KAROLIINA AUTIO

Efficacy and Safety of Oncolytic Vaccinia and Semliki Forest Virus in the Treatment of Canine and Feline Malignant Solid Tumours

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University of Helsinki  
Finland

EFFICACY AND SAFETY OF ONCOLYTIC VACCINIA AND SEMLIKI FOREST VIRUS IN THE TREATMENT OF CANINE AND FELINE MALIGNANT SOLID TUMOURS

Karoliina Autio

ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Veterinary Medicine of the University of Helsinki, for public examination in Walter Room, EE-Building, on 28 August 2015, at 12 noon.

Helsinki 2015
In memory of my beloved first dog Dixi
ABSTRACT

Cancer is one of the most common reasons for death in dogs, cats and humans. New therapeutic modalities are necessary to improve disease outcome. One promising approach is oncolytic virotherapy. Until now, the only oncolytic virus evaluated in a clinical trial in veterinary medicine has been canine oncolytic adenovirus, but a clinical trial has been started with oncolytic vaccinia virus (VV) in pet dogs. In cats, oncolytic viruses have not been evaluated in clinical settings. Tumour treatment in dogs and cats could also serve as a model for human cancer therapy.

The purpose of the thesis was to evaluate preclinically whether genetically modified oncolytic VV and Semliki Forest virus (SFV) could offer a new treatment modality for dogs and cats. Oncolytic VV was rendered tumour selective by a dual ablation of vaccinia growth factor and thymidine kinase, making it more cancer-specific than previously used VVs in veterinary research. To further increase the efficacy of the VV, an immunostimulatory gene, CD40L, was added to the virus backbone. Avirulent SFV A7(74) has a natural tropism for cancer cells and was not genetically manipulated.

Both viruses infected and killed tested cancer cell lines, and VV also infected most of the primary surgical tumour tissues tested. In the nude mouse xenograft model, double deleted VV (vvdd) significantly reduced tumour growth. Interestingly, when in intact monolayers, SCCF1 cells were not killed by VV, but secreted infectious, morphologically abnormal virions. One dog experienced a possible seizure after VV administration, but no other serious adverse events occurred. Vaccinia DNA declined quickly in the blood after virus administration, but was still detectable one week later by qPCR. Only samples taken directly after the VV infusion contained infectious virus, which was not found in any other blood, saliva, urine or faecal samples. Necropsies did not reveal any pathological changes associated with virus administrations.

In conclusion, our results show that oncolytic VV and SFV can infect and kill tested canine cancer cell lines, and that VV has antitumoral activity in the mouse xenograft model and it can infect fresh tumour biopsies. In addition, intravenous administration of the viruses did not induce life-threatening adverse events in healthy dogs. These agents thus warrant further evaluation in veterinary medicine.

Tämän tutkimuksen tarkoitukseena oli arvioida preklinisesti geneettisesti muunnellun onkolyyttisen vaccinia viruksen ja Semliki Forest viruksen (SFV) tehoa koirien ja kissojen syöpien hoidossa.

Tutkimuksessa käytettävää vaccinia viruksesta oli poistettu vacciniin kasvutekijä- ja tymidiinikinaasigeneeni, jolloin virus on syöpäspesifisempi kuin aikaisemmat eläinlääketieteellisissä tutkimuksissa käytetyt vaccinia virukset. Lisäksi vaccinia viruksen on lisätty immuunipuolustusta parantava CD40L-geeni. Avirulentti SFV A7/(74) puolestaan hakeutuu luonnostaan kasvainkudokseen ja lisääntyy siellä.


Tutkimustulostemme perusteella onkolyttinen vaccinia virus ja SFV infektoivat ja tapaovat testattuja koiran kasvainsolulinjoja ja lisäksi vaccinia virus hidasti syövän kasvua hiirimallissa ja infektoi potilaskoirista kerättyjä kasvaimia. Kumpikaan virus ei aiheuttanut henkäyhuhaavia haittavaikutuksia terveillä koirilla. Löydöstemme perusteella kumpaakin virusta kannattaa tutkia lisää koirien syöpien hoidossa.
ACKNOWLEDGEMENTS

The work described here was mainly carried out at the Department of Pathology and Transplantation Laboratory, Haartman Institute (Cancer Gene Therapy Group, CGTG), and the Department of Equine and Small Animal Medicine, Faculty of Veterinary Medicine, University of Helsinki, and in part at the Finnish Food Safety Authority Evira, Helsinki, and the A.I. Virtanen Institute for Molecular Sciences, Biotechnology and Molecular Medicine, University of Eastern Finland during 2010-2014. My sincere gratitude is owed to the countless individuals who contributed to these studies.

First, I warmly thank Akseli Hemminki who contacted me with his ambitious and fascinating idea of curing canine and feline cancer with oncolytic viruses. Akseli was the brains behind my thesis and made this interesting research project possible by taking me into his research group, presenting his enthusiastic views and supporting me throughout the project with encouraging and positive feedback.

My sincere gratitude is also owed to my supervisor, Docent Minna Rajamäki, who determinedly talked me into pursuing a PhD. I also thank the project leader, Professor Outi Vapaavuori. Both Minna and Outi showed an unwavering belief that I could complete this thesis.

Special thanks also go to my hands-on mentor Markus Vähä-Koskela. He was always there helping me with all the problems encountered in research. I identified him immediately as my soulmate, although our backgrounds were totally different, Markus being a great scientist and me an animal-loving clinician trying to comfort my patients.

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I thank my collaborators at A.I. Virtanen Institute, University of Eastern Finland: Janne Ruotsalainen, with whom I shared first authorship in the Semliki Forest virus paper, Professor Ari Hinkkanen and Minna Niittykoski. I greatly admire the professional but still relaxed atmosphere in your research group. I also thank Matti Waris from the University of Turku.
I warmly thank everyone at CGTG. You all made me feel welcome when I first came to the lab and had totally forgotten the basics of laboratory work in the last decade in which I had been working as a clinician. Special thanks to Suvi who started to work with vaccinia for her thesis at the same time with me. Suvi was always offering her help with the numerous and time-consuming tasks I was unable to finish since I had to be at the clinics. I am also grateful to Iulia, Kilian, Lotta, Sari and Marko for their generous assistance. All former and current technicians at CGTG, especially Kikka, Aila, Eerika, Elinä, Saija, Saila and Susanna, are thanked for their contributions. I owe a special thanks to Saila for helping me with vaccinia qPCR. Many thanks also to Akseli’s first-hand, Minna, for assistance with administrative and official issues.

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Although this work was mainly performed in the CTGT, I also wish to thank my colleagues Henna, Mimmi, Marika, Sanna, Susanne and Sari at the Department of Equine and Small Animal Medicine, Faculty of Veterinary Medicine, for friendship and support in both good and bad times. I also thank my boss, Professor Thomas Spillmann, for being very supportive and understanding of me dividing my time between clinical and scientific work. Professor Satu Sankari of the Central Laboratory and technicians Merja, Lilja and Johanna also deserve great thanks for fruitful co-operation. It has been a pleasure working with all of you!

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LIST OF ORIGINAL PUBLICATIONS

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**ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>A549</td>
<td>human lung adenocarcinoma epithelial cell line</td>
</tr>
<tr>
<td>A7(74)</td>
<td>avirulent Semliki Forest virus strain</td>
</tr>
<tr>
<td>Abrams</td>
<td>canine osteosarcoma cell line</td>
</tr>
<tr>
<td>ACE-1</td>
<td>canine prostatic carcinoma cell line</td>
</tr>
<tr>
<td>Ad5/3-hTERT</td>
<td>adenovirus type 5/3 expressing human telomerase reverse transcriptase</td>
</tr>
<tr>
<td>Ad5/3-hTERT-CD40L</td>
<td>adenovirus type 5/3 expressing human telomerase reverse transcriptase and CD40L</td>
</tr>
<tr>
<td>AdCD40L</td>
<td>adenovirus expressing CD40L</td>
</tr>
<tr>
<td>ALP</td>
<td>alkaline phosphatase</td>
</tr>
<tr>
<td>ALT</td>
<td>alanine aminotransferase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>B16-OVA</td>
<td>murine melanoma cell line expressing ovalbumin</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B cell lymphoma 2</td>
</tr>
<tr>
<td>BHK-21</td>
<td>baby hamster kidney cell line</td>
</tr>
<tr>
<td>BDIX</td>
<td>Berlin Duckrey IX inbret rat strain</td>
</tr>
<tr>
<td>BT4C</td>
<td>glioma induced by giving a single transplacental administration of N-ethyl-N-nitrosourea to pregnant BDIX rats</td>
</tr>
<tr>
<td>CAV</td>
<td>canine adenovirus</td>
</tr>
<tr>
<td>CD</td>
<td>cytosine deaminase</td>
</tr>
<tr>
<td>CDC</td>
<td>Centers for Disease Control and Prevention</td>
</tr>
<tr>
<td>CD40L</td>
<td>cluster of differentiation 40 ligand</td>
</tr>
<tr>
<td>CDRS</td>
<td>cytosine deaminase and somatostatin receptor gene</td>
</tr>
<tr>
<td>CEV</td>
<td>cell-associated extracellular enveloped virus</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CO₂</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>CPE</td>
<td>cytopathic effect</td>
</tr>
<tr>
<td>CPXV</td>
<td>cowpox virus</td>
</tr>
<tr>
<td>CT-2A</td>
<td>mouse astrocytoma cell line</td>
</tr>
<tr>
<td>D17</td>
<td>canine osteosarcoma cell line</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3’-diaminobenzidine</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTo9/06</td>
<td>feline mammary carcinoma cell line</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EEV</td>
<td>extracellular enveloped virus</td>
</tr>
<tr>
<td>EGFP</td>
<td>enhanced green fluorescent protein</td>
</tr>
<tr>
<td>EGRF</td>
<td>epidermal growth factor pathway</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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<tr>
<td>LIVP6.1.1</td>
<td>oncolytic vaccinia virus derived from Lister strain, Institute of Viral Preparations, Moscow, Russia</td>
</tr>
<tr>
<td>MDCK</td>
<td>Madin-Darby canine kidney cell line</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MOI</td>
<td>multiplicity of infection</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>MTD</td>
<td>maximum tolerated dose</td>
</tr>
<tr>
<td>MTS</td>
<td>3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium</td>
</tr>
<tr>
<td>MV</td>
<td>mature virus</td>
</tr>
<tr>
<td>NAb</td>
<td>neutralizing antibody</td>
</tr>
<tr>
<td>NaCl</td>
<td>sodium chloride</td>
</tr>
<tr>
<td>NIS</td>
<td>sodium iodide symporter gene</td>
</tr>
<tr>
<td>NR-417</td>
<td>antibody for intracellular mature vaccinia virus</td>
</tr>
<tr>
<td>OC-CAVE1</td>
<td>conditionally replicating canine adenovirus type 2</td>
</tr>
<tr>
<td>ONYX-015</td>
<td>oncolytic adenovirus replicating in and killing cells with p53 mutations by Onyx Pharmaceuticals</td>
</tr>
<tr>
<td>OPV</td>
<td>orthopoxvirus</td>
</tr>
<tr>
<td>OVA</td>
<td>ovalbumin</td>
</tr>
<tr>
<td>P-S</td>
<td>penicillin-streptomycin</td>
</tr>
<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered solution</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>p.i.</td>
<td>post-infection</td>
</tr>
<tr>
<td>pfu</td>
<td>plaque-forming unit</td>
</tr>
<tr>
<td>qPCR</td>
<td>quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>pRb</td>
<td>retinoblastoma protein</td>
</tr>
<tr>
<td>RGD</td>
<td>arginylglycylaspartic acid</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>SCCF1</td>
<td>feline squamous carcinoma cell line</td>
</tr>
<tr>
<td>SCID</td>
<td>severe combined immunodeficiency</td>
</tr>
<tr>
<td>SFV</td>
<td>Semliki Forest virus</td>
</tr>
<tr>
<td>SR</td>
<td>somatostatin receptor</td>
</tr>
<tr>
<td>ST-246</td>
<td>tecovirimat, an antiviral agent for orthopoxvirus infections</td>
</tr>
<tr>
<td>T-VEC</td>
<td>oncolytic herpes virus expressing granulocyte macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>TCID50</td>
<td>tissue culture infectious dose killing 50% of the cells</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
</tr>
<tr>
<td>TK</td>
<td>thymine kinase</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
<tr>
<td>Treg</td>
<td>T regulatory cell</td>
</tr>
<tr>
<td>U2OS</td>
<td>human osteosarcoma cell line</td>
</tr>
<tr>
<td>V-VET1</td>
<td>LIVP6.6.1</td>
</tr>
<tr>
<td>VA7-EGFP</td>
<td>replication-competent avirulent Semliki Forest virus expressing enhanced green fluorescent protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>VCOG-CTCAE</td>
<td>Veterinary cooperative oncology group - Common terminology criteria for adverse events</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>VGF</td>
<td>vaccinia growth factor</td>
</tr>
<tr>
<td>VIG</td>
<td>vaccinia immune globulin</td>
</tr>
<tr>
<td>vp</td>
<td>virus particle</td>
</tr>
<tr>
<td>VSV</td>
<td>vesicular stomatitis virus</td>
</tr>
<tr>
<td>VV</td>
<td>vaccinia virus</td>
</tr>
<tr>
<td>vvdd</td>
<td>double-deleted vaccinia virus with TK and VGF deletions</td>
</tr>
<tr>
<td>vvdd-hCD40L-tdTomato</td>
<td>double-deleted vaccinia virus expressing human CD40L and tdTomato</td>
</tr>
<tr>
<td>vvdd-luc</td>
<td>double-deleted vaccinia virus expressing Firefly luciferase</td>
</tr>
<tr>
<td>vvdd-tdTomato</td>
<td>double-deleted vaccinia virus expressing tdTomato</td>
</tr>
<tr>
<td>WV</td>
<td>wrapped virus</td>
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</table>
1 INTRODUCTION

As in humans, cancer is one of the most common reasons for death in dogs and cats. Surgery, chemotherapy and radiation therapy are the most prevailing treatments in veterinary oncology (Withrow et al. 2013), but in parallel with the human situation new approaches are needed, especially for advanced metastatic solid tumours, which are often incurable with traditional therapies. In addition, particularly in pets, maintaining the quality of life is very important and only minimal adverse events are accepted (Withrow et al. 2013).

Dogs and cats with spontaneous cancer serve as a good model for human cancers (Vail & MacEwen 2000, Hemminki et al. 2003, Ranieri et al. 2013, Wypij 2013, Laborda et al. 2014). Dogs share the same environment with their owners, their immune system is intact, their size is close to humans relative to laboratory rodents and cancer progression is spontaneous. These in addition to ethical reasons are key advantages over laboratory rodents. Like cancer in human patients but not in rodent models, cancer arising in dogs develops over several years, resulting in similar complexity, clonality and immune suppression as seen in man. The biological behaviour also has many similarities, including metastatic patterns, relapse and treatment resistance. In addition, the same cancer-associated genes and histological features have been found in both species (Vail & MacEwen 2000, Wang et al. 2013).

