL-12)	• Adenovirus (Lee et al. 2006) • HSV-1 (Varghese et al. 2006) • VSV (Shin et al. 2007)	Activates T-cells and NK cells
Interferon A1 or B1	• Adenovirus (Shashkova et al. 2008) • Vaccinia virus (Kirn et al. 2007) • VSV (Willmon et al. 2009) • Measles virus (Li et al. 2010)	Activates APCs and T-cells
4-1BB ligand	• Adenovirus (Huang et al. 2010) • Vaccinia virus (Kim et al. 2009)	Co-stimulates T-cells

1.4.1.1. GMCSF

So far the most studied and successful approach has been arming the viruses with granulocyte-macrophage colony-stimulating factor (GMCSF), a cytokine and leukocyte growth factor which has presented promising potential as an inducer of antitumor immunity (Dranoff 2003, Prestwich et al. 2009). It recruits monocytes, promotes the differentiation of progenitor cells into dendritic cells and macrophages and activates several types of immune cells such as NK cells. GMCSF also enhances host responses against the tumor through improved tumor antigen presentation by recruited dendritic cells and macrophages. Notably, systemic use of recombinant GMCSF for immune stimulation might cause side effects related to systemic exposure, while efficacy may remain limited due to low local concentration in tumors (Arellano et al. 2008). By producing GMCSF locally from the cancer cells, a high local concentration could be achieved while minimizing systemic exposure. Therefore, GMCSF is appealing for tumor immunotherapy and can be particularly useful in the context of oncolytic viruses. As shown in Table 1, GMCSF has been successfully paired with many oncolytic viruses, many of them being currently tested in clinical trials. In addition to previously reviewed clinical success of Pexa-Vec, GMCSF -encoding herpes simplex virus (talimogene laherparepvec, T-VEC;Amgen) has been reported to demonstrate regression of both injected and non-injected lesions in a Phase III trial (Kaufman et al. 2010). However, similarly to many other cytokines, GMCSF expression can also induce the proliferation of some suppressive cells such as myeloid derived suppressor cells (MDSC) (Kohanbash et al. 2013), and therefore some caution in the use of this cytokine might be needed. Notably, high systemic concentrations have been suggested to correlate with induction of potentially harmful myeloid-derived suppressor cells, while no such effect has been described to in situ GMCSF production with low systemic concentration (Serafini et al. 2004).

1.4.1.2. CD40L

Immunomodulatory molecule CD40L (also known as CD154) is a type II transmembrane protein expressed mainly on CD4+ T-cells. CD40L binds and interacts with the CD40 receptor expressed predominately on B-cells and antigen-presenting cells, such as macrophages and dendritic cells (Roy et al. 1993, Grewal et al. 1998). *CD40-CD40L* interactions are critical for development of humoral responses and adaptive cell-mediated immune responses (van Kooten et al. 2000). CD40 stimulation of antigen-presenting cells such as dendritic cells leads to their maturation and increased capacity to present antigens to

T-cells. Interactions trigger T-lymphocyte expansion and increase interleukin IL-12 production leading to a T helper 1 -type response (Grewal et al. 1996, Mackey et al. 1998). Recombinant soluble protein CD40L (rsCD40L) has also beem shown to have direct suppressive effects on CD40+ tumor cell proliferation *in vitro* (Tong et al. 2001) and *in vivo* (Hirano et al. 1999) and to induce apoptosis in CD40+ cancer cells (Davies et al. 2004). Clinical trials conducted with rsCD40L have generally been safe but systemic adverse events limited the dose that could be achieved locally, resulting in suboptimal efficacy. Although many examples of patients benefiting from treatment have been seen, the overall level of activity has been low (Vonderheide et al. 2001) Adenoviral vectors encoding CD40L have demonstrated promising results in preclinical studies (Loskog et al. 2005, Diaconu et al. 2012, Westberg et al. 2013) and in cancer patients (Malmstrom et al. 2010, Pesonen et al. 2012). However, when CD40L was combined with vesicular stomatitis virus, interference was seen as immunogenicity of the virus distracted immune responses away from priming of tumor-specific T cells, even in the presence of potent co-stimulatory signals. (Galivo et al. 2010).

1.4.2. Other approaches in cancer immunotherapy

Immunotherapy is a rapidly expanding field of research and in addition to virotherapy, many other approaches have been implemented, such as monoclonal antibody therapies, cancer vaccines and cell-mediated therapies (Mellman et al. 2011).

Monoclonal antibodies are used to treat many diseases, including some types of cancer. Over the past couple of decades, the US Food and Drug Administration (FDA) has approved more than a dozen monoclonal antibodies to treat solid and hematological malignancies (Cheever et al. 2009). A very important part of the immune system is its ability to maintain self-tolerance and control for the duration of immune responses in order to avoid tissue damage, and to do this, it uses immune checkpoints molecules that need to be activated or inactivated to start an immune response. Cancer cells can find ways to regulate these molecules as an immune resistance mechanism (Pardoll 2012). Because many of the immune checkpoints are modulated by ligand-receptor interactions, antibodies targeting these immune checkpoint molecules have been successfully developed against programmed cell death protein 1 (PD-1) and its ligand PDL1 (Tykodi 2014), and Cytotoxic T Lymphocyte Antigen 4 (CTLA-4) (Hodi et al. 2010). Ipilimumab (Yervoy; Bistrol-Myers Squibb), a CTLA-4 antibody was the first immunotherapeutic antibody achieving FDA approval for the treatment of metastatic melanoma. Ipilimumab blocks the interaction between CTLA-4 and B7 family accessory

molecules expressed on the surface of dendritic cells thus preventing the negative-feedback loop regulation of T-cells (Chambers et al. 2001, Mellman et al. 2011). In 2014, FDA approved two immune checkpoint molecules against PD-1, pembrolizumab (Keytruda; Merck & Co.) and nivolumab (Opdivo, Bristol-Myers). Targeting the PD-1 signalling pathway might have a more favorable safety profile as the interaction happens at the tumor site between the cancer cell and T-cells whereas ipilimumab releases the breaks of T-cell immunity mainly in the immunological synapse between T-cells and dendritic cells. Recent studies have also suggested that the combination of oncolytic virotherapy with immune checkpoint antibodies could reduce tumor burden by direct cell lysis and stimulate anti-tumor immunity (Engeland et al. 2014).

Identification of immunogenic tumor-antigens has led to rapid development of cancer vaccines as promising agents for cancer immunotherapy. The first success in cancer vaccines has been the market approval of sipuleucel-T (Provenge; Dendreon), an autologous dendritic cell vaccine designed to stimulate an immune response against metastatic, castration-resistant prostate cancer. Sipuleucel-T consists of patient's peripheral blood mononuclear cells, tumor-associated antigen (prostatic acid phosphatase) and GMCSF (Small et al. 2006, Kantoff et al. 2010).