Oncolytic virotherapy, where replication-competent viruses are armed with immunostimulatory transgenes, is a promising new treatment approach (Buller et al. 1988, McCart et al. 2001, Cerullo et al. 2012b, Elsedawy & Russell 2013, Gentschev et al. 2014). Before directly killing cancer cells, immunostimulatory genes are expressed by infected cells to awaken the host immune system, which is suppressed by the tumour microenvironment in progressing clinically evident tumours (Elsedawy & Russell 2013). Then, infected tumour cells are killed by oncolysis, releasing a broad variety of tumour antigens into the environment for the adaptive immune system to sample (Elsedawy & Russell 2013).

In 2006, the first oncolytic virus, oncolytic adenovirus ONYX-015, was approved on the market in China (Woller et al. 2014). Although the treatment response has been modest, multiple clinical trials investigating oncolytic viruses in different cancer types in humans are ongoing (National Cancer Institute 2014). Also undergoing development is oncolytic herpes virus T-Vec expressing granulocyte macrophage colony-stimulating factor (GM-CSF), which has demonstrated durable responses and improved survival in patients with advanced melanoma (Woller et al. 2014). In addition, vaccinia virus (VV) JX-594 has shown promise in early clinical trials and expresses the same transgene, GM-CSF, as T-Vec events (Mastrangelo et al. 1999, Park et al. 2008, Breitbach et al. 2011, Hwang et al. 2011, Heo et al. 2013). A clinical trial has
also been started in cancer-bearing dogs in the USA with an oncolytic VV called V-VET1 (Gentschev et al. 2013). However, the ultimate breakthrough with oncolytic viruses has yet to occur.

The aims of this thesis were to investigate whether genetically modified oncolytic vaccinia virus and Semliki Forest virus (SFV) would be efficient and safe oncolytic agents in preclinical evaluation for treatment of canine and feline otherwise incurable cancers.
2 REVIEW OF THE LITERATURE

2.1 VACCINIA VIRUS

Vaccinia virus (VV) is a typical Poxvirus belonging to Orthopoxvirus (OPV) genus, Poxviridae family and Chordopoxvirinae subfamily. Other well-known viruses in the same genus include variola virus and cowpox virus, which cause smallpox and cowpox, respectively.

The origin of VV is uncertain, but it is has been used to eradicate smallpox worldwide. In the 1800 century, when smallpox was common in society, it was noticed that milkers did not get smallpox. Jenner found out that these individuals had already had a disease called cowpox and concluded that cowpox prevents smallpox infection (Fenner & World Health Organization 1988). To prove his theory, Jenner inoculated cowpox in humans and was able to show that persons previously exposed to cowpox did not get smallpox. At the beginning of the 1900 century, Jenner isolated the virus from horses and started to use horsepox virus in smallpox vaccines. The smallpox vaccine based on horsepox virus was later used throughout Europe; however, during vaccination multiple different laboratory strains were developed (Fenner & World Health Organization 1988). The most commonly used strains are shown in Table 1.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Background</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Copenhagen</td>
<td>Northern European vaccine strain</td>
<td>Inherent tumour selectivity</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Used as smallpox vaccine but withdrawn due to adverse events</td>
</tr>
<tr>
<td>Lister</td>
<td>European vaccine strain</td>
<td>Inherent tumour selectivity</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Extensive use during smallpox eradication</td>
</tr>
<tr>
<td>Modified Ankara</td>
<td>Derived from Ankara strain through passage in avian cells</td>
<td>Does not replicate in mammalian cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Highly immunogenic: good vaccine</td>
</tr>
<tr>
<td>New York</td>
<td>Vaccine strain derived from Copenhagen strain</td>
<td>Does not replicate in mammalian cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Highly immunogenic: good vaccine</td>
</tr>
<tr>
<td>Tin Tan</td>
<td>Chinese vaccine strain</td>
<td>Used in China during smallpox eradication</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Oncolytic potential unknown</td>
</tr>
<tr>
<td>Wyeth / New York City Board of Health</td>
<td>Northern American vaccine strain</td>
<td>Minimal inherent tumour selectivity</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Slow replication in mouse tissue</td>
</tr>
<tr>
<td>Western Reserve</td>
<td>Laboratory strain derived from Wyeth through passage in mice brain</td>
<td>High tumour selectivity</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Strong oncolytic effect in mouse models</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Minimal use in humans</td>
</tr>
</tbody>
</table>
2.1.1 Biology of vaccinia virus
VV virion is a brick-shaped particle of approximately 360 x 270 x 250 nm and composed of approximately 75 proteins (Kroon et al. 2011). It has a single linear double-stranded DNA consisting of ca. 200 kilobase pairs (kb), and both ends have a hairpin loop. Genes in the centre encode conserved proteins vital for virus replication, whereas genes near the ends encode proteins associated with non-vital functions such as evading the host immune system and affecting virulence. Inverted terminal repeats are found at both ends of the genome. The genome of VV codes for more than 200 genes and can carry up to 25 kb of foreign transgenes.

VV replication happens in the cytoplasmic inclusions, where infecting virions are partly uncoated by cellular enzymes and then fully uncoated by viral enzymes released from the virion core. The replication cycle is divided into two parts controlled by either early or late gene products (Condit et al. 2006).

The virion has three different infectious forms: (1) mature virus (MV), (2) wrapped virus (WV) and (3) extracellular virus (EV) (Moss 2012). MV has a single lipid bilayer envelope and was previously called an intracellular mature virus (IMV) due to its intracellular location. WV has a double bilayer envelope and is located either intracellularly or extracellularly. Based on location, WV was previously divided further into three forms: (1) intracellular enveloped virus (IEV), (2) extracellular enveloped virus (EEV) and (3) cell-associated extracellular enveloped virus (CEV) (Ward & Moss 2001). IMV is the most common type of VV and is released outside the cell during oncolysis, whereas CEV and EEV bud out from the intact host cell.

Infection starts when the virus enters the cell by fusion with the host cell membrane. This requires an entry-fusion complex, which is also necessary for cell-to-cell spread of EV and suggests that the outer envelope is lost before fusion. After entering the cytoplasm, the early stage of transcription begins, involving proteins associated with uncoating of the viral DNA, DNA replication and intermediate transactivation for transcription of intermediate mRNA. Early viral genes prevent host cell functions almost completely for 4-6 hours after infection, allowing effective transcription and viral replication, which begins 1-2 hours after infection. Intermediate mRNA encodes late transactivators and results in late mRNA synthesis and production of membrane and early transcription factor structural proteins, which are added to the new virion.

Development of the crescent-shaped membrane structure composed of lipid and viral proteins is the first step in VV morphogenesis. As the crescents surround core proteins and enlarge, they become spherical immature viruses (IMs), which contain the genomic DNA. An immature virus becomes an infectious MV when crescents join together and form a core and become brick-shaped after proteolytic processes and disulphide bond formation (Satheshkumar et al. 2013). MVs are wrapped into two more membranes and transported to cell surface where the outer membrane fuses with the plasma
membrane. Retained or reattached virus is called CEV, whereas released virus is EEV. The whole replication cycle takes approximately 12 hours. The replication cycle of VV is presented in Figure 1.

VV can infect multiple species, including humans, cattle, rodents, monkeys and horses (Kroon et al. 2011). In the laboratory setting, VV is commonly investigated in mice and rabbits (Kotwal & Abrahams 2004), but VV can also infect dog and cat cell lines (Gentschev et al. 2009, Gentschev et al. 2012, Adelfinger et al. 2014).

VV and other OPV infections induce both humoral and CD4+ and CD8+ T cell response (Xu et al. 2004, Smith et al. 2013). Clearance of VV infection in an acute infection is suggested to occur mainly via virus-specific antibodies (Xu et al. 2004). In the antibody-depleted and gene knock-out mouse model, CD4+ T cells and major histocompatibility complex (MHC) class II-mediated immunity were crucial for VV clearance, whereas CD8+ T cell response determined how severe the VV infection was and how long the antibodies protected against a new OPV infection (Xu et al. 2004).

**Figure 1 Replication cycle of vaccinia virus.** Modified from Moss (2006) and McCart and Barlett (2009). GAGs, glycosaminoglycans; EEV, extracellular enveloped virus; IMV, intracellular enveloped virus (also called mature virus, MV); IV, immature virus; CEV, cell-associated enveloped virus. Wrapped virus (WV) includes IEV, EEV and CEV.
While VV is more immunogenic than smallpox virus, like all poxviruses, VV also carries an arsenal of immune evasion functions (Smith et al. 2013). For example, VV expresses vaccinia complement control protein, which blocks complement activation. In addition, EEV envelope expresses host complement control proteins making EEV less vulnerable to complement inactivation than IMV. VV also inhibits production, function and signal transduction of interferon and pro-inflammatory cytokines such as tumour necrosis factor (TNF) and interleukin (IL) 1 and 18 (Smith et al. 2013). Further, VV can prevent chemokine expression or its action by producing chemokine-binding proteins and prevent apoptosis by producing anti-apoptotic Bcl-2-like proteins and inhibiting caspase-1 in apoptosis. VV also down-regulates MHC class I expression in infected cells to avoid cytotoxic T cells and modulates natural killer cell function.

VV is very stable in the environment; infectious VV was recovered from a scab collected from a smallpox vaccination site and saved at room temperature for 13 years (Wolff & Croon 1968). Also, infectious smallpox virus was found in the household of a vaccinated person 10 days after he had left the house (Lederman et al. 2009). In addition, VV can remain infectious for 4 months at +4°C in water (Essbauer et al. 2007), and infectious VV has also been recovered from faeces of mice after 20 days’ incubation at room temperature (Abrahao et al. 2009).

2.1.2 INFECTIONS IN HUMANS

Although vaccinia virus is not a naturally occurring virus, zoonotic vaccinia virus outbreaks have been reported in Brazil since 1999 (Kroon et al. 2011). In Brazil, the disease is called bovine vaccinia and it induces pox-like exanthemetic lesions in the teats of dairy cows. Dairy workers get the infection from direct contact with infectious lesions. In addition to cattle and humans, VV has been detected in rodents, monkeys and horses, however, clinical signs may not always be seen (Kroon et al. 2011).

Healthy people usually do not become infected with vaccinia virus, but serious complications may occur in an immunocompromised person (Kroon et al. 2011). The incubation period in humans is estimated to be 3-5 days. The first clinical signs include itchy, nodular lesions commonly seen on the hands. Nodules become papules surrounded by oedema and inflammation, and then usually 12 days after the appearance of signs, they form necrotic painful ulcers. Finally, the ulcer is covered by a scab, which sloughs off within 4 weeks of the beginning of the disease.

Strong inflammation reaction may result in lymphangitis and lymphadenopathy and systemic influenza-like symptoms, including fever, headache and myalgia (Kroon et al. 2011). Systemic signs usually last 2-5 days, occurring 3-20 days after onset of skin lesions.

VV transmission is not well characterized, but it has been suggested that mice and rats are reservoirs in nature (Kroon et al. 2011). They ingest or
aspirate infectious scabs, milk, secretions or contaminated fomites, and transfer the virus to humans, cattle and wild animals via excrement, direct contact or predation. VV is very stable in the environment and does not require direct contact for transmission. Instead, because of its stability, it is likely that VV spreads through fomites.

2.1.3 TREATMENT OF VACCINIA VIRUS INFECTIONS
The same drugs are effective for all OPV infections and are listed in Table 2 (Kroon et al. 2011). Cidofovir (Vistiside®) slows down replication of several DNA viruses by inhibiting viral DNA polymerase, and it is the only drug licensed against OPV infections. Vaccinia immunoglobulin (VIG) is the treatment of choice for severe VV infection (Cono et al. 2003), but its availability may be restricted.

ST-246 (tecovirimat) prevents formation of extracellular virus particles of OPV and was effective in relieving smallpox symptoms in non-human primates infected with variola virus (Mucker et al. 2013). In addition, it has proved safe in healthy human volunteers (Chinsangaram et al. 2012), and was used experimentally in one patient with progressive vaccinia infection in combination with VIG (Lederman et al. 2012). In Finland, ST-246 was successfully used to treat cowpox in one patient (personal communication, Paula Kinnunen, The Finnish Defence Forces 2012). Pharmacokinetics of ST-246 has also been evaluated in dogs (Amantana et al. 2013), so it could be used in pet dogs if necessary.

2.1.4 SMALLPOX VACCINATION
Wild-type VV has been given to hundreds of millions of people for active immunization against smallpox. The vaccine is given percutaneously with a bifurcated needle. A papule forms at the inoculation site 2-5 days after vaccination. It first forms a vesicle and then a pustule, which dries out and forms a scab. Healing takes 14-21 days, and a pitted scar is left behind (Cono et al. 2003).

The most common adverse event is flu-like symptoms. Serious adverse events comprise myocarditis, pericarditis, cardiomyopathy, generalized vaccinia, eczema vaccinatum, progressing vaccinia and post-vaccinial encephalitis (Cono et al. 2003). Serious but not life-threatening adverse events occur in 48–935 cases, life-threatening adverse events in 14–52 cases and lethal adverse events in 1.1–1.5 cases per million vaccines (Cono et al. 2003). Adverse events are more likely in persons who have cardiac disease, eye disease treated with corticosteroids, congenital or acquired immune deficiency disorders and history or presence of eczema or other skin conditions. In addition, infants aged < 12 months and pregnant women are predisposed to adverse events. Thus, smallpox vaccination is not recommended for these
Table 2  Compounds with antiviral activity against vaccinia virus infection. Reprinted from Antiviral Research, Vol. 92, EG Kroon, BEF Moyta, JS Abrahao, FG daFoseca and S Trindade, Zoonotic Brazilian Vaccinia virus: From field to therapy, pp. 150-163. Copyright 2011, with permission from Elsevier.

<table>
<thead>
<tr>
<th>Therapeutic agent</th>
<th>Mode of action</th>
<th>Clinical trial</th>
<th>Adverse event</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell-penetrating peptides (CPP)</td>
<td>Inhibitor of viral entry</td>
<td>Yes. Currently licensed for the treatment of cytomegalovirus retinitis (Vistidine™). CMX001 is regulated for smallpox under the “Animal Rule” efficacy</td>
<td>Resistant strains may emerge; Nephrotoxicity (can be alleviate with the use of probenecid). CMX001 is not toxic and is orally available</td>
</tr>
<tr>
<td>Cidofovir Nucleotide analogues / DNA replication inhibition</td>
<td>Yes. Currently licensed for the treatment of cytomegalovirus retinitis (Vistidine™). CMX001 is regulated for smallpox under the “Animal Rule” efficacy</td>
<td>Resistant strains may emerge; Nephrotoxicity (can be alleviate with the use of probenecid). CMX001 is not toxic and is orally available</td>
<td></td>
</tr>
<tr>
<td>STI-571 (Gleevec™) Inhibitors of virus release and spread</td>
<td>Not for poxvirus treatment. Licensed for the treatment of certain types of cancer</td>
<td>Inhibition of the physiological functions by epidermal growth factor receptor and Abl family kinases in non-infected cells</td>
<td></td>
</tr>
<tr>
<td>Gefitinib (Iressa™) Inhibitors of virus release and spread</td>
<td>Not for poxvirus treatment. Licensed in US and Europe for the treatment of patients with non-small cell lung cancer (NSCLC)</td>
<td>Inhibition of the physiological functions by epidermal growth factor receptor and Abl family kinases in non-infected cells</td>
<td></td>
</tr>
<tr>
<td>ST-246 Inhibitors of virus release and spread (acts on F13L ORF of VACV)</td>
<td>FDA phase II clinical trials against smallpox virus</td>
<td>Resistive strains may emerge</td>
<td></td>
</tr>
<tr>
<td>RNA interference (RNAi) Inhibition of protein expression</td>
<td>No</td>
<td>Not described</td>
<td></td>
</tr>
<tr>
<td>Imiquimod Immune Response Modifiers (IRMs)</td>
<td>Licensed for the treatment of genital warts caused by human papillomavirus. A few studies conducted for the treatment of poxvirus infections</td>
<td>Moderate/severe local inflammatory response</td>
<td></td>
</tr>
<tr>
<td>Ethnopharmacological compounds Unknown</td>
<td>No</td>
<td>Not tested in vivo</td>
<td></td>
</tr>
</tbody>
</table>

individuals. VV can potentially induce an infection in a healthy person (Kotwal & Abrahams 2004).

Smallpox vaccination was discontinued in Finland in 1980 (Finnish National Institute for Health and Welfare), and is not recommended in Europe since the risk of getting VV infection is minimal when correct laboratory working instructions are followed and the risk of adverse events from the vaccine outweighs the benefits (Kotwal & Abrahams 2004). However, in USA vaccination is recommended for people working with VV or in the military.
The vaccine should be re-administered every 3-10 years to maintain antibodies (Center for Disease Control and Prevention), implying that most Finns and other Europeans are not protected.

Wild-type VV usually spreads through direct contact with skin lesions and eyes, but does not pass through healthy skin (Kotwal & Abrahams 2004). Aerosol infections are unlikely (Cono et al. 2003). People in the smallpox vaccine risk group should not work with VV, although not all VV specialists think that pregnancy or mild skin disease prevents working with VV if safety directions are followed (Kotwal & Abrahams 2004).

2.1.5 VACCINIA-RABIES RECOMBINANT VACCINE

*Thymidine kinase (TK)*-deleted Copenhagen strain VV expressing rabies virus glycoprotein has been used in a vaccinia-rabies recombinant vaccine for oral immunization of wild foxes and raccoons in Europe and North-America (Brochier et al. 1996, Roess et al. 2012). Multiple studies have showed that the vaccine is safe in the target species and also in non-human primates (Brochier et al. 1996). The risk of spreading the virus into the environment is also minimal since the virus is not capable of horizontal transmission to other animals (Brochier et al. 1996). The potential risk would be recombination of the recombinant virus with a wild OPV if they infect and replicate in the same cell of the same host animal (Brochier et al. 1996).

More than 200 million oral rabies vaccine baits have been distributed into wildlife (Roess et al. 2012). To evaluate the risks of bait vaccines for humans and pets, a national surveillance was carried out in USA in 2001-2009 during which more than 80 million bait vaccines were distributed in 18 states (Roess et al. 2012). Six persons reported mild dermal symptoms, such as rash, itching, redness and swelling, after handling the rabies baits, and two individuals had more severe vaccinia infection (Rupprecht et al. 2001, Centers for Disease Control and Prevention (CDC) 2009). The first patient was on immunosuppressive medications for inflammatory bowel disease and she had fresh abrasions in her right arm and wrist that got contaminated with the liquid from the ruptured rabies bait vaccine (Centers for Disease Control and Prevention (CDC) 2009). Four days later, she developed poxlesion, erythema and oedema in the right hand. Due to underlying immunosuppressive disease and progression of the vaccinia infection, she received two doses of VIG and an investigational antiviral agent, ST-246. Four weeks after the incidence, her skin lesions had cleared and her inflammatory bowel disease was under control.

The second patient was a pregnant woman with epidermolytic hyperkeratosis (Rupprecht et al. 2001). Her dog bit her when she was removing a ruptured bait vaccine from the dog’s mouth. Three days after the incident, she noticed two blisters on her arm, which progressed to painful, erythematous, oedematous cellulitis and necrosis of the pustules. Necrotic areas were surgically removed, but she then developed generalized exfoliation.
Review of the literature

on her face and neck. One month after the dog bite, her lesions had healed and her pregnancy continued normally. Notably, the risk of getting VV infection from the rabies vaccines is very low since >200 million vaccinia baits have been distributed into nature (Roess et al. 2012). Adverse events reported in dogs eating vaccinia baits are mild and include lethargy, vomiting and diarrhoea (Roess et al. 2012).

The VV used in rabies vaccines is the Copenhagen strain and it has only a TK-deletion, compared with our virus, which has both TK- and VGF-deletions, making it more cancer-specific.

2.1.6 POXVIRUS INFECTIONS IN CATS

Poxvirus infections are not very common in cats, and only some 400 cases of Cowpox virus (CPXV) infections have been described in this species (Sandvik et al. 1998). The infection may actually be more common but may go unrecognized by veterinarians and owners. The symptoms consist of multiple skin lesions primarily seen on the head, oral cavity, neck, forelimb or paws, conjunctivitis and purulent ocular discharge. Rare systemic infections can be fatal in an immune-deficient cat or if co-infections occur or internal organs are infected. Infection is usually transmitted via hunting by infected rodents, which serve as a reservoir for CPXV (Pfeffer et al. 2002). At least one case report exists in which CPXV was transmitted from a cat to a human (Hawranek et al. 2003). Cat-to-cat transmission is probably rare (Bennett et al. 1989). In contrast to CPXV, Lister strain VV showed low infectivity in cats and VV infection seems to be self-limiting (Bennett et al. 1989).

2.1.7 VACCINIA VIRUS VECTORS IN CANCER THERAPY

VV has oncolytic properties, and thus, use of VV in cancer therapy is discussed in Section 2.3 Oncolytic viruses.

Local immunotherapy with genetically attenuated VV vector NYVAC or canarypox virus expressing human or feline IL2 was used in cats with fibrosarcoma to prevent tumour recurrence after surgery and iridium-based radiotherapy (Jourdier et al. 2003). The tumour recurrence during a one-year follow-up was 39% in cats receiving NYVAC expressing human IL2 and 28% in cats receiving canarypox virus expressing feline IL-2 immunotherapy in addition to conventional therapy, compared with 61% in cats without immunotherapy. In fact, canarypoxvirus expressing feline IL-2 is currently commercially available in Europe for the treatment of feline fibrosarcomas.

2.2 SEMLIKI FOREST VIRUS

Semliki Forest virus (SFV) is an enveloped, positive strand RNA virus belonging to Alphavirus genus and Togaviridae family. SFV was first isolated
from mosquitoes in the Semliki Forest of Western Uganda in 1942. Since then, multiple laboratory strains have been developed, the most common of which are presented in Table 3 (Griffin 2007).

SFV, like other alphaviruses, are transmitted by mosquitoes and can infect many vertebrate hosts. SFV is mainly found in Africa in mosquitoes, including Aedes africanus and aegypti, but is also present in parts of Asia and possibly Central and Southern Europe (Lundström 1999). As a neurotropic and neuroinvasive virus, SFV-induced encephalitis has been reported in humans, horses and monkeys, and experimentally, mice, rats, guinea pigs, rabbits and hamsters have been infected (Griffin 2007).

Table 3 Most common Semliki Forest virus laboratory strains.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Background</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>A7</td>
<td>Mozambique</td>
<td>Avirulent for an adult mouse, lethal encephalitis in suckling mice, resulting in death in 3-5 days</td>
</tr>
<tr>
<td>A7(74)</td>
<td>Mozambique</td>
<td>The least virulent strain derived from A7. Passaged through neonatal mouse brain. Lethal in mice infected ≤12 days</td>
</tr>
<tr>
<td>V12</td>
<td>Uganda, passed in mice in Bethesda</td>
<td>Lethal encephalitis in mice, but young guinea pigs and rabbits survive with or without clinical signs</td>
</tr>
<tr>
<td>V13</td>
<td>Uganda, passed in mice in Bethesda</td>
<td>Lethal encephalitis in mice, but young guinea pigs and rabbits survive with or without clinical signs</td>
</tr>
<tr>
<td>L10</td>
<td>Uganda, passed in mice in London</td>
<td>Most virulent strain: lethal in mice in 4 days, and young guinea pigs and rabbits may get clinical symptoms, while adults will not</td>
</tr>
<tr>
<td>m⁹</td>
<td>Uganda, passed in mice in London</td>
<td>Avirulent, derived from L10 by chemical mutagenesis</td>
</tr>
</tbody>
</table>

2.2.1 BIOLOGY OF SEMLIK FOREST VIRUS

SFV virions are icosahedral and the diameter is 60-70 nm. The genome is 11-12 kb in size and two-thirds of it codes for non-structural proteins at the 3'-end and one-third for non-structural proteins at the 5'-end (Quetglas et al. 2010).

Alphaviruses attach to cells via their E2 glycoprotein (Griffin 2007). There are probably multiple receptors that SFV uses, but the first one identified was the MHC class I molecule (Griffin 2007). However, SFV infection can occur without it. Virus enters the cell through endocytosis, and viral envelopes fuses with the endosome, which is induced by E1 glycoprotein and requires low pH (Quetglas et al. 2010). The nucleocapsid is broken and the viral RNA is released into the cytoplasm, where RNA replication takes place (Quetglas et al. 2010).

First, complementary minus-sense genomic RNA is produced in the first 3-4 hours after infection, leading to amplification of viral genome and capsid protein formation. Synthesis and glycosylation of envelope proteins take place in the endoplasmic reticulum, and glycosylation is completed in the Golgi complex. Viral DNA is packed into the capsid, which buds out from the cell.
plasma membrane with envelope proteins. Release of progeny virus typically occurs 4-6 hours after infection (Griffin 2007, Quétglas et al. 2010). The replication cycle of SFV is presented in Figure 2.

SFV infection induces both cellular and humoral immune response (Griffin 2007). SFV is sensitive to type I interferons (IFNs), which are part of the innate immunity against viral infection (Fragkoudis et al. 2007). IFN limits virus replication before specific immune response and is induced by inhibiting mRNA translation and later replication steps. Antiviral antibodies are usually detected in experimentally infected adult mice within 3-4 days of virus inoculation (Fazakerley et al. 1993). Virus-reactive lymphocytes appear in lymph nodes and circulation, and mononuclear cells in the site of infected tissue during the same time period in alphavirus infections (Griffin 2007).

SFV is not very stable and infectious titre decreased by half in 3 days at 22°C in water suspension in the dark (Benbough 1969), and the virus is inactivated in pH below 6 (White et al. 1981).

2.2.2 PATHOGENESIS IN VERTEBRATES

Pathogenesis of alphavirus in vertebrates is presented in Figure 3. SFV is transmitted into the vertebrate host via the bite of an infected mosquito. Virus-contaminated saliva is deposited extravascularly into the host. The virus titre is highest immediately after the mosquito is infected, and declines within 1-2 weeks, but the mosquito stays infected for the rest of its life (Griffin 2007).

The virus spreads from the subcutis to muscle, where it replicates and then travels into the circulation, or alternatively, infects Langerhans cells in the skin.
and then spreads to local lymph nodes and into the circulation (Griffin 2007, Ryman & Klimstra 2008). In mice, significant viraemia is detected 24 hours after intraperitoneal injection of SFV, but declines after 48 hours and disappears from the blood in 4 days (Fazakerley et al. 1993). SFV probably enters the central nervous system (CNS) across cerebrovascular endothelial cells, but may travel through the choroid plexus and olfactory nerves as well (Fazakerley 2002). In the CNS, SFV infects mainly neurons and oligodendrocytes and incidentally meningeal, edendymal or choroid plexus cells (Fazakerley 2002). It spreads in the CNS from one cell to another and through the cerebrospinal fluid.

In brains, virulent SFV strains, such as L10, induce panencephalitis, leading to death before the host immune system has time to stop the infection (Fazakerley et al. 1993). Lethality of avirulent strains A7 and A7(74) depends on the age of the mouse (Bradish et al. 1971, Fazakerley et al. 1993). In neonatal mice, infection is lethal, but if infection occurs when mice are 14 days or older, they will survive and infection will remain local in the perivascular areas. Since the CNS is still maturing in neonatal mice, the neurons undergoing axogenesis and synaptogenesis are still sensitive to avirulent SFV infection (Fazakerley 2002).

Neuronal death occurs in neonatal mice probably by apoptosis, whereas in older mice mature neurons are more resistant to infection (Fazakerley 2002). For example, A7(74) can replicate in mature neurons, but new virions cannot bud out from the cell (Fazakerley 2002). In an immunodeficient mouse, this can lead to persistent infection, but in an immunocompetent animal the host
immune system will clear out the virus. Infection of oligodendrocytes may result in demyelination (Griffin 2007).

Little is known about alphavirus infections in dogs. In an early study, SFV was given to 5- to 6-week-old healthy mongrel puppies via various routes, including intraperitoneal, intranasal, intradermal, intracardial and intracerebral (Reagan et al. 1953). Each group had two puppies, except for the intraperitoneal group, which had only one puppy. Intraperitoneal and intracranial infection resulted in neurological signs in the puppies 7-10 days after virus administration, after which the dogs were sacrificed. The brains were collected and suspended, and the suspension was given intracranially to mice, which showed neurological signs 3-4 days after infection.

2.2.3 INFECTIONS IN HUMANS

SFV infections are quite common in humans in Africa based on serological surveys, which suggest that approximately 50% of the African population have neutralizing antibodies (NABs) against SFV (Mathiot et al. 1990). Most of the infections are symptomatic or mild and may be mixed with malaria or influenza (Mathiot et al. 1990).

One publication describes an outbreak of SFV infection in the Central African Republic in 1987 in which SFV induced a febrile illness especially in European soldiers recently arrived from France (Mathiot et al. 1990). Clinical signs included fever, headache, myalgia and arthralgia. SFV was isolated in the sera of 22 patients. During the same time period SFV was isolated from mosquitoes. *Aedes africanus* was the most common mosquito species affected in the area, but several others were infected as well, including *Aedes*, *Anopheles*, *Conquilletidia*, *Culex*, *Eretmapodities* and *Manosonia* species. In addition, four patients were evaluated for SFV antibodies 6-7 months after the disease; two had titres of 128, and the other two 32 and 64.

Apart from this outbreak, only one publication of human illness was located in the literature. The publication describes a patient working in the laboratory with Osterrieth strain SFV (Willems et al. 1979). She had a one-year history of chronic purulent bronchitis, after which she developed severe headache and fever progressing to neurological signs, generalized seizure, coma and death. SFV-induced encephalitis was confirmed in autopsy.

Treatment of SFV infections consists of supportive therapy if the patient suffers from severe symptoms since no antiviral drugs against SFV exist (Krauss et al. 2003).

2.2.4 SEMLIKI FOREST VIRUS VECTORS IN CANCER THERAPY

SFV, like other alphaviruses, is an attractive virus as a gene therapy vector in addition to oncolytic effects of the virus (Quetglas et al. 2010). Like VV, SFV has a broad tropism and can infect and replicate efficiently in multiple cell types from various host origins, including insects, avian and mammalian
species. Also like VV, SFV replicates fast and produces high levels of new virions. In contrast to VV, SFV has relatively small RNA, but it can be relatively easily manipulated, and viral RNA is infectious (Quetglas et al. 2010). Cancer vaccines expressing tumour-associated antigens and genes inducing apoptosis, or immunotherapy with vectors expressing cytokines or inhibiting angiogenesis are under investigation. The downside of alphavirus vectors is that the gene expression is transient. In cancer therapy, however, this may not be a problem, as the aim is to destroy the tumour as quickly as possible (Quetglas et al. 2010).