Other promising approach to immunotherapy involves engineering patients' own immune cells, such as T-cells and NK cells to recognize and attack tumors. This approach is called adoptive cell therapy. Therapeutic cells are harvested and prepared in the laboratory prior to re-infusion into patients (Dudley et al. 2003). Immune cells can be selected for desired properties and enriched to high numbers in the laboratory prior to infusion. Additionally concomitant treatments are used to enhance the therapy, including preconditioning chemotherapy, radiation and systemic IL-2 infusion. This approach has been especially successful in melanoma. Rosenberg et al. extracted tumor-infiltrating lymphocytes (TILs) from patients with metastatic melanoma and re-administration of these expanded T-cell products back into the donor patients resulted in remarkable responses and even complete regressions (Rosenberg et al. 2011).

Another approach is to collect circulating lymphocytes from blood and via retroviral transduction engineer these naïve T-cells to express artificial T-cell receptors (TCRs), which can recognize their specific tumor antigens bound to MHC-molecules. For example, melanoma antigens MART-1 and gp100 have been successfully targeted with this approach

(Johnson et al. 2009) but the drawback of TCR approaches is that the use is HLA-restricted and TCR therapies may also cause autoimmune reactions due to off-target toxicity (Hinrichs et al. 2014). These problems can be partially circumvented by using novel artificial receptors called chimeric antigen receptors (CARs) which target native cell-surface antigens in an MHC-independent manner. This approach is not dependent on HLA and is more flexible with regard to targeting molecules. For example, single chain antibodies can be used and CARs can also incorporate co-stimulatory domains. The archetypal CAR molecule consist of an antigen-binding variable fragment from monoclonal antibodies fused to intracellular T-cell signaling domains from CD3ζ, CD28, 4-1BB, and/or other signaling molecules (Bridgeman et al. 2010). However, the targets of CAR cells need to be on the membrane of target cells, while TCR modifications can utilize intracellular targets. Up to date CARs have shown a lot of promise especially in the treatment of B cell leukemia´s, targeting the differentiation antigen CD19 on the surface of malignant cells. Recently, CARs have also been combined with oncolytic virotherapy (Nishio et al. 2014).

2. AIMS OF THE STUDY

1. To evaluate transduction and oncolytic efficacy of different capsid modified adenoviruses in pancreatic cancer models *in vitro* and *in vivo*, and to study the utility of silica implants as carrier vehicles for adenovirus *in vivo* (I).

2. To generate a double-deleted vaccinia virus carrying tdTomato fluorophore for imaging and immunostimulatory CD40L or GMCSF molecule for improved efficacy due to the immune-response and apoptosis prompted by the immunomodulating molecule (II-III).

3. To reveal and characterize the exceptional life cycle of vaccinia virus in feline SCCF1 cells, in which infection leads to assembly of incomplete but infectious particles (IV).

3. MATERIALS AND METHODS

3.1. Cell lines

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

Table 1. List of human, hamster, feline and mouse cell lines used in the studies

Cell line	Description	Species	Used in
293	embryonic kidney cells	human	I
A549	lung adenocarcinoma	human	I, II, III, IV
Hs 766T	pancreatic cancer	human	I
SW 1990	pancreatic cancer	human	I
Capan-2	pancreatic cancer	human	I
HPAC	pancreatic cancer	human	I
Panc-1	pancreatic cancer	human	I
M4A4-LM	breast cancer	human	II
EJ	bladder carcinoma	human	II
PC3MM2	prostate cancer	human	II
CV-1	kidney epithelial cells	african green monkey	II, III
Ramos-Blue	B-cell cell line	human	II
B16-ova	melanoma cell line	murine	II
Vero	kidney epithelial cells	african green monkey	II, III
TF1	erythroleukemic cells	human	III
HaK	renal cancer	hamster	III
Hap-T1	pancreatic cancer	hamster	III
SCCF1	squamous cell carcinoma	feline	IV

All cell lines were maintained in the conditions recommended by the manufacturer.

3.2. Human specimens

Fresh pancreatic cancer tissue samples were obtained with signed informed consent and ethical committee permission from patients undergoing surgery at Helsinki University

Central Hospital. Histology analysis of the samples was studied by pathologists from the hospital.

3.3. Adenoviruses

Replication deficient and competent viruses were amplified on A549 and 293 cells and purified on double cesium chloride gradients following standard protocols (Luo et al. 2007). The presence of gene deletions and insertions and the absence of wild-type virus were confirmed by polymerase-chain-reaction (PCR). Main features of the replication deficient viruses are described in Table 2 and features of the replication competent viruses used in the study I have been listed on Table 3.

Table 2. List of replication deficient adenoviruses used in study I

Virus	E1	Fiber	Reference
Ad5luc1	Luciferase	Wild type	(Kanerva et al. 2002)
Ad5/3luc1	Luciferase	5/3 serotype chimerism	(Kanerva et al. 2002)
Ad5lucRGD	Luciferase	RGD motif in HI loop	(Dmitriev et al. 1998)
Ad5.pK7	GFP + luciferase	7 lysine residues at C-terminus	(Wu et al. 2002)
Ad5RGD.pK7	GFP + luciferase	RGD motif in HI loop and 7 lysine residues at C-terminus	(Wu et al. 2002)

Table 3. List of replication competent adenoviruses used in study I

Virus	E1	E3	Fiber	Reference
Ad300wt	Wild type	Wild type	Wild type	ATCC[1]
Ad5/3-Δ24	24 bp deletion (Δ24) in the constant region 2 of the E1A gene	Wild type	5/3 serotype chimerism	(Kanerva et al. 2003)
Ad5-Δ24RGD	Δ24	Wild type	RGD motif in HI loop	(Suzuki et al. 2001)
Ad5-Δ24.pK7	Δ24	Wild type	7 lysine residues at C-terminus	(Ranki et al. 2007)
Ad5-Δ24E3	Δ24	Wild type	Wild type	(Suzuki et al. 2001)
Ad5/3-Δ24-GMCSF	Δ24	gp19k/6.7K deleted, hGMCSF inserted	5/3 serotype chimerism	(Koski et al. 2010)

[1] Wild type serotype 5 adenovirus (Ad300wt) was purchased from American Type Culture Collection (Manassas, VA, USA).

3.4. Vaccinia viruses

The viruses used in the study were all cancer specific double deleted vaccinia viruses (vvdd) of Western Reserve strain and displaying total deletion of vaccinia growth factor (VGF) and partial deletion of thymidine kinase (TK) gene. All viruses have also insertion of lacZ gene in VGF site coding inactive but immunogenic beta-galactosidase enzyme. Main features of vaccinia viruses used in studies II-IV are listed on Table 4.

Viruses were amplified on A549 cells and purified over a sucrose cushion, and plaque forming unit virus titers (pfu/ml) were determined by plaque assay as described previously (McCart et al. 2001). The presence of the inserted genes was verified by PCR, fluorescent microscope and FACSarray.

Table 4. List of vaccinia viruses used in studies II-IV.