2.3 ONCOLYTIC VIRUSES

Oncolytic viruses are cancer-specific viruses that kill cancer cells usually via immunogenic apoptosis, necrosis/necroptosis, pyroptosis or autophagy, where the cell is destroyed through the actions of lysosomes (Guo et al. 2014). In classic apoptosis, the cell membrane stays intact and antigens or danger signals are not released outside the cell, whereas in immunogenic apoptosis, necrosis and pyroptosis the cell membrane breaks down and cell content is released outside (Guo et al. 2014). Oncolytic viruses may have natural tropism for cancer cells, but commonly they have been genetically modified so that infection and replication take place only in cancer cells. Efficacy of oncolytic viruses can be increased by adding immunostimulatory genes to the viral genome. However, the immunotherapeutic effect is not only caused by transgenes, but also by the host’s antitumour immune response elicited when tumour cells die and release danger signals and tumour-associated antigens to dendritic cells (DCs) (Guo et al. 2014). The principles of oncolytic virus infection are shown in Figure 4.

The first approved oncolytic virus was recombinant human adenovirus type 5 (ONYX-015®) (Woller et al. 2014). It has been on the market in China since 2006 for the treatment of head and neck cancer. Various other oncolytic viruses that have been or are being evaluated in clinical trials in humans include vaccinia virus, other adenoviruses, herpesvirus, reovirus and vesicular stomatitis virus (National Cancer Institute 2014).

T-Vec (previously known as OncoVEXGM-CSF) is an attenuated herpes simplex virus 1 that expresses GM-CSF (Woller et al. 2014). The phase III trial did not improve overall survival for patients with advanced, unresectable melanoma, but a durable response rate was seen in 16% of patients relative to 2% of controls. Interestingly, 11% of the patients had a complete response. The most common adverse events included fatigue, chills and fever.

Reolysin is a reovirus that has been evaluated in phase II trials in patients with head and neck cancer, ovarian cancer and pancreatic cancer (Guo et al. 2014). Reolysin in combination with paclitaxel and carboplatin induced one complete and 6 partial responses in 17 patients with head and neck cancer, and
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Oncolytic virus replicates in tumour tissue (A) but not in normal tissue (B). DAMPs, damage-associated molecular pattern molecules; APCs, antigen-presenting cells.

thus, is currently being evaluated in a phase III trial (Guo et al. 2014).

In veterinary medicine, use of oncolytic adenovirus has been reported in tumour-bearing pet dogs (Laborda et al. 2014), and pet dogs with cancer are being recruited for a clinical trial with VV (Gentschev et al. 2013, Genelux Inc. 2013).

2.3.1 CHARACTERISTICS OF VACCINIA VIRUS AS AN ONCOLYTIC AGENT

VV has many features of a successful oncolytic virus (McCart et al. 2001, Guo et al. 2005, Kim et al. 2006, Kirn & Thorne 2009). VV is highly immunogenic and produces a strong cytotoxic T cell and antibody response. It also quickly destroys infected cells; the first infectious viruses are produced in 8 hours and infected cells die in 48-72 hours.

VV spreads efficiently through the blood to the body and has a broad host range, including humans and numerous mammals; however, Chinese hamster ovary cells are not affected (Mcfadden 2005). VV has natural tropism for cancer cells probably due to a leaky vasculature. In addition, vaccinia virus has
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a large genome, which can accommodate multiple foreign genes to make new recombinant viruses.

Finally, vaccinia virus does not integrate DNA into the host chromosome, and a variety of antiviral agents are available to treat the infection if necessary. Both of these features increase the safety of the virus.

Serious side effects are rare, and extensive experience with using the wild-type vaccinia virus as a live vaccine in the smallpox eradication programme for millions of patients has proven its safety in clinical settings (Kirn & Thorne 2009).

2.3.2 ONCOLYTIC DOUBLE-DELETED VACCINIA VIRUS (VVDD)

Oncolytic vaccinia virus can be genetically modified to improve tumour specificity (McCart et al. 2001). These manipulations have resulted in the development of a tumour-selective double-deleted vaccinia virus (vvdd), which is even safer than the wild-type vaccinia virus (McCart et al. 2001).

*TK* deletion renders the virus specific for cells highly active in the epidermal growth factor pathway (EGRF) since thymidine triphosphatase is not present in non-dividing cells (McCart et al. 2001). EGRF-ras is upregulated in many solid tumours, but not in hematopoietic cancers (Elsedawy & Russell 2013), and is also expressed in canine and feline tumours (Bergvist & Yool 2010).

During vaccinia infection vaccinia growth factor (VGF) stimulates the cells around infected cells to divide, allowing nucleotides for virus synthesis. Deletion of VGF results in selective replication in actively dividing cancer cells with an excess of nucleotides (McCart et al. 2001). In mouse models, vvdd was shown to be significantly more attenuated than single gene deleted viruses (McCart et al. 2001).

2.3.3 ONCOLYTIC VVDD EXPRESSING HUMAN CD40 LIGAND AND TDTOMATO (VVDD-HCD40L-TDTOMATO)

To further improve efficacy of vvdd, the virus has been armed with human CD40 ligand (hCD40L). Construction of vvdd-tdTomato and vvdd-hCD40L-tdTomato is presented in Figure 5. CD40L is a member of the TNF family. It enhances the antigen-specific T cell response by activating antigen-presenting cells (Bereta et al. 2004). Anti-proliferative, and pro-apoptotic effects of CD40L have also been reported in human CD40+ bladder, cervical and ovarian carcinoma cell lines (Vardouli et al. 2009). Apoptosis could be either caspase-8- and/or caspase-9-dependent in CD40+ cells (Ullenhag & Loskog 2012). Further, CD40L is an important danger signal in thrombocytes and is released when blood vessels are injured, resulting in release of inflammatory cytokines, which may allow local infiltration of immune cells into the tumour (Ullenhag & Loskog 2012, Westberg et al. 2013).

Intratumoural adenovirus gene therapy coding for hCD40L (AdCD40L) was recently used in the treatment of 19 dogs with malignant melanoma as an
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**Vvdd-tdTomato**

**Vvdd-hCD40L-tdTomato**

Figure 5  Schematic drawing of virus construct of vvdd-tdTomato and vvdd-hCD40L-tdTomato (with permission of Parviainen et al. 2014). ITR, inverted terminal repeats; TK*, tyrosine kinase open reading frame.

intratumoural injection (Westberg et al. 2013). As a proof of concept, complete response was seen in 5 and partial response in 8 dogs, including response shown in non-injected metastatic lesions. Infiltration of T and B lymphocytes was detected in the tumour tissues, confirming immune stimulation in 8 of 11 dogs.

AdCD40L was also used in the treatment of humans with bladder cancer, and it induced significant local immune stimulation with increased numbers of T cells and reduced numbers of tumour cells (Malmström et al. 2010). In the same study, AdCD40L treatment was associated with a decreased number of T regulatory cells (Tregs). An immunosuppressive environment is necessary for tumour immune evasion and key players include CD4+ Tregs and myeloid-derived suppressor cells, which either directly inhibit other immune cells or secrete immunosuppressive substances that prevent effector cell function or DC maturation (Ullenhag & Loskog 2012). In addition to decreased Treg counts, gene therapy with CD40L has promoted a myeloid cell population shift towards anti-tumoural M1 macrophages and granulocytic cells instead of immunosuppressive myeloid cells, thus reducing immunosuppression associated with the tumour (Liljenfeldt et al. 2014).

tdTomato is a red fluorescent protein used in imaging of gene expression, protein localization and protein-protein interactions in vivo (Singer et al. 2010). Virus-expressing tdTomato can be detected in infected cells, which helps in monitoring virus distribution, thus increasing the sensitivity of biosafety evaluation of the treatment agent. tdTomato imaging can also be useful in understanding the magnitude and persistence of anti-tumour effects in live animals.

Parviainen et al. (2014) have previously evaluated Western Reserve vvdd expressing tdTomato and human (vvdd-hCD40L-tdTomato) or murine (vvdd-mDC40L-tdTomato) CD40 ligand in cell cultures and murine models (Parviainen et al. 2014). Functional CD40L was expressed both in vitro and in tumours treated with the CD40L-expressing virus, but was not detected in the serum of the treated mice. Anti-tumoural efficacy was increased in a CD40-sensitive xenograft model and the presence of lymphocytes, B cells, DCs and macrophages suggests anti-tumoural immunity induced by CD40L.
2.3.4 ONCOLYTIC VACCINIA VIRUSES IN HUMAN MEDICINE

Three oncolytic vaccinia viruses are currently under investigation in clinical trials (National Cancer Institute 2014). JX-594 and GLONC-1 have TK deletion and JX-929 also has VGF deletion.

2.3.4.1 JX-594

The best-known oncolytic vaccinia virus in humans is JX-594, a TK-deleted Wyeth strain vaccinia virus expressing human GM-CSF and LacZ genes. Arming the virus with GM-CSF improves the efficacy of the virus by not only increasing the amount of granulocytes and macrophages produced from hematopoietic tissue but also recruiting them to the site of production, activating them and inducing anti-tumoural activity (Mastrangelo et al. 1999). In addition, GM-CSF stimulates proliferation and maturation of dendritic cells. LacZ expresses β-galactosidase, which is a functionally inactive but immunologically active protein, thus making detection of the virus possible by immunohistochemistry (Mastrangelo et al. 1999). It has also been used to assess immune response to the gene product (Mastrangelo et al. 1999).

JX-594 has been evaluated in patients with cutaneous (Mastrangelo et al. 1999) and metastatic melanoma (Hwang et al. 2011), refractory or metastatic liver cancer (Park et al. 2008, Heo et al. 2013) and metastatic solid tumours (Breitbach et al. 2011), and is currently being tested in other tumour types as well (National Cancer Institute 2014).

In the first published study, JX-594 was administered intralesionally twice weekly for 6 weeks for patients with cutaneous melanoma with a dose of 10^4 - 2 x 10^7 pfu/lesion, corresponding to 10^4 - 8 x 10^7 pfu/session (Mastrangelo et al. 1999). One patient with only cutaneous metastasis had a complete response, one patient had a partial response and three patients had mixed responses. Two of the patients failed to respond to treatment. Adverse events included mild flu-like symptoms resolving in 24 hours, and local inflammation with dermal pustule formation was also seen. Biopsies confirmed infiltration of CD4+ and CD8+ lymphocytes, histiocytes and eosinophils and all of the patients formed antibodies to vaccinia virus.

In a dose escalation study, JX-594 was given intratumourally at a dose of 10^8 - 3 x 10^9 pfu to patients with refractory primary or metastatic liver cancer every 3 weeks (Park et al. 2008). Both patients receiving the highest dose had grade III hyperbilirubinaemia, and thus, the maximum tolerated dose (MTD) was 10^9 pfu per person. All patients experienced mild to moderate flu-like symptoms, and a transient decrease in lymphocytes, platelets and red blood cells was seen in the first 3 days (Park et al. 2008). Neutrophilia associated with GM-CSF expression was detected in four of six patients with MTD. Treatment resulted in partial response in three patients, whereas six patients had stable disease and one patient progressive disease. Treatment response was not evaluated in four patients. Jx-594 genomes were detected in the blood.
immediately after virus injection in all patients and then again 3-22 days later in 12 of 14 patients, consistent with virus replication. Virus was also detected in the distant, non-injected tumour, suggesting viral spreading through the blood.

To evaluate the optimal effective dose of the virus, 30 liver cancer patients were randomized to two treatment groups receiving either low-dose (10^8 pfu) or high-dose (10^9 pfu) JX-594 intratumourally three times two weeks apart (Heo et al. 2013). Survival of the patients receiving the high dose was significantly better than in the low-dose group (11.1 vs. 6.7 months). Mild flu-like adverse events were monitored in all patients, and one patient suffered from severe vomiting and nausea requiring extended hospitalization. In addition, one patient had multiple skin pustules first noticed 4 days after the treatment, but which had disappeared within 6 weeks. Lymphopenia was reported in 4 of 30 patients and decreased haemoglobin in 3 of 30 liver cancer patients treated with JX-594. In addition, 9 of 14 patients had neutrophilia. Genomes of JX-594 were again detected in the blood, and in three patients they remained for up to 15-36 days.

Hwang et al. (2011) further evaluated intralesional administration of low-dose JX-594 given once weekly over 6 weeks to patients with metastatic melanoma, and demonstrated viral replication, tumour necrosis, perivascular lymphocyte infiltration and expression of both transgenes in tumour biopsies. In addition, Breitbach et al. (2011) showed that intravenous administration of JX-594 also resulted in cancer-specific infection and replication of the virus in the biopsies obtained 8-10 days after infusion of JX-594. The virus dose in that study was 10^5/kg up to 10^9 pfu per person.

In the both studies, side-effects associated with virus administration were mild and consisted mainly of flu-like symptoms, including fever, chills, fatigue, headache, nausea, vomiting, anorexia and myalgia. However, three patients receiving the virus intravenously or in the liver developed pustules (Breitbach et al. 2011, Heo et al. 2013). Infectious JX-594 was not detected in urine or throat swabs from 14 patients (Park et al. 2008), but throat swabs from some patients revealed virus (EudraCT number 2009-011121-13).

Due to the promising results, multiple clinical trials have been started in colorectal cancer, paediatric solid tumours and liver cancer (National Cancer Institute 2014).

2.3.4.2 JX-929

JX-929 (vvdd-CDSR) is a modification of JX-594, but it is a more potent Western Reserve strain and has both TK and VGF deletions (Zeh et al. 2015). As a transgene, JX-929 has cytosine deaminase (CD) and somatostatin receptor (SR) genes. CD is an enzyme that activates non-toxic prodrug 5-fluorocytosine to cytotoxic drug 5-fluorouracil in infected cancer cells, where replication of the virus takes place. SR is used to image the virus by octreotide scintigraphy.
In the phase I dose-escalating study, the virus was given intratumourally to patients with solid tumours (Zeh et al. 2015). The results showed that the virus infects and destroys cancer cells and triggers anti-tumour immunity. The maximum tolerated dose was not reached in 16 patients recruited to the trial, and the maximum feasible dose was determined to be $3 \times 10^9$ pfu.

The most common adverse events included fever and/or chills within 24 hours of virus injection. Fifteen of 17 patients had fever, malaise and/or pain 5-15 days after virus administration associated with an inflammatory reaction to the virus. One patient suffered grade 3 pain at the tumour site that was thought to be associated with virus-induced inflammation; it resolved in 2 days with narcotic pain medications.

In laboratory tests, mild elevations in liver values were detected in addition to increases in lactate dehydrogenase, which may have indicated tumour necrosis. Haematology showed a relative lymphopenia compared with baseline values, but no other obvious changes.

All patients had a significant increase in VV neutralizing antibodies. Viral genomes were detected in the blood of nine patients, all of whom received $\geq 10^8$ pfu. Viral genomes re-emerged into the circulation in only two patients at later time-points. Seven patients had their tumours biopsied 8 days after virus injection, and infectious virus was recovered in four patients. In addition, one VV genome was detected in one biopsy. In two patients, virus was also recovered from non-injected lesions, suggesting systemic spreading of the virus. Saliva and urine were collected to determine shedding of the virus, but none of the samples was positive for infectious virus or VV genomes.

### 2.3.4.3 GL-ONC1

A third oncolytic vaccinia virus in clinical trials is GL-ONC1, which is a TK-attenuated Lister strain vaccinia virus expressing green fluorescent protein (GFP), beta-galactosidase (LacZ) and beta-glucuronidase (GusA) (Jaime et al. 2012).

In a phase I trial in which the virus was given intravenously to patients with solid tumours, nine patients had stable disease for 8-48 weeks, but no complete or partial responses were seen. Dose-limiting toxicity was grade 3 in aspartate transaminase in one of six patients receiving $10^9$ pfu. Other adverse events in 27 patients were mild flu-like symptoms, as with JX-594 and JX-929, including fever, fatigue, musculoskeletal pain, nausea and vomiting. In addition, virus-associated skin rash was seen in two patients; GFP expression was detected in the skin and infectious virus was isolated from the lesions.

Infectious virus was also measured from blood, urine, faeces and sputum, and one patient had positive results for 11 days. Antibody titre increased in all except one patient. Researchers were also able to show virus in one tumour biopsy by immunohistochemistry, confirming that the virus was able to reach the tumour after intravenous infusion.
Clinical trials are currently ongoing in head and neck cancer, pleural cavity cancers and peritoneal carcinomatosis (Genelux Inc. 2013, National Cancer Institute 2014).

2.3.5 ONCOLYTIC SEMLIKI FOREST VIRUS

Oncolytic SFV has only been evaluated in preclinical trials. Replication-deficient SFV expressing LacZ was first reported to infect and kill seven human prostatic cancer cell lines and human prostatic tissue explants (Hardy et al. 2000). The infected cells detached from the culture surfaces and the basement membrane, and approximately half of the cells died 24-72 hours after infection.

Replication-competent vector VA7-EGFP from an avirulent A7(74) strain expressing enhanced green fluorescent protein was shown to infect and kill multiple tumour cell lines, including rat gliosarcoma and mouse neuroblastoma (Vähä-Koskela et al. 2003) and human melanoma, lung carcinoma and colon adenocarcinoma in vitro (Vähä-Koskela et al. 2006). Oncolytic capacity was also detected in human melanoma xenografts in severe combined immunodeficiency (SCID) mice with a single virus injection of $10^6$ pfu intraperitoneally, intratumourally and intravenously (Vähä-Koskela et al. 2006). However, some cancer cells were resistant to the virus, and after an initial response new tumour nodules were formed.

Efficacy of A7(74) and VA7-EGFP was evaluated in a subcutaneous A549 human lung adenocarcinoma model in immunodeficient nude mice, and a rat BT4C glioma model in immunocompetent BDIX rats (Määtta et al. 2007). Intratumoural injection of SFV induced very strong antitumoural response, whereas efficacy decreased when virus was given intravenously. When viruses were given intraperitoneally, no response was seen. The authors concluded that the difference in antitumoural efficacy between different routes was at least partly due to a strong type I IFN response. In the rat glioma model, an initial antitumoural response was also seen after intratumoural virus injection, but the tumours then started to regrow. Loss of antitumoural efficacy may have been due to NAbs.

VA7-EGFP also induced oncolysis in several osteosarcoma cell lines, and a survival benefit was seen in an orthotopic osteosarcoma nude mouse model after intratumoural virus administration (Ketola et al. 2008). However, none of the mice was cured, and the authors suggested arming the virus with transgenes to improve efficacy. In addition, VA7-EGFP was assessed in an orthotopic lung cancer tumour model in immunodeficient nude mice (Määttä et al. 2008). A significant improvement was observed in survival time when the virus was delivered intratumourally, but this was not the case after systemic delivery. This raised speculation about whether innate immune defence limits infectious capacity of the virus.

Further, VA7 vectors proved effective in killing several human glioma cell lines and resulted in a good antitumoural response in both subcutaneous and
orthopaedic human glioma xenografts in nude mice after intravenous administration (Heikkila et al. 2010).

Later, it was reported that oncolytic efficacy of VA7 is prevented by IFN-β (Ruotsalainen et al. 2012). VA7 was able to infect and kill mouse glioma cells GL261 and CT-2A in vitro, but when confluent, GL261 was resistant to infection. If cells were treated with mouse IFN-β before infection with SFV, the virus was unable to replicate in the cells. Neither of the SFV vectors was able to infect GL261 and CT-2A tumours in an orthotopic xenograft nude mouse model. Pre-treatment of the mice with immunosuppressive cyclophosphamide or rapamycin did not improve their survival, although serum-neutralizing antibodies were significantly decreased. Thus, tumours that have mutations preventing type I IFN production or response appear to be sensitive to SFV vectors, while cells maintaining type I IFN response are resistant.

2.3.6 ONCOLYTIC VIRUSES IN VETERINARY MEDICINE

Multiple oncolytic viruses, such as adenovirus (Hemminki et al. 2003, Ternovoi et al. 2005, Le et al. 2006, Alcayaga-Miranda et al. 2010, Laborda et al. 2014), VV (Gentschev et al. 2009, Gentschev et al. 2010, Gentschev et al. 2012, Patil et al. 2012), myxoma virus (Urbasic et al. 2012), canine distemper virus (Suter et al. 2005) and parvovirus (Nyky et al. 2010), have been studied in canine cell lines and tumour xenografts. In addition, some of them, e.g. vesicular stomatitis virus (VSV) (LeBlanc et al. 2013) and VV (Gentschev et al. 2013), have been or are currently being evaluated in clinical studies in dogs suffering from cancer. In cats, only two publications on oncolytic viruses are available (MacNeill et al. 2012, Adelfinger et al. 2014).

2.3.6.1 Oncolytic vaccinia virus in preclinical studies in dogs

An unarmed oncolytic vaccinia virus from a Lister strain containing inactivated $TK$, GLV-1h68, has presented oncolytic efficacy in canine mammary adenoma and carcinoma cell lines and xenografts in nude mice (Gentschev et al. 2009, Gentschev et al. 2010). GLV-1h109, a derivate of GLV-1h68 armed with an anti-VEGF (vascular endothelial growth factor) single-chain antibody, also showed oncolytic potency against canine soft tissue sarcoma and prostatic carcinoma in an in vivo model and induced intense intratumoural infiltration of host immune cells in tumour-bearing mice (Gentschev et al. 2012, Patil et al. 2012). However, an unarmed less virulent Lister strain virus, LIVP1.1.1, showed even better oncolytic efficacy against canine soft tissue sarcoma in preclinical studies (Gentschev et al. 2012), thus resulting in isolation of LIVP6.1.1 (Gentschev et al. 2013).

LIVP6.1.1 is a wild-type Lister strain vaccinia virus with no genetic manipulations, but $TK$ is naturally inactivated and the virus has different
mutations than GLV-1h68 (Gentschev et al. 2013). LIVP6.1.1 is able to kill canine soft tissue sarcoma, melanoma, osteosarcoma and prostatic sarcoma cell lines, and efficacy has been shown in soft tissue sarcoma and prostatic carcinoma xenografts as well (Gentschev et al. 2013). Due to promising results in preclinical trials, LIVP6.1.1, also called V-VET1, is currently being evaluated in clinical trials in pet dogs with naturally occurring cancer (Gentschev et al. 2013, Genelux Inc. 2013).

2.3.6.2 Oncolytic adenovirus in dogs

Adenoviruses are the best-evaluated oncolytic viruses in dogs. A conditionally replicating canine adenovirus type 2, OC-CAVE1, was reported to infect, replicate in and kill canine cancer cells in osteosarcoma laboratory cell line and tumour biopsy obtained from a naturally occurring canine osteosarcoma (Hemminki et al. 2003). In addition, OC-CAVE1 had antitumoural efficacy against canine osteosarcoma xenograft in a nude mouse model. Also human adenovirus type 5 was able to infect and replicate in canine mesenchymal and epithelial cancer cell lines, including osteosarcoma, mammary carcinoma and melanoma cells (Ternovoi et al. 2005).

Further, efficacy of canine adenovirus type 2- and 5-based oncolytic viruses was reported in canine osteosarcoma and melanoma in other preclinical studies (Le et al. 2006, Alcayaga-Miranda et al. 2010, Laborda et al. 2014). In order to test the viruses in pet dogs, initial toxicity of osteosarcoma-targeted canine adenovirus (CAV)-2 was evaluated in six healthy laboratory dogs (Smith et al. 2006). Only mild adverse events, such as diarrhoea, were detected. In haematology, neutrophil count decreased in all dogs; 3 dogs had grade 1 and 1 dog grade 4 neutropenia. Lymphocytes increased in 5 of the 6 dogs, and in 3 dogs the values were above the reference range. The dogs were euthanized 4 days after the virus administration; three dogs already had increased serum NAbs. Viral genomes were found in the blood until euthanasia, and low numbers of genomes were detected in some faecal and urine samples. Liver and spleen had the highest viral copy numbers, but low numbers were also detected in lung, lymph node, bone marrow and muscle, suggesting that the virus can spread effectively in the body and infect multiple tissues.

Recently, pRb-responsive, RGD-modified and hyaluronidase-armed canine oncolytic adenovirus was administered intratumourally to six pet dogs (Laborda et al. 2014). Two dogs had appendicular osteosarcoma, and other tumours included sweat gland adenoma, mast cell tumour, fibrosarcoma and neuroendocrine hepatic carcinoma. Sweat gland adenoma and mast cell tumour showed partial response, but progressed again 11 months after virus treatment. Fibrosarcoma was surgically removed 15 days after virus treatment and the dog stayed tumour-free for at least 8 months. Although no evidence of virus-associated adverse events were seen, one dog died due to disseminated intravascular coagulation following tumour lysis and one dog had a self-
inflicted wound that could have been related to tumour necrosis induced by the virus. All dogs, except one, had increased CAV-2 antibodies. The dog with the highest baseline CAV-2 titres had evidence of viral replication and necrosis in both inoculated and non-inoculated tumours, suggesting that high antibody titres may not prevent oncolytic potency of the virus. qPCR revealed adenovirus genomes in the blood soon after virus inoculation, but these decreased over time. The last positive sample was collected one week after virus treatment.

2.3.6.3 Vesicular stomatitis virus in dogs

A genetically modified VSV expressing IFN-β and NIS-protein for nuclear imaging, VSV-IFNβ-NIS, was also evaluated in a dose escalation study in five healthy Beagle dogs (LeBlanc et al. 2013). The highest dose induced severe liver toxicity and shock, leading to euthanasia of the dog. Other adverse events included fever, nausea, lymphopenia and oral lesions. Oncolytic VSV is currently undergoing a phase 1 trial in human liver cancer patients (National Cancer Institute 2014).

2.3.6.4 Other oncolytic viruses in dogs and cats

Other oncolytic viruses that have been studied in canine cancers include reovirus (Hwang et al. 2013, Hwang et al. 2014, Igase et al. 2015), myxoma virus (Urbasic et al. 2012), canine distemper (Suter et al. 2005) and parvovirus (Nykky et al. 2010).

Canine mast cell tumour cell lines were highly sensitive to reovirus-induced oncolysis (Hwang et al. 2013), but this was detected in only 4 of 10 canine lymphoma cell lines (Hwang et al. 2014). Tumour regression was shown both in the mast cell tumour and lymphoma xenograft mouse model and in primary canine mast cell tumour biopsies in vitro (Hwang et al. 2013, Hwang et al. 2014). Recently, oncolytic potency of reovirus was also demonstrated in canine mammary gland tumor, osteosarcoma and malignant melanoma cell lines (Igase et al. 2015).

Myxoma virus was able to kill multiple canine cancer cell lines grown from surgical biopsies (Urbasic et al. 2012), whereas distemper virus, evaluated in cervical tumour-derived cell lines and in T and B cell lymphoma biopsies, induced apoptosis (Suter et al. 2005). In addition, parvovirus was also able to induce apoptosis in canine fibroma cells (Nykky et al. 2010).

One publication has reported the use of myxoma virus in two feline carcinoma cell lines (MacNeill et al. 2012). Myxoma virus is a rabbit-specific pathogen that is non-pathogenic in cats and other vertebrates (Bull & Dickinson 1937), and thus, is a potential candidate as an oncolytic agent in cats. The tested myxoma virus was able to transduce and induce apoptosis in feline squamous cell carcinoma SCCF1 cells and feline mammary carcinoma.
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STUDE cells 48 hours after infection (MacNeill et al. 2012). Although SCCF1 was less sensitive for viral oncolysis than the other feline carcinoma cell line STUDE, isolated from mammary tumour, myxoma virus was able to enter and induce early protein expression in SCCF1 cells. It also induced a cytopathic effect (CPE) in SCCF1 cells, but CPE was not seen until 24 hours after infection compared with STUDE cells, where CPE was detected already 12 hours post-infection (p.i.). The virus was able to produce infectious virions in both cell lines, although the amount of virions was less than with a fully-permissive cell line.

Recently, a new recombinant oncolytic VV strain, GLV-5b451, was investigated in feline mammary carcinoma cell line DT09/06 isolated from a patient cat (Adelfinger et al. 2014). The virus was derived from LIVP 6.1.1 and expresses the anti-VEGF single-chain antibody GLAF-2 against feline mammary carcinoma. glaf-2 gene was inserted into the J2R thymidine kinase coding locus, and it was under the control of the VV synthetic early-late promoter. The virus efficiently killed feline mammary carcinoma cells and significantly regressed tumour growth in a nude mouse xenograft model. The virus also induced VEGF-antibody synthesis, which caused a reduction of VEGF in the tumours and inhibited angiogenesis.

2.3.7 SAFETY OF USING ONCOLYTIC VACCINIA AND SEMLIKI FOREST VIRUS IN VETERINARY MEDICINE

Safety of humans is an important consideration when treating pets with potential human pathogens. Vvdd is cancer-specific so it should not replicate in normal tissues or cause disease in healthy individuals, but smallpox vaccine, which is not cancer-specific, can cause adverse events in immunosuppressed people including cancer patients. Oncolytic VV has only been evaluated in cancer patients, and thus, the possibility of adverse events, especially in other immunosuppressed individuals, should not be ignored. In addition, VV is very stable in organic material, unlike SFV.

SFV infection is usually mild in humans and transferred by mosquitoes living in tropical areas (Mathiot et al. 1990), so outside tropical regions SFV infection is not a concern, although aerosol infection is possible (Krauss et al. 2003). In epidemic areas, a mosquito bite during viraemia could result in SFV transmission.

Saliva from VV-treated dogs may pose a risk via shedding and licking, especially for immunosuppressed individuals with skin lesions. The risk of complications should be low, as vvdd is much less pathogenic than the wild-type VV used in smallpox vaccination. In addition, virus expressed tdTomato and β-galactosidase can be used for monitoring of excretions and possible pox lesions.

Since pet dogs urinate and defecate outside and it is difficult to completely collect the urine, urine from virus-treated dogs may pose an environmental
hazard. The risk is minimal for faeces, which can be easily collected and disposed after inactivation with disinfectants, heat or UV light.