Virus	Marker gene	Transgene	Used in	Reference
vvdd-luc	luciferase		IV	(Guse et al. 2010)
vvdd-tdTomato	tdTomato		II, III, IV	Study II
vvdd-tdTomato-hCD40L	tdTomato	hCD40L	II	Study II
vvdd-tdTomato-mCD40L	tdTomato	mCD40L	II	Study II
vvdd-tdTomato-hGMCSF	tdTomato	hGMCSF		Study III

3.4.1. Construction of vvdd-tdTomato and vvdd-tdTomato-hCD40L/mCD40L/hGMCSF

The novel viruses vvdd-tdTomato, vvdd-tdTomato-hCD40l, vvdd-tdTomato-mCD40l and vvdd-tdTomato-hGMCSF were generated using standard vaccinia virus preparation methods. Briefly, the tdTomato gene was first cloned in to pSC65 plasmid under the control of the pE/L promoter to create pSC65-tdTomato. After that, hCD40l, mCD40l or hGMCSF cDNA was cloned under the control of pE/L promoter to create pSC65-tdTomato-hCD40l, pSC65-tdTomato-mCD40l or pSC65-tdTomato-hGMCSF shuttle plasmid. Correct insertion of the transgenes in the cloning plasmid were verified by restriction enzyme analysis and sequencing. These plasmids were co-transfected in CV-1 cells with vvdd-luc and successfully

recombinated viruses were picked if they were positive for red fluorescent and negative for luciferase.

3.5. *In vitro* studies

3.5.1. Cytotoxicity assays (I-IV)

To study cell killing efficacy of oncolytic viruses, 10 000 cells were seeded on 96-well plates and infected in triplicates with different concentrations of virus suspended in growth medium (GM) containing 2% fetal calf serum (FCS). After 1 hour, medium was changed and replaced with 5% FCS GM. Progress of infection was monitored daily and the MTS [3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] cytotoxicity assay was performed as recommended by kit manufacturer (Cell Titer 96 Aqueous One Solution, Promega).

3.5.2. Gene transfer assays (I, IV)

For *in vitro* gene transfer assays on study I, cells were infected with replication-deficient viruses for 30 minutes at room temperature suspended in growth medium with 2% FCS. Cells were washed once and complete medium was added. After incubation at 37°C for 24 hours, luciferase (Luciferase Assay System, Promega) was measured with TopCount luminometer (PerkinElmer).

For *ex vivo* gene transfer assays on study I, human pancreatic cancer samples were cut into pieces (~200 mg) and homogenized. Samples were infected with 2.5 x 10^8 viral particles and after 1 hour incubation at 37°C, the medium was replaced with fresh growth medium. Tissues were collected after 24 hours, and lysed with Cell Culture Lysis Buffer (Promega). Samples were freeze-thawed three times and luciferase was analyzed from supernatant. Protein concentration was determined by using a detergent compatible protein assay kit (Bio-Rad).

For transduction assays in study IV, 100,000 cells were plated in 24-well plate and infected in triplicates with virus suspended in different concentrations in growth medium containing 2 % FCS for 30 min. Luciferase activity was measured from lysed cells according to the manufacturer's instructions (Luciferase Assay System, Promega, Madison, WI, USA). Transduction of tdTomato was also confirmed by infecting cells with 0.2, 1 or 5 MOI and visualizing the tdTomato expression 24 hours later with fluorescent microscope.

3.5.3. Expression and biological activity of transgenes

To analyze the transgene expression, cells were seeded on 24-well plates and infected with different concentrations of virus suspended in growth medium containing 2% FCS. After thirty minute incubation, cells were washed and medium was replaced with complete growth medium. Supernatant wad collected on different time points and analyzed according to the manufacturer's manual (FACSarray for hGMCSF or soluble hCD40l). Murine CD40l expression was checked with mouse sCD40l Elisa kit according to the manufacturer´s protocol (Bender Medsystems).

To confirm the biological activity of hGMCSF, supernatant of vvdd-tdTomato-hGMCSF infected cells was studied on TF1 cells, which require GMCSF to maintain their viability. Supernatant was collected 48 post infection, filtered and applied on TF1 cells. Commercial hGMCSF (2 ng/ml) was used as a positive control and TF1 cells without hGMCSF supplementation were used as a negative control. Viability of the cells was measured with MTS as described previously.

3.5.4. Immunogenicity of cell death (II)

Calreticulin exposure on the cell surface as well as ATP and HMGB1 release have been recently proposed as *in vitro* measurable indication markers for an immunogenic cell death. HMGB and ATP release to the supernatant as well as calreticulin exposure on the cell surface were analyzed following 12 hours after infection with vvdd-hCD40L-tdTomato or vvdd-tdTomato. Calreticuline exposure was measured by flow cytometry by staining the cells with anti-calreticulin antibody (primary antibody) and Alexa-Fluor 488 IgG (secondary antibody). Extracellular ATP was measured with ATP Determination Kit (MolecularProbes; Invitrogen) and the amount of released HMGB1 was analyzed with ELISA kit (IBL International).

3.5.5. Human-derived lymphocyte and human peripheral blood mononuclear cell (PBMC) stimulation (II)

To confirm the biological activity of hCD40l in human cells, A549 cell monolayers were infected with vvdd-tdtomato-hCD40l and supernatant was collected and filtered. Ramos-Blue cell line stably expresses an NFkB/AP-1-inducible SEAP (secreted embryonic alkaline phosphatase) and can therefore be used as a reporter cell line for stimulation with CD40l. Cells were stimulated with supernatant for 24h stimulation and the levels of SEAP can were measured using QUANTI-Blue™, a medium that turns blue in the presence of SEAP. Results were read with microplate reader at the wave length of 450nm.

To see if CD40l produced by the virus could stimulate human peripheral monocytes (PBMCs), 24-well plates of A549 cells were infected with vvdd-tdTomato or vvdd-hCD40L-tdtomato and lysed cells were collected on three days post infection. Isolated human PBMCs were grown as according to the instructions of manufacturer (Tebu-bio) and PBMCs were stimulated by adding the lysate on top of them and supernatants were analyzed for stimulation markers human TNF alpha, IL-1alpha, IL-6, IL-10 and RANTES by FACSarray.

3.5.6. Electron microscopy (IV)

Two separate methods for EM specimen preparation, negative staining and thin sectioning, were implemented on study IV.

Negative staining samples were prepared by collecting supernatant of infected cells and purifying the viral particles through sucrose cushion as described previously (Guse et al. 2010). One droplet of sample was absorbed on formva-carbon coated EM specimen, absorbing for 1 minute. The suspension was then blotted away by Whatman filter paper, and sample was stained for 30 seconds by adding 2% Potassium phosphotungstate (KPT), pH 7.2 on top of the sample. Prior to the electron microscope for observation, the stain was blotted away and the sample was air-dried for 2 minutes.

Resin embedded thin section samples were prepared by fixing the infected cells directly on culture dish with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 30 minutes. Fresh glutaraldehyde fixative was added on top of the cells and fixation was continued for additional half an hour. Cells were post-fixed by 2% osmium tetroxide for 1 hour, dehydrated in series of ethanol and embedded in LX-112 resin. Ultra-thin sections were cut at thickness of 60-80 nm and stained with uranyl acetate and lead citrate in Leica EMstain automatic stainer (Leica microsystems, Austria) according to the manufacturer´s recommendations.

3.6. *In vivo* studies

All animal protocols were reviewed and approved by the Experimental Animal Committee of the University of Helsinki and the Provincial Government of Southern Finland. Mice and Syrian Golden hamsters were purchased from Taconic at 4-5 weeks of age and quarantined for at least one week before starting the experiments. Animal were housed under standard conditions, fed *ad libitum* and the health status of the animals was monitored daily.

3.6.1. Animal models in study I

3.6.1.1 Survival experiments

Peritoneal pancreatic cancer was established in severe combined immunodeficiency (SCID) mice by injecting $7x10^6$ Panc-1 cells intraperitoneally. On day 4 mice were treated intraperitoneally with 1 x 10^9 virus particles of replication-competent, Δ24-modified oncolytic adenoviruses or wild type Ad300wt and monitored for survival. Two groups of mice received 40 mg / kg gemcitabine alone or in combination with Ad5/3-Δ24. In addition, silica gel was used in delivery of Ad5/3-Δ24 and compared to the virus injected group. Virus embedded with silica was prepared and characterized as described previously (Kangasniemi et al. 2009). Implants were implanted surgically and mock mice received a silica implant without the virus. Another survival experiment was performed in immunocompetent Syrian hamster. Peritoneal pancreatic cancer was established by injecting $3x10^6$ Hap-T1 cells intraperitoneally and the animals were treated with $5x10^{10}$ vp of Ad5/3-Δ24-GMCSF as an injection or as surgically implanted silica implant. Survival of the animals was followed daily.

3.6.1.2 Effect of pre-existing antibodies on gene transfer in vivo

For *in vivo* gene transfer study, immunocompetent ICR mice were preimmunized with Ad5/3luc1. After 4 weeks, mice received 1 x 10^{10} viral particles of Ad5/3luc1 as an injection or in a silica implant. Livers and pancreases were collected on day 8 and DNA was extracted using the QIAamp DNA mini kit (Qiagen). Adenoviral load was quantified by qPCR as genome copy numbers. PCR amplification was based on primers and probe targeting the E4 region. β-actin was used for normalization and E4 and β-actin standard curves were generated by using serial dilutions of E4-gene containing plasmid AdEasy (Quantum) and mouse whole genomic DNA (BD Biosciences).

3.6.1.3 Cytokine analysis.

Cytokines IL-6, IFN-γ and RANTES were analyzed as indicators of pro-inflammatory responses using BD Cytometric Bead Array (CBA) Human Soluble Protein Flex Set (Becton Dickinson) according to manufacturer's instructions.

3.6.1.4 Neutralizing antibody titer

Serum samples were incubated at 56 °C for 90 min for complement inactivation and dilution series (1:4 to 1:16 384) were prepared in serum-free DMEM. Ad5/3luc1 was mixed with serum dilutions and incubated at room temperature for 30 min. 96-well plates were seeded

with 1 x 10^4 293 cells and infected with 100 VP/cell. Cells were incubated for 24 hours, lysed and luciferase activity was measured with Luciferase Assay System (Promega) according to the manufacturer´s instructions. The amount of NAbs in the serum was determined with the dilution which resulted to the inhibition of gene transfer.

3.6.2. Animal models in study II

3.6.2.1 Subcutaneous tumor growth inhibition experiments

Subcutaneous EJ or A549 tumors were established by injecting 10^6 cells into flanks of nude Naval Medical Research Institute (NMRI) mice. When tumors reached the sizo of approximately 5x5 mm, virus was injected with vvdd-tdTomato-hCD40L either intratumorally ($5x10^4$ pfu) or intravenously (10^5 pfu) and the growth of the tumors was monitored every other day. In the second experiment, total of 2,5x105 B16-ova cells were injected subcutaneously to induce tumors into flanks of immune competent C57Bl/6 mice. 10^7 pfu of vvdd-tdTomato or vvdd-tdTomato-mCD40L was injected intratumorally and tumor growth was measured every other day. Animals were euthanized and samples were collected on day 18. Simultaneously part of the animals were monitored for survival up to day 60.

3.6.2.2 Bioluminescence imaging

For bioluminescence imaging, subcutaneous M4A4-LM3 breast tumors were injected with vvdd-tdTomato or vvdd-tdTomato-hCD40L and images were captured on different approximately twice a week using the IVIS imaging series 100 system (Xenogen, Alameda, CA).

3.6.2.3 FACS array and flow cytometry

To assess human CD40L concentrations in blood or in tumors blood samples were taken 3 and 13 days post injection and on day 13, also tumors were collected and lysed with ultrasonification. Samples were analyzed with FACSarray for hCD40L quantification as described previously.

Tumors, spleens and lymph nodes from B16-ova bearing mice were collected and smashed into single-cell suspension for flow cytometry analysis. Cells were stained according to manufacturer instructions following antibodies; 553066 – CD3, 553033 – CD8, 561736 – CD19, 55093 – CD11b (Macrophages, MDSC), 553801 – CD11c (DC), 560756 – NKp46

and 550954 – CD4 antibody (BD Biosciences). Samples were analyzed with BD Accuri C6 flow cytometer and results were plotted with FacsAccuri C6 software (BD Biosciences).

3.6.3. Animal models study III

3.6.3.1. Subcutaneous tumor growth inhibition and rechallenging experiment

Subcutaneous Hap-T1 tumors were established by injecting 10^7 cells subcutaneously into both flanks of Syrian hamsters. Tumors were injected intratumorally with 10^7 pfu of vvdd-tdTomato, vvdd-tdTomato or PBS and tumor growth was monitored every other day. On day 32, six animals from each group were euthanized and their spleens were collected for co-culturing with tumor cells. Hamsters cured with vvdd-tdTomato or vvdd-tdTomato-hGMCSF were re-challenged with the same tumor (HapT1) or a different hamster renal cancer tumor (HaK). Naïve hamsters were used as control groups for tumor growth and the growth was followed every other day.

3.6.3.2. Histology and immunohistology

Tumors from each group were collected and fixed with 10% buffered formalin immediately after euthanizing the animals. After 24 hour fixation, samples were stored in 70% ethanol. Fixed tumors were embedded with paraffin, cut into sections (3-5 μm) and stained with hematoxylin-eosin, rabbit anti-human CD3 antibody (Dako) or mouse anti-human calprotectin antibody (AbDserotec).

3.6.3.3 Viral DNA load in tumors

Tumors were collected on day 8 and DNA was extracted using the QIAamp DNA mini kit (Qiagen). Vaccinia virus was quantified by qPCR as genome copy numbers. PCR amplification was based on primers and probe targeting the viral HA J7R gene. Hamster GAPDH was used for normalization and E4 and β-actin standard curves were generated by using serial dilutions of HA containing plasmid and hamster whole genomic DNA extracted from cultured cells

3.6.3.4. Cytotoxicity, migration and proliferation of splenocytes **ex vivo**

To study the induction of cytotoxic lymphocytes induced by virus treatment, spleens were collected from the PBS or virus treated animals. Spleens were homogenized, filtered and cultured for 24 hours. $5x10^4$ Hap-T1 or HaK cells were seeded on 96-well plates and different

ratios of splenocytes were added on top of the cells. Cell viability was measured by MTS assay after 24 hours as described previously.

Migration of splenocytes was assessed with QCMTM Chemotaxis Cell Migration assay (Millipore). HapT1 cells were infected with vvdd-tdTomato or vvdd-tdTomato-hGMCSF and 24 hours later supernatant was collected and filtered. Supernatant was placed as an attractant of the receiver plate and CFSC-labelled splenocytes were added on top of the filter plate. Migrated splenocytes were quantified with FACS Accuri C6 flow cytometer (BD Biosciences) following 20h of incubation.