In humans, infectious VV was detected in urine, faeces and sputum in one of 27 patients receiving oncolytic VV (Jaime et al. 2012), but was not detected in urine or throat swabs from 14 patients treated with other oncolytic VV (Park et al. 2008) or in urine or saliva of 16 patients receiving vvdd (Zeh et al. 2015). However, infectious oncolytic VV has been detected in throat swabs (EudraCt 2011-000051-16), and thus, infectious VV could also be found in canine secretions and possible spreading of the virus via secretions should not be neglected.

In theory, recombination of the virus in the environment is possible if the cell is co-infected with vvdd and another orthopoxvirus at the same time, although double-strand DNA viruses are usually stable (Brochier et al. 1996). Since the vvdd-tdTomato-hCD40L virus does not contain any genes increasing pathogenicity and transgenes make it more immunogenic, the new virus would be less likely to induce infection than wild-type orthopoxviruses.

The biggest risk for VV spreading would be a superficial ulcerated tumour or a skin lesion since pox viruses usually spread with direct contact through skin lesions and eyes. VV does not pass healthy skin (Kotwal & Abrahams 2004) and aerosol infection is unlikely (Cono et al. 2003). Pox-like skin lesions have been reported in a few human cancer patients receiving oncolytic VV (Breitbach et al. 2011, Heo et al. 2013), and classic vaccinia necrosum was reported in VV-injected skin lesions in cancer patients receiving vvdd (Zeh et al. 2015). Skin lesions were also reported in laboratory Beagles receiving an intradermal inoculation of TK-deleted VV (Appel & Paoletti 1988). The dogs were in close contact with dogs not receiving the virus, and none of the latter group had induction of antibodies or skin lesions, suggesting that the virus does not spread from inoculated dogs to non-inoculated dogs.
3 AIMS OF THE STUDY

The primary goal of this study was to evaluate preclinically whether genetically modified double-deleted oncolytic vaccinia virus (vvdd) and Semliki Forest virus (SFV) could be used in the treatment of canine and feline cancers.

Specific aims of the study were as follows:

1. To test the effect of vvdd and vvdd-expressing human CD40 ligand on dog cancer cell lines and tumour tissues and to determine the efficacy of vvdd in nude mouse models with tumour xenografts (I).

2. To test the effect of vvdd and vvdd-expressing human CD40 ligand on feline squamous cell carcinoma (SCCF1) cell lines and to evaluate production of oncolytic vaccinia virus in them (II).

3. To determine the safety and biodistribution of vvdd-expressing human CD40 ligand in healthy dogs (III).

4. To test the effect of attenuated SFV on dog cancer cell lines and to determine the safety and biodistribution of attenuated SFV in healthy dogs (IV).
4 MATERIALS AND METHODS

4.1 CELL LINES

Two canine osteosarcoma cell lines (Abrams and D17), one prostatic carcinoma cell line (ACE-1) and one feline squamous cell carcinoma cell line (SCCF1) were used in the study. In addition to cancer cell lines, the Madin-Darby canine kidney (MDCK) cell line served as a control for normal cells.

Abrams was kindly provided by Dr. D. Vail (University of Wisconsin-Madison, WI, USA), D17 by Dr. R. Alemany (Catalan Institute of Oncology, Barcelona, Spain), ACE-1 and SCCF1 by Dr. T. Rosol (Ohio State University, Columbus, OH, USA), and MDCK by Dr. H. Sariola (University of Helsinki, Finland).

Other cell lines used included Vero cells originating from African green monkey kidney epithelium, U2OS originating from a human osteosarcoma and BHK-21 originating from baby hamster kidney cell line, all received from the American Type Culture Collection (Manassas, VA, USA). In addition, A549 cells originating from human lung adenocarcinoma epithelium were obtained from the National Cancer Institute (Bethesda, MD, USA) and murine melanoma cell line B16-OVA was kindly donated by Dr. R. Vile (Mayo Clinic, Rochester, MN, USA).

All cells were cultured in 5% CO2 at 37°C in recommended growth media purchased from Lonza (Verviers, Belgium) (I-III) or Sigma Aldrich (St. Louis, MO, USA) (IV) and supplemented with 10% foetal calf serum (FCS), 1% L-glutamine and 1% penicillin-streptomycin (P-S), unless otherwise specified. Cells for SFV experiments had only 5% FCS and were complemented with 25 mM HEPES. Cell lines are summarized in Table 4.

4.2 VIRUSES

The VVs used were all cancer-specific vvdd of the Western Reserve strain and have total deletion of the VGF gene and partial deletion of the TK gene. All viruses also had insertion of the LacZ gene in the VGF site coding the inactive but immunogenic beta-galactosidase enzyme. Furthermore, vvdd-luc had insertion of luciferase, vvdd-tdTomato tdTomato and vvdd-CD40L-tdTomato human CD40L and tdTomato genes. The viruses were engineered as previously described (Guse et al. 2010, Parviainen et al. 2014).

Vvdd-luc was grown on Vero cells, and vvdd-tdTomato and vvdd-hCD40L-tdTomato on A549 cells. Viruses were purified using sucrose gradient centrifugation, and plaque assay was used to measure virus titres (Earl et al. 1998). Titring of vvdd-luc was performed on Vero cells, and titring of vvdd-tdTomato and vvdd-CD40L-tdTomato on A549 cells.
Materials and methods

Table 4  Cell lines used in Studies I-IV.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Origin</th>
<th>Growth media</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abrams</td>
<td>Canine osteosarcoma</td>
<td>DMEM</td>
<td>I, IV</td>
</tr>
<tr>
<td>ACE-1</td>
<td>Canine prostatic carcinoma</td>
<td>F12-K</td>
<td>I</td>
</tr>
<tr>
<td>D17</td>
<td>Canine osteosarcoma</td>
<td>DMEM</td>
<td>I, IV</td>
</tr>
<tr>
<td>MDCK</td>
<td>Madin-Darby canine kidney cell line</td>
<td>EMEM</td>
<td>I</td>
</tr>
<tr>
<td>SCCF1</td>
<td>Feline squamous cell carcinoma</td>
<td>DMEM with 4.5 g/l glucose</td>
<td>II</td>
</tr>
<tr>
<td>A549</td>
<td>Human lung carcinoma</td>
<td>DMEM</td>
<td>I, II, III, IV</td>
</tr>
<tr>
<td>U2OS</td>
<td>Human osteosarcoma</td>
<td>DMEM</td>
<td>I, II, III, IV</td>
</tr>
<tr>
<td>Vero</td>
<td>African green monkey kidney epithelium</td>
<td>DMEM with 4.5 g/l glucose, DMEM with 1 g/l glucose</td>
<td>I, II, III, IV</td>
</tr>
<tr>
<td>B16-OVA</td>
<td>Murine melanoma cell line expressing ovalbumin</td>
<td>DMEM</td>
<td>II</td>
</tr>
<tr>
<td>BHK-21</td>
<td>Baby hamster kidney cells</td>
<td>DMEM</td>
<td>IV</td>
</tr>
</tbody>
</table>

DMEM, Dulbecco’s Modified Eagle Medium growth media
EMEM, Eagle’s minimal essential medium
F-12K, Kaighn’s Modification of Ham’s F-12 Medium

Table 5  Table of viruses used in Studies I-IV.

<table>
<thead>
<tr>
<th>Virus name</th>
<th>Virus</th>
<th>Transgene</th>
<th>Study</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vvdd-luc</td>
<td>TK- and VGF-deleted Western Reserve vaccinia virus</td>
<td>Luciferase</td>
<td>I</td>
<td>Guse et al. 2010</td>
</tr>
<tr>
<td>Vvdd-tdTomato</td>
<td>TK- and VGF-deleted Western Reserve vaccinia virus</td>
<td>tdTomato</td>
<td>I, II, III</td>
<td>Parviainen et al. 2014</td>
</tr>
<tr>
<td>Vvdd-hCD40L-tdTomato</td>
<td>TK- and VGF-deleted Western Reserve vaccinia virus</td>
<td>human CD40L, tdTomato</td>
<td>I, II, III</td>
<td>Parviainen et al. 2014</td>
</tr>
<tr>
<td>Ad5/3-hTERT-CD40L</td>
<td>Serotype 5 adenovirus with knob replacement from serotype 3 adenovirus</td>
<td>Human telomerase reverse transcriptase and CD40L</td>
<td>II</td>
<td>Diaconu et al. 2012</td>
</tr>
<tr>
<td>Ad5/3-hTERT</td>
<td>Serotype 5 adenovirus with knob replacement from serotype 3 adenovirus</td>
<td>Human telomerase reverse transcriptase</td>
<td>II</td>
<td>Diaconu et al. 2012</td>
</tr>
<tr>
<td>A7(74)</td>
<td>Attenuated Semliki Forest virus</td>
<td></td>
<td>IV</td>
<td>Bradish et al. 1971</td>
</tr>
<tr>
<td>VA7-EGFP</td>
<td>Attenuated Semliki Forest virus</td>
<td>Enhanced green fluorescent protein</td>
<td>IV</td>
<td>Vähä-Koskela et al. 2003</td>
</tr>
</tbody>
</table>

Ad5/3-hTERT-CD40L and Ad5/3-hTERT were engineered and produced according to a previous description (Diaconu et al. 2012). The VV for the dog study was produced in GMP-quality A549 cells, and infectious titre was measured by median tissue culture infective dose (TCID50) in A549 cells (Hemminki et al. 2011).
The replication-competent, attenuated A7(74) strain of SFV was kindly provided by Dr. R. E. Shope (Yale Arbovirus Research Unit, Yale School of Medicine, New Haven, CT, USA) and generated as described elsewhere (Bradish et al. 1971). The replication-competent, attenuated VA7-EGFP expressing enhanced green fluorescent protein used in in vitro studies has also been described previously, as has generation of the infectious cDNA clone and expression vector (Vähä-Koskela et al. 2003). Viruses were amplified in baby hamster kidney BHK-21 cells, and infectious titre was measured by plaque titration (Ruotsalainen et al. 2014). A list of the viruses used in Studies I-IV is presented in Table 5.

Inactivation of vvdd-luc was performed with ultraviolet light following a previously described protocol (II) (Tsung et al. 1996). The virus was first incubated at room temperature for 10 min in 10 μg/ml psoralen in Hanks balanced solution with 0.1% FCS and then irradiated for 3 min in a CL-100 UV cross-linker (UVP, Cambridge, UK) with 365 nm ultraviolet A light.

4.3 IN VITRO EXPERIMENTS

4.3.1 TRANSDUCTION ANALYSIS (I, II)
Transduction of vvdd-luc was performed by plating 100 000 cells per well in a 24-well plate in growth medium (GM) with 5% FCS. After a 48-h incubation, the cells were washed once with 2% FCS GM and infected in triplicate with different concentrations of virus in 2% FCS GM for 30 min. Infected cells were then washed once with 10% FCS GM and incubated for 4 h. Finally, cells were lysed and luciferase activity was measured according to the manufacturer’s instructions (Luciferase Assay System, Promega, Madison, WI, USA). Transduction of viruses expressing tdTomato was performed similarly, except that cells were incubated for 24 h and fluorescence of tdTomato was monitored by fluorescence microscope (Olympus 1X71 inverted microscope, Olympus, Tokyo, Japan) at 8, 12 and 24 h p.i.

4.3.2 CYTOTOXICITY ASSAYS (I, II, IV)
Cell viability after vvdd-tdTomato and vvdd-hCD40L-tdTomato viral infection was measured using a colorimetric cell lysis test (MTS). Ten thousand cells per well were plated on 96-well plates in 5% FCS GM and incubated for 48 h, after which wells were infected in triplicate with different concentrations of virus for one hour in 2% FCS GM. After that, 5% FCS GM was added to the wells, and cells were incubated until cell viability was measured by MTS according to the manufacturer’s instructions (Cell Titer 96 AQueous One Solution Proliferation Assay, Promega). Inactivated vaccinia virus was used as a non-replicating control. Inactivation of the virus was performed as described elsewhere (Guse et al. 2010).
For infection with SFV (IV), twenty-five thousand cells per well were plated on 48-well plates. The next day, the cells were infected in triplicate with VA7-EGFP at 1, 0.1 and 0.01 multiplicity of infection (MOI). Virus infection was followed by fluorescence of EGFP, and cytopathic effect was monitored by using a phase contrast microscope (Zeiss Axio Observer Z1, Zeiss, Oberkochen, Germany) at 24 and 48 h after infection. Immediately after that, crystal violet staining was performed to confirm cytopathicity, and the plates were imaged with a digital camera. Then, crystal violet was dissolved in room temperature in 250 μl of 10% acetic acid for 20 min in a shaker to quantify cell viability. Absorbance of the dissolved dye from the samples transferred to a 96-well plate was measured at a wavelength of 595 nm with a plate reader (VICTOR2, PerkinElmer, Waltham, MA, USA). Growth media served as a negative control.

4.3.3 VIRAL REPLICATION KINETICS ANALYSIS (I, II)
Two hundred thousand cells were plated on 24-well plates in 5% FCS GM and infected the next day with vvdd-tOMato and vvdd-hCD40L-tOMato in triplicate at 0.01 pfu per cell for one hour in 2% FCS GM. Then, 5% FCS GM was added to the wells, and cells were detached by scraping them with the bigger end of a pipet tip and collected with GM 24, 48 and 72 h after and frozen at -80°C. After three freezing-thawing cycles, supernatant was separated from cell pellet by centrifuging at 200 G for 5 min and collected, and a standard plaque forming assay (Earl et al. 1998) was performed in duplicate to determine the amount of infectious virus.

4.3.4 CLONAL ASSAY (I, II)
Clonal assay was performed for ACE-1 and SCCF1 cell lines. Ten thousand and 50,000 cells grown on 6-well plates and infected after a 48-h incubation for one hour with 10 pfu per cell in 2% FCS GM. Cells were collected with supernatant at 24 and 72 h by scraping from the wells. One hundred thousand, 1000, 100 and 10 cells were plated in triplicate on 6-well plates and standard plaque forming assay was performed after 6-7 days to count the plaques.

4.3.5 QUANTIFICATION AND INFECTIVITY OF THE PRODUCED VIRIONS IN IN VITRO SCCF1 CELLS (II)
VV was extracted and purified from infected A549 and SCCF1 cells to monitor the amount of virus produced by the cells. To determine the production rate of secreted virions, the supernatant of the infected SCCF1 cells was also collected at different time-points for plaque assay. Collected supernatant was also used to infect either SCCF1 or A549 cells to observe the efficacy of secondary infection, and a transduction assay was performed as described above.
4.3.6 NEUTRALIZATION ASSAY IN SCCF1 CELLS (II)

Supernatant collected from VV-infected SCCF1 cells was incubated for one hour at 37°C with or without NR-417 monoclonal anti-vaccinia antibody (BeiResources, Manassas, VA, USA), after which a standard plaque assay was performed to determine the amount of neutralized virus.

4.3.7 ELECTRON MICROSCOPY OF SCCF1 CELLS (II)

A negative staining of whole-mounted viral particles and a thin sectioning of resin-embedded cells with viruses were used in this study.