Activation of splenocytes was assessed by measuring their proliferation rate *ex vi*vo. Proliferation of the cells was assessed by measuring the amount of BrdU attached to newly synthesized DNA following 24 hours of culturing on 96-well plates (BrdU cell proliferation ELISA kit, Abcam).

3.7. Statistics

All values are indicated as mean standard error of the mean (SEM). Tumor sizes as a function of time were compared by Mann-Whitney test (MedCalc software) and p-values of <0.05 were considered statistically significant. A single preplanned comparison of mean tumor volume was done by using a Fisher´s exact test (MedCalc software). Survival was analyzed using Kaplan-Meier test or log-rank t-test (SPSS 11.5 and GraphPrism) Differences between groups in *in vitro* assays were calculated using two-tailed Student's t-test (GraphPrism software).

4. RESULTS AND DISCUSSION

4.1. Capsid-modified oncolytic adenoviruses show enhanced transduction and oncolytic effect in pancreatic cells and tissues (I)

One limiting factor in the most commonly used Ad5-based vectors is their dependence on the coxackie and adenovirus receptor CAR expression on the cell surface of cancer cells. CAR has been shown to be expressed in low levels in many cancer types (Bauerschmitz et al. 2002) and thus there is a need for transductional targeting. Adenovirus 3 serotype has been shown to have a distinct tropism in human primary cancer cells as it binds to DSG-2 receptor, and therefore Ad5/3 chimeric serotype virus was constructed by substituting the entire fiber knob of serotype 5 with the adenovirus serotype 3 knob domain (Kanerva et al. 2002). Other capsid modifications we were interested in studying were the incorporation of Arg-Gly-Asp (RGD)-peptide modification, targeting the virus binding to αvβ-class integrins which are also highly expressed in many tumors (Wu et al. 2002). Another capsid modification was the incorporation of linker sequences (pk7), which target the binding to heparan sulfate proteoglycans (HSPGs), also expressed in a variety of cancers (Ranki et al. 2007, Hammond et al. 2014).

First, a panel of capsid-modified adenoviruses were tested *in vitro* and the 5/3 chimerism was shown to have superior gene transfer efficacy compared to Ad5 in pancreatic cancer cell lines Capan-2 and HPAC. RGD and pk7-modified viruses were able to improve the transduction in Capan-2 and SW-1990 cell lines whereas in the CAR-positive, non-malignant 293 cells the difference was less than 7-fold (Figure 2, study I). The same phenomenon was seen in primary pancreatic adenocarcinoma samples, as Ad5/3 chimeric virus had the best transduction efficacy in all four samples (Figure 1, study I). However, given the variation between the samples, in clinical settings it might be rational to analyze patient tumors before the treatment so that the optimal virus could be selected for each patient.

Furthermore, replication-competent versions of corresponding viruses featuring a 24 base pair deletion in E1A region were studied for their ability to replicate in and lyse cancer cells. As many cancer cells have a defect in the Rb/p16 pathway leading to pRb overexpression, the deletion in E1A region of the virus restricts the virus replication to tumor cells in which the E1A protein binding to Rb is not needed. The Rb/p16 pathway has been shown to be abrogated in virtually all pancreatic carcinomas (Schutte et al. 1997). In cytotoxicity assays,

Ad5/3 was most oncolytic in three out of four pancreatic cell lines (Figure 3, study I). These results are in line with previous studies with Ad5/3 chimerism in other cancer types, supporting its excellent utility in cancer gene therapy (Kanerva et al. 2003, Guse et al. 2007, Koski et al. 2010).

4.2. Capsid modified adenoviruses increase survival *in vivo* in combination with gemcitabine or silica gel (I)

One of the obstacles in virotherapy is the rapid clearance of the viral vectors by the activated immune system and therefore new delivery methods are needed for gene delivery to sufficiently large areas of the tumor. Silica-sol-gels can immobilize drugs, enzymes, antibodies and even whole cells without loss of their biological activity and they have been successfully been used as combinations with many different drugs (Coradin et al. 2006). Previously it has also been shown that they are a suitable candidate for adenoviral delivery. Kangasniemi et al. reported extended release of capsid-modified oncolytic adenoviruses from silica-implants *in vivo*, and treatment of mice with pancreatic cancer doubled their survival. In addition, silica gel-based delivery slowed down the development of anti-adenoviral antibodies (Kangasniemi et al. 2009). Encouraged by these results, this approach was studied further in pancreatic cancer models in combination with gemcitabine, which is the standard of care treatment for pancreatic cancer (Bernhard et al. 2008). Possible reasons for synergy between Ad5/3-Δ24 and gemcitabine include the chemosensitizing activity of E1A (Lee et al. 2003) and/or altered replication kinetics. Previously, gemcitabine has been used successfully in combination with Ad5/3-Δ24 in ovarian cancer (Raki et al. 2005).

In vivo, all viruses were able to increase the survival of the mice over mock, gemcitabine or wild type virus alone and combining the Ad5/3 with gemcitabine seemed to result in the best survival, although the difference was not significant (Figure 4, study I). Ad5/3 covered with silica resulted in equally good survival data as with an injected virus proving the applicability of the silica as a delivery method. Keeping in mind that this experiment was done in nude mice, we did not expect to see a dramatic difference in the efficacy. In addition, the use of silica as a delivery method resulted in a more safe and favorable liver to pancreas gene transfer ratio (Figure 5, study I).

4.3. Using silica implants for virus delivery reduces the antiviral immune response (I)

We also hypothesized that shielding the virus with the silica might protect the adenovirus from the vigorous immune response against the virus and contribute to the immunological and efficacy perspectives. To study the function of silica-coated adenovirus, an efficacy experiment was performed in immune competent Syrian hamsters. Hamsters have been shown to be semi-permissive for adenovirus replication and responsive to human GMCSF so in this animal model we decided to use our clinically most relevant virus Ad5/3-Δ24-GMCSF (Koski et al. 2010). Virus injections eventually resulted in better survival than virus coverd with silica, one at least partial explanation being the surgery scar metastasis problem related to the implantation we encountered due to the aggressive nature of the used cell line (Figure 4d, study I). In addition, immunological parameters were assessed by measuring inflammatory cytokines and neutralizing antibodies, which were less pronounced in silica-groups. (Figure 6, study I). Induction of high IL-6 levels have been suggested to be related to adenovirus-mediated toxicity (Nemunaitis et al. 2007) and lower Nab induction might lead to prolonged systemic circulation of the virus and therefore contribute to the accessibility of the virus into tumors (Petry et al. 2008).

4.4. Development of an oncolytic vaccinia virus with tdTomato and CD40L and characterization of the virus *in vitro* (II)

The immune system is often thought of as key player in successful oncolytic virus delivery and efficacy. Recent clinical and preclinical studies emphasize that there is a significant interplay between virotherapy and immunotherapy approaches and have revealed potential synergy between these two promising therapeutic strategies. The potential of antitumor immune activation after oncolytic virus treatment has been further exploited to improve therapy by incorporating a range of immunostimulatory genes into a number of viruses (Prestwich et al. 2009).

CD40-mediated cancer therapy has been under development since it became clear that CD40 plays a profound role in the stimulation of adaptive immune responses. CD40L, also known as CD154, is a membrane glycoprotein predominantly expressed on the surface of CD4+ T-cells. Its receptor CD40 is expressed on the surface of B-cells and antigen presenting cells and the interaction leads to activation of both humoral and adaptive cell-mediated immune responses (Grewal et al. 1998, Mackey et al. 1998). Furthermore the interaction stimulates

antigen presentation and cytokine production by macrophages and dendritic cells, triggers T-cell expansion and increases IL-2 production leading to engagement of cytotoxic T-cells necessary for anti-tumor immune response. Given these effects, we hypothesized that engineering vaccinia virus to express the potent T-cell co-stimulatory molecule CD40-ligand would function as a multifunctional agent capable of inducing tumor-specific oncolysis and strong anti-tumor immune responses.

The virus generated for this study features four genetic modifications. Thymidine kinase and vaccinia growth factor were disrupted in order to generate double deleted vaccinia virus for increased safety and cancer selectivity (McCart et al. 2001). Notably, in contrast to previous Western Reserve strain based vaccinia designs which have only featured an insertion into TK, we completely deleted the TK region to avoid the possibility of back-recombination which could result in a wild type TK gene. The virus backbone also has a cDNA expressing the tdTomato fluorochrome driven by vaccinia p7.5 promoter incorporated in its genome for imaging the virus *in vitro* and *in vivo* (Figure 1, Study II). TdTomato is an attractive option compared to the most commonly used fluorophores such as green fluorescent protein (GFP) as it possess a greater tissue penetration and photostability (Winnard et al. 2006, Shaner et al. 2008). The imaging transgene is potentially important for biosafety and patient safety reasons. In clinical settings, detection of viral shedding or interpretation of the nature of skin lesions in trial patients would benefit from imaging capability. Human CD40L (hCD40l) was inserted under the control of vaccinia pE/L promoter. For a better understanding of CD40L effects, corresponding virus encoding murine CD40L (mCD40l) was generated for studies in syngeneic immunocompetent animal models. Correct deletions and insertions of the new viruses were confirmed by PCR and human CD40L expression was studied *in vitro* by infecting A549 cells with the virus and measuring the CD40L concentration over time. As expected, a dose-dependent increase of secreted hCD40L was observed (Figures 2a, Study II).

Next, we assessed whether the oncolytic activity of the newly generated virus had remained unaltered. Cytotoxicity was assessed in A549 (CD40-), M4A4-lm3EJ (CD40+) and B16-ova cells and we did not observe any significant difference between the two viruses in these cell lines indicating that the expression of the transgene did not significantly alter the biology of the virus (Figure 2, Supplementary Figure 1, Study II). Recent observations have suggested that the immunogenicity of cell death can significantly influence subsequent anti-tumor immune response and the overall efficacy of a drug. Calreticulin exposure as well as ATP and

HMGB1 release have been recently proposed as *in vitro* measurable indication of an immunogenic cell death (Hannani et al. 2011). Assessment of these markers showed a trend of upregulation of these markers in CD40+ EJ-cell line (Figure 3, Study II). Notably, these assays were challenging to perform in the context of vaccinia virus because of the robust lytic effect which can "dilute" the effects of CD40L. As we were therefore only able to analyze relatively early time points, it has been reported that the optimally HMGB1 and ATP should be measured at later time points (Guo et al. 2005).

4.5 CD40L encoding virus displays anti-tumor efficacy and tumor-restricted replication which can be followed by fluorescent imaging *in vivo* (II)

Pharmacokinetics of the virus was also studied in tumor bearing mice *in vivo*. TdTomato expression was imaged in nude mice bearing M4A4-LM2 tumors, showing even and comparable tdTomato expression between groups injected with vvdd-hCD40L-tdTomato or control virus. Quantification of CD40l from tumors and blood revealed high levels of hCD40L in the tumors while no CD40l was detected from the blood, supporting the desired outcome of local, tumor-restricted expression of the transgene (Figure 4b-c, Study II).

Next, further *in vivo* experiments were performed to study the anti-tumor efficacy of these newly generated viruses. Nude mice bearing A549 tumors or EJ tumors were injected intratumorally and the growth of the tumors were followed every other day. In the CD40l-positive EJ xenograft model, our CD40l virus showed superior anti-tumor activity compared to parental virus but in A549 cells this advantage was lost (Figure 5, Study II). As systemic administration provides the opportunity to treat both the primary tumor and any metastatic deposits simultaneously, so the same experiment was performed with intravenous injections of the virus. However, no difference was seen between the viruses although anti-tumor efficacy was observed (Figure 6, Study II). Systemically administered virus is often rapidly cleared from the circulation and this phenomenon can happen even in a immune deficient model because of complement activation, antiviral cytokines, tissue-resident macrophages. Also non-specific uptake by other tissues might compromise the successful delivery of the virus (Ferguson et al. 2012). If decreased amount of virus was delivered intravenously, it might be that CD40l failed to reach the optimal therapeutic level which would have been needed for seeing significant differences between armed and unarmed virus in this experiment. Notably, in both models the parental virus showed a potent anti-tumor activity in

both cell lines, most likely due to the efficient oncolytic potency of vaccinia virus which was not controlled by the functional immune system.

4.6. CD40L encoding virus induces immune responses in human immunological cells and in immunocompetent mouse model (II)

Since human CD40l is not active in mice and thus limits the repertoire of immunological *in vivo* studies, the effect of hCD40l on human immunological cells was studied *in vitro*. The first assay was done using human B-cell derived cell line (Burkitt´s lymphoma), in which CD40L can activate NF-κB inducible reporter gene expression. As expected, filtered media derived from the infected cells was able to significantly enhance the activation of these cells. In the second assay, human primary lymphocytes were stimulated with lysate from infected cells and a trend of more immunogenic cytokine expression profile, characteristic to Th1-type response, was seen (Figure 7, Study II).

As immunotherapies that rely on priming specific CD8 (+) T-cells against melanocyte and melanoma antigens can generate significant therapeutic responses, we hypothesized that this virus could enhance virotherapy with concomitant priming of melanoma-specific T-cells. Therefore, vvdd-tdTomato-mCD40L virus was assessed in B16-ova melanoma model which expresses chicken ovalbumin gene. Mice treated with vvdd-tdTomato showed significantly better tumor growth control and prolonged survival compared to the unarmed virus (Figure 8, Supplementary Figure S3, Study II). Analysis of immune cell filtration to tumors revealed a trend of increased infiltration of lymphocytes, although significant differences were only seen in the amounts of NK-cells, dendritic cells and B-cells (Figure 9, Study II). However, also an increased proportiond of myeloid-derived suppressor cells were seen in tumors emphasizing the fact that immunostimulation tends to induce also immunosuppression, and finding the balance between anti-tumoral and anti-viral immune response is one of the key determinants of the outcome in immunovirotherapy. Previously, CD40L encoding, non-replicating recombinant vaccinia viruses have been studied as vaccines and shown to robustly boost the immune response, supporting the use of an oncolytic platform in the context of CD40L (Bereta et al. 2004, Feder-Mengus et al. 2005).