The negative staining method was used to investigate surface structures of purified, whole-mounted VV particles in the supernatant of infected SCCF1 and A549 cells. The method detects viral particle as a whole in three dimensions.

First, the supernatant collected from infected cells was filtered through a sucrose cushion to purify the virus particles as described elsewhere (Guse et al. 2010). Then, one droplet (10-20 μl) of virus suspension was applied on top of the formvar-carbon coated electron microscopy (EM) specimen supporting grids. After absorbing for one minute, the suspension was blotted away with Whatman filter paper and one droplet (30-40 μl) of negative stain and 2% potassium phosphotungstate (KPT) at pH 7.2 was added on top of the grids for 30 s to stain the virus. Finally, the stain was blotted away and the grids were air-dried for 2 min and loaded into EM for inspection. The virions are predominantly attached to the grid with the largest, flat surface and face up, which is typical for asymmetrical particles.

Resin-embedded thin-section EM was used to study the internal structure of viral particles and infected host cells. Unlike the negative staining method, thin sections allow evaluation of VV structures in any orientation since the embedding and cutting direction are random relative to the particles.

Initially, VV-infected SCCF1 and A549 cells were fixed immediately out of incubator with 2.5% glutaraldehyde in 0.1 M phosphate buffer at pH 7.4 for 30 min 48 h p.i. on-site in the culture dish. The cells were then scraped off the dish, centrifuged and collected as a small pellet of 2-3 mm³. For further fixation, fresh glutaraldehyde fixative was added on top of the pellet for half an hour. After that, the pellets were post-fixed for one hour with 2% osmium tetroxide, dehydrated in series of ethanol and embedded in LX-112 resin. Ultra-thin sections were cut at a thickness of 60-80 nm, mounted on 200 mesh EM specimen supporting grids, stained with uranyl acetate and lead citrate in an automatic stainer (Leica EMStain, Leica microsystems, Austria) according to the manufacturer’s instructions and examined by EM.

Examination of both negative stained specimen and thin section was performed by JEM 1400 transmission electron microscope (TEM) (JEOL, Tokyo, Japan) at 80 kV. Side-mounted Morada TEM digital camera (Olympus Soft Imaging Solution GMBH, Munster Germany) was used to take digital electron micrographs. Brightness and contrast of the images were adjusted by
iTem software from the camera manufacturer, and Adobe Photoshop software was used for analysis.

### 4.3.8 CELL DEATH TYPE ASSESSMENT (I)

For the assessment of the type and degree of cell death in association with viral infection, D17 osteosarcoma monolayers were infected with vvdd-luc at 0.1 pfu per cell. The cells were collected at 24 and 48 h after infection with trypsin digestion and centrifuged at 61 g for 10 min. The supernatant was replaced by phosphate-buffered saline (PBS). After this procedure had been repeated twice, the resulting cell pellet was fixed for 24 h in 4% paraformaldehyde in PBS pH 7.4 and then paraffin wax-embedded, or fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for TEM. Uninfected cells served as the control.

The paraffin-embedded cell pellet was sectioned in slices of 3-5 μm and stained with haematoxylin and eosin (HE). To recognize cleaved caspase-3-positive apoptotic cells, immunohistological stain was performed with rabbit anti-cleaved caspase-3 antibody (Cell Signaling Technology, Inc., Danvers, MA, USA) (see Section 4.4.3. Post-mortem examination, histology and immunohistology).

Cell pellets were washed for 90 min in 0.1 M sodium cacodylate buffer, post-fixed in 1% osmium tetroxide(aq) and routinely resin-embedded for TEM. Semi-thin sections of 0.75 μm were stained with toluidine blue and used to recognize areas of interest to prepare 80-nm ultrathin sections. Philips 208S Transmission Electron Microscope (Koninklijke Philips Electronics N.V., Eindhoven, the Netherlands) was used to examine the samples at 80 kV.

### 4.4 IN VIVO EXPERIMENTS

### 4.4.1 ETHICAL CONSIDERATIONS

The in vivo mice studies were approved by the National Animal Experiment Board of the Regional State Administrative Agency of Southern Finland (no. ESLH-2009-06696/Ym-23). The VV dog experiment and the SFV experiment were approved by the same agency (nos. ESAVI/4953/04.10.03/2011 and ESAVI/3231/04.10.07/2013, respectively).

All animals were purpose-bred and the dogs were retired breeding dogs slated for euthanasia. The dogs had been vaccinated regularly against canine distemper, hepatitis, parvovirus, parainfluenza, leptospirosis and rabies and had been dewormed regularly.

All animals were housed in isolation wards under standard condition and they had free access to water at all times. Mice were fed ad libidum, whereas the dogs were fed twice daily with commercial laboratory animal food. The
4.4.2 NUDE MOUSE XENOGRAFT MODEL (I)
Six 4-week-old nude HsdCpb: NMRI – Fox1 Nu/Nu female mice purchased from Harlan Laboratories (Horst, the Netherlands) were each injected with $1 \times 10^7$ canine prostatic carcinoma cells (ACE-1) subcutaneously in both flanks under sedation to induce tumours. When the tumours were approximately 5 mm in diameter, each tumour was injected $10^5$ pfu of vvdd-luc or placebo (PBS), and the injection was repeated in 10 days. The size of the tumours was measured in two dimensions three times a week, and the mice were euthanized when the sum of the longest diameter of both tumours was 25 mm or the tumours became ulcerated. The schematic drawing of the xenograft model is presented in Figure 6.

![Figure 6](image-url)  
**Figure 6**  
Experimental xenograft model in nude female mice. Ten million ACE-1 cells were injected subcutaneously in both flanks in each mouse. Mice were treated twice within 10 days with oncolytic vaccinia virus or placebo. vvdd-luc, lucifarese-expressing double-deleted oncolytic vaccinia virus; pfu, plaque-forming units.

4.4.3 POST-MORTEM EXAMINATION, HISTOLOGY AND IMMUNOHISTOLOGY (I)
The heart, lung, liver, spleen, kidney, and all tumours from ACE-1 xenograft mice were collected for histological examination. Tissues were fixed in 10%...
Materials and methods

buffered formalin, routinely paraffin wax-embedded, cut in tissue sections of 3-5 μm and stained with HE or immunohistological stains.

Immunohistology was used to visualize virus infection and apoptotic cells by using a rabbit anti-vaccinia antibody (Quartett, Berlin, Germany), a rabbit anti-β-galactosidase antibody (EMD Millipore Corp., Temecula, CA, USA), and a rabbit anti-cleaved caspase-3 antibody (Cell Signaling Technology, Inc., Danvers, MA, USA).

The Lab Vision™ Biotinylated Goat anti-Rabbit HRP kit (Thermo Fisher Scientific, Runcorn, UK) was used as the detection system. Staining was visualized with 3,3’-diaminobenzidine (DAB) or Polydetector HRP Green Substrate-Chromogen kit (Bio SB Inc., Santa Barbara, CA, USA) with Harris haematoxylin counterstaining. The vvdd-luc-infected D17 cell line served as a positive control and sections without primary antibody and tumours of the untreated mice as negative controls.

4.4.4 FUNCTIONALITY OF HUMAN CD40L IN DOGS (I)

Canine peripheral blood mononuclear cells (PBMCs) were collected from left-over buffy coat from volunteer blood donors after separation of red blood cells and plasma in the Canine Blood Bank, Veterinary Teaching Hospital of the University of Helsinki following a previously described protocol (Schreuer & Hammerberg 1996).

A549 cells were infected with Ad5/3-hTERT-CD40L, and the control was Ad5/3-hTERT. The supernatant was collected and filtered to purify CD40L produced by the virus. Canine PBMCs were cultured with the filtered supernatant in Roswell Park Memorial Institute (RPMI) medium (Lonza). IL-8 was measured by ELISA (Canine CXCL8/IL-8 Quantikine, R&D Systems, Minneapolis, MN, USA) following the manufacturer’s instructions at 24, 48, 72 and 96 h after co-culture.

4.4.5 BIOPSY TRANSDUCTION ASSAY (I)

Fresh biopsy samples of naturally occurring suspected tumours in pet dogs were collected during surgery or after euthanasia in 10% FCS DMEM with 1% L-glutamine and 2% P-S. The study protocol was approved by the Ethics Committee of the Campus of Viikki, University of Helsinki, Finland, and all owners signed informed consent forms.

Biopsy samples were sliced into 2 x 2 x 2 mm pieces, and three pieces from each were transferred into one well in 24-well plates. Each well was infected with 5 x 10⁶ pfu of vvdd-hCD40L-tdTomato, and the samples were incubated in 5% CO₂ at 37°C. Transduction of tdTomato was observed by fluorescence microscope (Olympus 1X71 inverted microscope, Olympus) at 1, 2 and 4 days after infection.
4.4.6 DOG MODELS (III, IV)

4.4.6.1 Dogs (III, IV)
Two healthy adult male HsdRcc:DOBE Beagle dogs were used for the vaccinia experiment, and two females of the same breed were used in the SFV experiment. The dogs were purchased from Harlan Laboratories (Gannat, France).

4.4.6.2 Study design (III, IV)
Dog 1 in the vaccinia virus experiment (III) (dog VV1) was used for the evaluation of acute toxicity and received a single infusion of vvdd-tdTomato-CD40L. Dog 2 in the vaccinia virus experiment (dog VV2) was used to evaluate chronic toxicity and received four infusions of vvdd-tdTomato-CD40L once a week. Dog VV1 was euthanized 24 h after virus infusion and dog VV2 one week after the 4th virus infusion. The dogs were randomly chosen for each group.

Dogs 1 and 2 in the Semliki Forest virus experiment (IV) (dog SFV1 and SFV2) received one infusion of SFV and were euthanized 3 weeks later. All dogs had full necropsy performed by a study pathologist. The study plan and virus doses are presented in Table 6.

The dose of the VV (III) was based on the phase I dose escalation study with another oncolytic VV, JX-594, in patients with primary or metastatic liver cancer (Park et al. 2008). The highest dose in the study was $3 \times 10^9$ pfu per injection, which is approximately $4 \times 10^7$ TCID$_{50}$/kg in a 75-kg patient assuming that pfu and TCID$_{50}$ units are roughly equivalent. The dose we chose to use in our study was $1.2 \times 10^8$ TCID$_{50}$/kg, which is three times the maximum dose used in human patients (Park et al. 2008).

In the SFV study, we used a low dose, injecting both dogs with $10^6$ pfu. In mice, guinea pigs and rabbits, commonly used doses are between $10^4$ and $10^6$ pfu, and in a susceptible mammalian host, attenuated SFV may reach peak titres of up to $10^8$ pfu/ml, even following a minute inoculum. The size of the animal does not seem to matter for the penetrance of viraemia (Bradish et al. 1971, Bradish & Allner 1972, Fleming 1977, Jagelman et al. 1978, Fazakerley et al. 1993).

The virus was diluted in 50 ml of saline (Baxter, Norfolk, UK) and was given over 15 min (200 ml/h) as an intravenous infusion into a peripheral vein. The SFV infusion line ran through a tempered water bath (ca. $37^\circ$C) before entering the vein (IV). Before virus administration, an intravenous catheter was placed and 10 ml of saline was administered through it to ensure vein access. The saline bag and infusion line were flushed with 25 ml of saline at the same infusion rate after virus administration to confirm that the intended virus dose was delivered. In all, the infusion lasted approximately 23 min.
In the SFV study, we simulated the virus administration protocol afterwards to confirm that the dogs received live virus. The virus diluted in saline was kept on ice for 3 h 25 min, which was the maximum time from virus preparation until it was delivered, and then directed at the infusion speed through a 37°C water bath. The outcoming solution was collected for virus titrating.

The dogs were monitored for adverse events according to the Veterinary cooperative oncology group - Common terminology criteria for adverse events (VCOG-CTCAE) (Veterinary cooperative oncology group 2004). The monitoring schedule is shown in Table 7.

Table 6  
Study plan and virus doses of the dogs receiving oncolytic vaccinia or Semliki Forest virus.

<table>
<thead>
<tr>
<th>Dog</th>
<th>Dose no.</th>
<th>Dose (TCID$_{50}$ or pfu/kg)</th>
<th>Weight (kg)</th>
<th>Total dose</th>
<th>Administration schedule</th>
<th>Endpoint/ Euthanasia</th>
</tr>
</thead>
<tbody>
<tr>
<td>VV1</td>
<td>1</td>
<td>1.2 x 10$^8$</td>
<td>12.8</td>
<td>4 x 10$^9$ TCID$_{50}$</td>
<td>Single infusion</td>
<td>24 h p.i.</td>
</tr>
<tr>
<td>VV2</td>
<td>1</td>
<td>1.2 x 10$^8$</td>
<td>14.0</td>
<td>1.7 x 10$^9$ TCID$_{50}$</td>
<td>Infusions once per week over 4 weektimes</td>
<td>1 wk after 4$^{th}$ virus infusion</td>
</tr>
<tr>
<td></td>
<td>2-4</td>
<td>0.8 x 10$^8$</td>
<td></td>
<td>1.1 x 10$^9$ TCID$_{50}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SFV1</td>
<td>1</td>
<td>~1.5 x 10$^5$</td>
<td>6.7</td>
<td>10$^6$ pfu</td>
<td>Single infusion</td>
<td>3 wk p.i.</td>
</tr>
<tr>
<td>SFV2</td>
<td>1</td>
<td>~7.8 x 10$^4$</td>
<td>12.9</td>
<td>10$^6$ pfu</td>
<td>Single infusion</td>
<td>3 wk p.i.</td>
</tr>
</tbody>
</table>

VV1, Dog 1 in vaccinia experiment; VV2, Dog 2 in vaccinia experiment; SFV1, Dog 1 in Semliki Forest virus experiment; SFV2, Dog 2 in Semliki Forest virus experiment p.i. post-infusion

Table 7  
Clinical examination schedule of the dogs receiving oncolytic vaccinia or Semliki Forest virus during the first 24 hours after virus administration.

<table>
<thead>
<tr>
<th>Time</th>
<th>RR</th>
<th>HR</th>
<th>MM</th>
<th>CRT</th>
<th>T</th>
<th>BP</th>
<th>Swelling</th>
<th>Itching</th>
<th>Vomiting</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>15 min</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>30 min</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>45 min</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>1 h p.i.</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>2 h p.i.</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>4 h p.i.</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>8 h p.i.</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>11 h p.i.</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>24 h p.i.</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
</tbody>
</table>

RR, respiratory rate; HR, heart rate; MM, colour of mucous membranes; CRT, capillary refill time; T, rectal temperature; BP, blood pressure: systolic/diastolic/mean p.i. post-infusion
4.4.6.3 Sample collection for clinical pathology (III, IV)

Blood was collected from the jugular, cephalic or saphenous vein for haematology into tubes containing potassium (K2) EDTA, and for clinical chemistry and neutralizing antibody measurements into serum tubes according to Table 8A (III) and 8B (IV).

Table 8 Sample collection schedule for the study of dogs receiving (A) oncolytic vaccinia (III) or (B) Semliki Forest virus (IV).