Despite the promise of CD40L as an immune-stimulatory agent, combination of oncolytic virus and CD40L can be tricky and immunostimulatory modification of a virus does not inevitably enhance the antitumoral effect. For example, in a previous study CD40L has been

reported to also suppress vaccinia virus when inserted in a replication-incompetent virus (Ruby et al. 1995). Although our virus was able to retain its oncolytic properties, it is important to keep in mind that immunostimulatory molecules might have counteracting properties as well and the biologic effects depend on the diverse effects of CD40L protein. In another study, vesicular stomatitis virus encoding CD40L failed to demonstrate superior efficacy than its unmodified equivalent when the virus was injected intratumorally, and was actually even less effective than a non-replicating adenoviral vector expressing CD40L. In this case, early nonspecific T-cell activation initiated by replicating VSV-CD40L distracted the immune responses away from priming of tumor-specific T cells, even in the presence of potent co-stimulatory signals. Vaccinia is highly immunogenic and therefore the same phenomenon might have been seen also in our study to some extent, as despite the prompted immune responses we did not see increased amounts of ova-spesific T-cells in the virus-treated tumors (data not shown). However, results from oncolytic adenovirus encoding CD40L have given promising results in preclinical studies (Diaconu et al. 2012), in advance therapy access program (Pesonen et al. 2012) and in clinical trials (Malmstrom et al. 2010). Thus, CD40L remains as a promising transgene candidate for immunovirotherapy. In conclusion, these results emphasize how important it is to compare, select, and optimize different immunomodulators in different viral platforms and their combinations for induction of antitumor immunity.

4.7. Development of an oncolytic vaccinia virus with hGMCSF and characterization of the virus *in vitro* (III)

To date, GMCSF has been the most studied and promising immunostimulatory gene inserted into clinically advanced oncolytic viruses. The interest for GMCSF derives from its strong ability to generate systemic adaptive antitumor immunity by inducing the recruitment and differentiation of activating DCs in the tumor microenvironment (Dranoff et al. 1993). Clinical activity of GMCSF as an immune stimulant in tumor cell and dendritic cell vaccines in humans is well established (Arellano et al. 2008), but systemic use of high dose GMCSF can be toxic and also have undesirable effects on tumor immunity. Therefore, arming a potent oncolytic vaccinia virus with human GMCSF as transgene could be used to circumvent this, leading to beneficial high local doses of GMCSF with minimal systemic exposure.

Combining an oncolytic vaccinia virus with GMCSF expression holds great promise, and many different oncolytic viruses encoding GMCSF have been used to date. Wyeth strain vaccinia virus vector encoding GMCSF (Pexa-Vec) has shown promising results in clinical trials (Heo et al. 2011, Heo et al. 2013) but in contrast to Pexa-Vec, we wanted to test a Western Reserve strain as a backbone, as it has displayed greater inherent tumor selectivity and oncolytic potency over the other strains *in vitro* (Thorne et al. 2007). The virus was constructed as the CD40L virus in the previous study, utilizing vvdd-tdTomato as a backbone virus and placing hGMCSF transgene under the pE/L promoter. Vvdd-tdTomato-GMCSF showed potent oncolytic efficacy in cancer cells, and was as effective as the parental virus *in vitro* (Figure 1, Study III). Secretion of virally produced GMCSF from infected cancer cells confirmed the functionality of the virus, and bioactivity of the GMCSF was assessed in human lymphocyte cell line TF1, the viability of which is dependent on functional human GMCSF. Viability of TF1 cells cultured with virally-produced GM-CSF remained high and even better than control cells with a commercial supplement (Figure 2, Study III).

4.8. GMCSF encoding virus showwed anti-tumor efficacy in immunocompetent Syrian Hamsters and protected the animals from subsequent tumor re-challenge (III)

Evaluation of human GMCSF in preclinical cancer models is difficult because the human GMCSF is not active in mice. Rabbits have been used for preclinical testing of Pexa-Vec demonstrating cytotoxic T-lymphocyte induction and increased neutrophil, monocyte and basophil concentrations in peripheral blood (Kim et al. 2013). We aimed to establish Syrian hamsters, being permissive to vaccinia (Tysome et al. 2012) and sensitive to human GMCSF (Cohen et al. 1988), for a feasible model for the virus.

Immunocompetent Syrian hamsters carrying syngeneic subcutaneous HapT1 tumors were treated with vvdd-tdTomato-hGMCSF or unarmed parental virus and antitumor activity was measured by following the tumor growth. Both viruses showed strong antitumor activity leading to complete eradication of the tumors in both groups. Next, the same animals were re-challenged with the same or another, previously unencountered cell line, and we observed that animals previously treated and cured with GMCSF-encoding virus were able to reject the tumors with higher efficiency (Figure 3, Study III). This phenomenon has also been seen in studies with oncolytic adenovirus encoding human GMCSF (Cerullo et al. 2010), confirming that the viral platform coupled with immunostimulatory molecules is a promising way to

induce tumor-specific immunity. Additionally, presence of the virus in tumors was confirmed by qPCR and immunohistochemical analysis revealed that tumors exhibit extensive necrosis and pronounced heterophil infiltration, possibly due to the oncolysis or GMCSF expression (Supplementary Figures 2 and 3, Study III) (Papatriantafyllou 2011, Hemminki et al. 2013). To further characterize the activation of the immune system by GMCSF, we collected the splenocytes, consisting of a range of important immune mediator cell populations such as T and B lymphocytes, dendritic cells and macrophages, for immunological studies. Splenocytes of the animals were cultured *in vivo* and showed increased cell killing activity, as well as proliferation and migration capacity (Figures 4 and 5 Study III).

In conclusion, the Syrian hamster model can be used for characterizing both viral replication and host immune response although it is important to keep in mind that there is no perfect model for studying the immune-mediated antitumor activity of oncolytic viruses, given the differences in the immune systems of humans and rodents. In addition, one major hurdle with hamsters is the lack of reagents which would be required for more detailed immunological studies. Overall, results with Western Reserve strain double deleted vaccinia virus encoding GMCSF were in line with results obtained with the leading agent of the field, Wyeth Strain single-deleted vaccinia virus (Kirn et al. 2009). Good safety data has been reported in clinical trials featuring GMCSF-armed oncolytic vaccinia viruses (Kaufman et al. 2010, Heo et al. 2011, Heo et al. 2013), and in line with these results, our virus appeared to be efficient, safe, and capable of inducing tumor-specific anti-tumor immune responses. Together, these results confirm that the combination of vaccinia and GMCSF is a promising candidate for clinical translation.

4.9. Incomplete but infectious vaccinia virions are produced in the absence of oncolysis in feline SCCF1 cells (IV)

In addition to clinical translation to humans, vaccinia virus is also being developed for virotherapy in domestic animals and currently clinical trials are ongoing at least in pet dogs (Patil et al. 2012). Notably, large animals can offer more translatable data to human trials compared to rodents, giving an additional rational to study oncolytic viruses in both humans and pets. Vaccinia is known for its natural tropism for cancer cells and its broad host range and thus to our surprise, it failed to lyse feline SCCF1 cells during the preclinical screening of potential feline and canine cell lines for virotherapy. Our findings suggested that SCCF1

cells produce a morphologically abnormal virus which is nevertheless infective, revealing a new side of the virus-host cell interactions and intracellular biology of vaccinia virus.

After 3 days, infected cells maintained their viability significantly better compared to A549 cells and infected cells persisted even up to 10 days without complete lysis (Figure 1a-c, Study IV). Despite this, it turned out that cells were not resistant to vaccinia infection, as successful virus entry and early gene expression were shown *in vitro* (Figure 1d, Supplementary Figure 1 and 2, Study IV). The replication cycle was not completely blocked and interestingly these cells constantly secreted infectious particles which were able to re-infect SCCF1 or A549 cells and could be neutralized with an anti-vaccinia antibody (Figure 2, Study IV).

We wanted to characterize the nature of these particles and performed electron microscopy analysis from both the secreted particles and the infected cells. Interestingly, electron microscopy analysis of purified, secreted virus particles revealed notable differences between particles produced by SCCF1 versus A549 cells. A549 cells secreted mature, enveloped virions featuring typical characteristics for vaccinia virus. Clearly different particles were being produced by SCCF1 cells, when only misshaped immature virions, loosely packed without outer envelopes were detected (Figure 3, Study IV). The same pattern was seen in thin-section samples of infected cells. Infected A549 cells produce the whole range of viral particles at different maturation stages, but inside the SCCF1 cells the picture was different: the replication factories were inconspicuous and only few immature viruses, few of them having DNA injected to form nucleoids, were seen. Even at the peripheral region where viruses are going to be released, there were only enlarged IVs inside cytoplasm without any membranous envelope (Figures 4 and 5, Study IV).

The life cycle of vaccinia virus is well characterized (Moss 2006). The replication, maturation and morphogenesis of vaccinia virus within a host cells is a process consisting of successive intermediate steps thus producing a series of middle stage products. Many viral protein defects have been identified to affect replication, maturation and morphogenesis of vaccinia virus but all the core proteins and membrane proteins needed for virion maturation are encoded by the viral genome (Wang et al. 1995, Rodriguez et al. 1997, Traktman et al. 2000). Our data indicated that the similar effects can be brought out by the host cells and suggests that some cellular co-factor target of a viral protein affecting the replication of the virus is missing or defective in the SCCF1 cell line. Careful inspection of the morphology of

the cells in these studies did not reveal any apparent changes in the cells, as they were only seen to feature characteristics typical for cancer cells (Supplementary Figure 2, Study IV). Recently rabbit poxvirus, myxoma virus, was shown to cause cytopathic effects in the SCCF1 line. Notably, although cytopathic effects were seen, replication of the virus was slower compared to the positive control cell line and there was no statistically significant difference in cell death compared to mock-infected cells (MacNeill et al. 2012). Additional studies in other feline cell lines would be needed to confirm if this finding was restricted to this cell line alone or possibly in feline cells in general. However, identifying the mechanism could provide new and interesting insight into both viral and cellular biology revealing molecular mechanisms underlying host cell infection and viral replication as well as virulence and the life cycle of vaccinia virus.

5. SUMMARY AND CONCLUSIONS

Oncolytic viruses have several unique properties in comparison to small molecular drugs as they are able to replicate in and lyse cancer cells. In addition, many cell signaling pathways which are activated and promote the growth of tumor cells also favor the growth and replication of viruses. The field of oncolytic virotherapy is constantly expanding and many viruses are currently being tested clinically as a novel strategy for the treatment of cancer. However, their efficacy as single agents has been modest. The overall goal of this thesis was to develop new approaches for improved efficacy of genetically engineered oncolytic viruses.

An important determining factor for the successful entry of adenoviruses is the expression of the coxsackievirus and adenovirus receptor (CAR) on the tumor cell surface. However, cancer cells often express only low levels of CAR and the poor transduction efficacy is one of the major hurdles related to oncolytic adenoviruses. To circumvent this, several capsid modifications were tested in pancreatic cancer models *in vitro* and *in vivo*. The preclinical data obtained shows that capsid modified, replication competent adenoviruses such as Ad5/3-Δ24 have advantageous properties for treatment of pancreatic cancer, with or without concomitant gemcitabine. In addition, new delivery methods for oncolytic viruses are needed, since the viruses tend to be rapidly recognized by the immune system leading to quick clearance of the virus from systemic circulation. A silica-gel based delivery method for adenovirus was assessed *in vivo* and it was shown to induce lower pro-inflammatory cytokine responses and neutralizing antibody titers while the survival benefit compared to injected virus was not compromised.

Although oncolytic viruses were originally designed to function as tumor-lysing therapeutics, they have been proven to initiate systemic antitumor immune responses and can be used for tumor-restricted delivery of anti-tumoral therapeutic genes. Two other studies focused on arming oncolytic vaccinia virus with immunomodulatory molecules. The first investigated molecule was CD40l, an important modulator of adaptive immune responses. Another aspect of the study was to generate a virus encoding tdTomato for imaging purposes and test its applicability *in vivo*. As a result, vvdd-tdTomato-CD40l was generated. The virus displayed efficient lysis of human tumor xenografts in nude mice and was capable of stimulating human immune cells *in vitro*. Successful imaging experiments supported the applicability of tdTomato as a feasible fluorochrome for imaging the virus *in vivo*. In a syngeneic immune

competent mouse model, vaccinia virus encoding murine CD40L also successfully controlled the tumor growth and induced anti-tumor immune responses by recruiting immunological cells to the tumor site.

The second investigated immunostimulating molecule was GMCSF. GMCSF has been combined with many oncolytic viruses but the mechanism of action is not yet completely known and difficult to study, as immunocompetent animal models sensitive to human GMCSF are sparse. Interestingly, human GMCSF is active in hamsters and therefore we constructed vvdd-tdTomato-hGMCSF and studied the efficacy and immunological responses in Syrian hamsters. Virus treatments were able to completely eradicate syngenic tumors and provided partial or even complete protection from re-challenging with same or allogeneic tumors. This phenomenon was even stronger in animals treated with vvdd-tdTomato-hGMCSF, suggesting that certain levels of T-cell memory had perhaps been developed during the therapy.

In the last study, some new insights to the biology on oncolytic vaccinia virus were revealed. We were able to show that the life cycle of vaccinia was compromised in feline SCCF1 cells, but despite the imperfect formulation of the virions fully infectious virions were released from the cells. These results indicate that cellular factors might have a crucial role in the maturation steps of the virus, whereas previous studies have focused mainly on viral defects.

In conclusion, the studies presented in this thesis contribute to the understanding of the complex phenomenon of oncolytic viruses. Combination of selective replication, lysis, and localized immune response make oncolytic viruses a powerful and promising alternative for the treatment of cancer. In the future, coupling oncolytic virotherapy with for example tumor antigen vaccination, immune checkpoint inhibitors and adoptive cell therapy could lead to the generation of multimodal therapeutics needed for improved therapeutical outcomes in cancer patients.

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PART C – ORIGINAL PUBLICATIONS