<table>
<thead>
<tr>
<th>Week</th>
<th>Day</th>
<th>Laboratory measurements</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>A.</td>
<td>1</td>
<td>0 CBC, chem, NAbs, urinalysis, blood, saliva, urine, faeces</td>
<td>Virus infusion</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>CBC, blood, saliva, urine, faeces</td>
<td>Euthanasia dog VV1</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>CBC, blood, saliva, urine, faeces</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>CBC, blood, saliva, urine, faeces</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>7 CBC, chem, Nabs, urinalysis, virus</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>CBC, blood, saliva, urine, faeces</td>
<td></td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>CBC, blood, saliva, urine, faeces</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>14 CBC, chem, NAbs, urinalysis, blood, saliva, urine, faeces</td>
<td></td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>CBC, blood, saliva, urine, faeces</td>
<td></td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>CBC, blood, saliva, urine, faeces</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>21 CBC, chem, NAbs, urinalysis, blood, saliva, urine, faeces</td>
<td>Euthanasia dog VV2</td>
</tr>
<tr>
<td></td>
<td>23</td>
<td>CBC, blood, saliva, urine, faeces</td>
<td>Euthanasia SFV dogs</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>CBC, blood, saliva, urine, faeces</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Week</th>
<th>Day</th>
<th>Laboratory measurements</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>B.</td>
<td>1</td>
<td>0 CBC, chem, NAbs, urinalysis, blood, urine, faeces</td>
<td>Virus infusion</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>CBC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>CBC, blood, urine, faeces</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>CBC, blood, urine, faeces</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>7 CBC, chem, urinalysis, blood, urine, faeces</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>CBC, NAbs, blood, urine, faeces</td>
<td></td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>CBC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>14 CBC, chem, urinalysis, blood, urine, faeces</td>
<td></td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>CBC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>CBC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>21 CBC, chem, NAbs, urinalysis, blood, urine, faeces</td>
<td>Euthanasia SFV dogs</td>
</tr>
</tbody>
</table>

CBC, complete blood count; Chem, clinical chemistry; NAbs, neutralizing antibodies; Blood, saliva, urine, faeces; for virus detection
VV1, Dog 1 in vaccinia experiment; VV2, Dog 2 in vaccinia experiment; SFV, Semliki forest virus dogs
Haematology was analysed by an automated haematology analyser (ADVIA 2120i Hematology System, Siemens Healthcare Diagnostics Inc., Tarrytown, NY, US). Serum was separated after a minimum 30-min incubation at room temperature by centrifuging for 10 min at 2400 G and analysed by a clinical chemistry analyser (Konelab 30i, ThermoFisher Scientific, Vantaa, Finland). Table 9 shows the haematology and clinical chemistry parameters measured.

4.4.6.4 Sample collection for virus detection and cytokines (III, IV)

In the vaccinia experiment (III), three millilitres of blood was collected into EDTA tubes for virus detection and cytokine measurements before and immediately after virus administration, and then 1, 2, 4, 8, 11 and 24 h and 2, 4 and 7 days after each virus infusion.

In the SFV experiment (IV), four millilitres of blood was collected into serum tubes for virus detection before and 2, 4 and 22 h and 2, 4, 7, 9, 14 and 21 days after virus infusion.

Saliva for the vaccinia virus experiment (III) was collected by holding two cotton tips in each side of the mouth for 30 s. Five millilitres of urine was collected by cystocentesis for each sample, and 10-15 g of faeces was collected rectally or from the kennels when fresh not contaminated sample was available and the rectum contained no faeces (III, IV). The collection schedule is presented in Table 8A (III) and 8B (IV).

Blood samples for vaccinia virus detection (III) were frozen first at -18°C and transferred the same day to -80°C until analysis. Serum for SFV detection
(IV) was separated as described above, and all samples were frozen and stored following the same protocol as for the samples for vaccinia virus detection.

4.4.6.5 qPCR for vaccinia virus (III)

Gentra Puregene Blood Kit (QIAGEN, Germantown, MD, USA) was used to extract DNA from whole blood following the manufacturer’s instructions. Saliva collected by cotton swabs was diluted in 2500 μl of phosphate-buffered saline (PBS, BioWhittaker, Lonza), then the sample was shaken and the cotton swabs were squeezed towards the tube walls to get all of the fluid into the tube. Gentra Puregene Blood Kit for Body Fluids (QIAGEN) was used for DNA extraction according to the manufacturer’s instructions using 1 ml of diluted saliva. DNA from urine was extracted by the same method, using 1 ml of urine, and for faeces, 180-220 mg. Faecal samples were first handled by QIAamp DNA Stool kit (QIAGEN GmbH, Hilden, Germany) by using Buffer ASL and InhibitEX tablets to lyse bacteria and other pathogens and to absorb inhibitors, and then Gentra Puregene Tissue Kit (QIAGEN) was used according to the manufacturer’s instructions. The same kit was used for necropsy samples using 50-100 mg of each tissue. Finally, extracted DNA concentration was measured by spectrophotometry (Nanodrop 8000, Thermo Scientific, Wilmington, DE, USA). To quantify the method, we used samples from healthy dogs as negative controls, and for positive controls the same samples were spiked with VV. Collection of saliva, urine and faeces for qPCR is presented in Figure 7.
Materials and methods

Table 10  **Primers and probes used in vaccinia virus qPCR.**

<table>
<thead>
<tr>
<th>Gene target</th>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA J7R</td>
<td>OPHA-probe</td>
<td>AGT GCT TGG TAT AAG GAG</td>
</tr>
<tr>
<td></td>
<td>OPHA-F89</td>
<td>GAT GAT GCA ACT CTA TCA TGT A</td>
</tr>
<tr>
<td></td>
<td>OPHA-R219</td>
<td>GTA TAA TTA TCA AAA TAC AAG ACG TC</td>
</tr>
<tr>
<td>Canine actin</td>
<td>Probe</td>
<td>TCC TGG CCT CAC TGT CCA CCT TCC AGC</td>
</tr>
<tr>
<td></td>
<td>FW</td>
<td>GCG CAA GTA CTC TGT GTG GAT</td>
</tr>
<tr>
<td></td>
<td>RW</td>
<td>GTC GTA CTC CTG CTT GTG GAT</td>
</tr>
</tbody>
</table>

Table 11  **Limit of detection and quantification for vaccinia genome qPCR.**

<table>
<thead>
<tr>
<th>Secretion</th>
<th>Detection limit</th>
<th>Quantification limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole blood</td>
<td>1 copy/30 μl</td>
<td>1 copy/3 ml</td>
</tr>
<tr>
<td>Urine</td>
<td>1 copy/100 μl</td>
<td>1 copy/10 ml</td>
</tr>
<tr>
<td>Faeces</td>
<td>1 copy/5 mg</td>
<td>1 copy/500 mg</td>
</tr>
<tr>
<td>Saliva</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

Polymerase chain reaction (PCR) was based on primers and probe targeting the HA J7R gene (Applied Biosystems UK, Cheshire, UK). Ten microlitres of extracted DNA, 12.5 μl of 2X LightCycler 480 Probes Master Mix (Roche, Mannheim, Germany), 0.25 μl of each 50 μM primer, 0.4 μl of 0.1 μM probe and 0.16 μl of nuclease-free water were used for each qPCR reaction.

Quantitative PCR (qPCR) was performed by LightCycler 480 II (Roche Diagnostics GmbH, Mannheim, Germany) under the following conditions: 2 min at 50°C, 10 min at 95°C, 50 cycles of 15 s at 95°C and 1 min at 60°C and 10 min at 40°C. All samples were run in triplicate. A standard curve was created for absolute quantification by using HA-vaccinia plasmid kindly donated by Professor Olli Vapalahti, Haartman Institute, University of Helsinki. Water was used as a negative control. In addition, TaqMan Exogenous internal positive control (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA, USA) was used to ensure that there were no PCR inhibitors. Standard curve and qPCR reaction were run with the same protocol for canine β-actin to quantify the result for canine genomic DNA. Probes and primers were purchased from Oligomer (Helsinki, Finland) and are presented in Table 10. The limit of detection and quantification for analysis are shown in Table 11. We were unable to define limits for DNA detection or saliva quantification since the amount of saliva in the sample was low.

4.4.6.6  **Detection of infectious virus (III, IV)**

Plaque assay was performed for all saliva and urine samples positive for vaccinia DNA and for selected blood samples to detect infective virus (III). Four hundred thousand A549 and Vero cells per well were plated on a 6-well plate in GM with 10% FCS. The cells were incubated for 24 h and then infected
with 200 μl of sample diluted with 6 ml of growth media (1 ml/well). To maximize infection, infected cells were centrifuged at 600 g for 20 min at room temperature and then incubated for 30 min in 5% CO2 at 37°C. GM with 10% FCS was then added, and the plates were incubated for 3 days. Transduction was detected with fluorescence microscope on days 1 and 3, and plaques were stained with Coomassie blue on day 3.

PBS and the baseline samples from the Beagles taken before virus infusion served as negative controls. Positive controls included blood, saliva and urine from healthy dogs and PBS in which virus was added right before infection.

In addition, TCID50 with 10^{-2}-10^{-9} dilutions was performed on selected blood samples in Vero cells. The results were read on day 10.

In the SFV experiment (IV), saline containing virus used to simulate virus administration protocol and sera collected from the dogs were titrated for SFV A7(74) on Vero cells according to a previously published protocol (Ruotsalainen et al. 2014). Serial dilutions of samples were prepared in cold medium, and the stock A7(74) virus served as a positive control.

The protocol for virus titration from urine and faecal samples was modified from Buonagurio et al. (1999) (IV). Urine samples were diluted 1:10 and 1:100 in DMEM with 1 g/l glucose and virus titration was done on Vero cells as for sera. Faeces (0.1-0.2 g) were mixed with 10x (w/v) DMEM and centrifuged at 3000 g for 10 min at 4°C before virus titration. The supernatant was diluted in growth media 1:10 and 1:100 for titration after sterile filtering with a 0.22 μm PES membrane syringe filter (Syringe Filter, Porvair Science, Leatherhead, UK). Baseline samples and growth media served as negative controls. Faecal samples for recovery controls were prepared by adding 10^6 pfu of SFV A7(74) per 0.1 g of baseline faecal samples of both dogs diluted in 10x (w/v) amount of medium. After that, the mixtures were vortexed, sterile-filtered, serial-diluted (from 10^{-1} to 10^{-6}) to growth medium and titrated as described above.

### 4.4.6.7 Cytokine measurement (III)

Cytokines were measured from the plasma collected from EDTA tubes by using Procarta Immunoassay Magnetic Bead kits (Affymetrix Panomix, Santa Clara, CA, USA) and are presented in Table 12. To eliminate infectious virus before analysis, 50 μl of QuantiGene Plex Assay Kit Lysis Mixture (Affymetrix Panomix) was added to 100 μl of plasma. Fluorescence was measured by a Bio-Plex 200 System (Bio Rad, Hercules, CA, USA) device according to the manufacturer's instructions.

### 4.4.6.1 Neutralizing antibodies (III, IV)

NAbs for VV virus (III) were measured from the serum samples from dog VV2 collected 2, 3 and 4 weeks after first virus infusion and in dogs receiving SFV
Materials and methods

Table 12  Cytokines measured from the Beagles receiving vaccinia virus.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Abbreviation</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interferon γ</td>
<td>IFN-γ</td>
<td>Immunostimulatory and immunomodulatory effects, Inhibit viral replication directly, promote apoptosis in infected cells</td>
</tr>
<tr>
<td>Interleukin-2</td>
<td>IL-2</td>
<td>Growth, proliferation and differentiation of T cells</td>
</tr>
<tr>
<td>Interleukin-6</td>
<td>IL-6</td>
<td>Pro-inflammatory, recruit lymphs &amp; neuts; induced by vaccinia early gene products</td>
</tr>
<tr>
<td>Interleukin-8</td>
<td>IL-8</td>
<td>Neutrophil chemotactic factor: migrate toward the site of infection + phagocytosis</td>
</tr>
<tr>
<td>Interleukin-10</td>
<td>IL-10</td>
<td>Anti-inflammatory</td>
</tr>
<tr>
<td>Interleukin-12</td>
<td>IL-12</td>
<td>T cell stimulation</td>
</tr>
<tr>
<td>Tumour necrosis factor-α</td>
<td>TNF-α</td>
<td>Pro-inflammatory, recruit lymphs &amp; neuts; induced by vaccinia early gene products</td>
</tr>
<tr>
<td>Monocyte chemotactic protein-1</td>
<td>MCP-1</td>
<td>Chemotactic factor for monocytes</td>
</tr>
</tbody>
</table>

Lymphs, lymphocytes; neuts, neutrophils

(IV) from samples collected 2, 9 and 21 days after virus infusion (Table 8A and B).

For VV NAb measurement (III), 50 000 A549 were plated per well in DMEM with 10% FCS on a 96-well plate. To inactivate complement, serum was heated the next day for 90 min at 56°C, and dilution serial of 1:4 was prepared in plain DMEM. Diluted serum and 48 pfu of vvdd-luc at a concentration of 4 x 10^3 pfu/ml were incubated for 60 min at room temperature on a shaker with a slow rate.

GM was then removed and the cells were washed, after which 100 pfu of vvdd-luc incubated in serum dilutions was added in triplicate to each well. After incubation for 60 min in 5% CO2 at 37°C, 100 μl of DMEM with 10% FCS was added to the wells, and the plates were incubated for 20 h in 5% CO2 at 37°C. GM was then removed, cells were lysed and luciferase activity measured with Luciferase Assay System (Promega) using TopCount luminometer (PerkinElmer, Waltham, MA, USA) according to the manufacturer’s instructions. The NAb titre for VV was defined as the highest dilution of the virus preventing ≥50% of the luciferase expression (Mastrangelo et al. 1999).

SFV NAbs were measured according to Ruotsalainen et al. (2012). In brief, the serum samples were diluted 1/2, 1/5, 1/25, 1/125, 1/625 and 1/3125 into DMEM, and mixed with 50 pfu of the A7(74) virus in 1:1 (100 μl:100 μl). The mixture was incubated on ice for approximately 30 min and then plaque was titrated on Vero cells in 12-well plates in duplicate. DMEM was used as a negative control and polyclonal rabbit anti-SFV antibody in DMEM as a positive control. Fluorescence was monitored by fluorescence microscope (Zeiss Axio Observer Z1). The highest dilution of the virus that completely prevented plaque formation (50 pfu SFV A7(74)) was defined as the NAb titre.
4.4.6.2 Necropsy (III, IV)

Complete necropsy was performed on all dogs by a board-certified study pathologist. Samples for histological evaluation were collected from the tissues listed in Table 13. Tissues were fixed in neutral-buffered 10% formalin, trimmed, embedded in paraffin, sectioned at 4 μm thickness, routinely processed and stained with HE.

Samples from the same organs were collected in liquid nitrogen for detection of the virus by qPCR in the vaccinia study (III), whereas only selected organs were collected in dry ice in the SFV experiment (IV) (Table 14). In addition, in the VV study (III) tissue samples were collected for TEM examination and were fixed in 5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2-7.4).

Table 13 Tissue list for histopathology collection from the dogs receiving oncolytic vaccinia (VV)(III) and Semliki Forest virus (SFV) (IV). Abbreviations in parentheses mean that tissue was collected only in that study.

<table>
<thead>
<tr>
<th>Adrenal Glands (cortex and medulla)</th>
<th>Macroscopic Lesions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aorta (thoracic) (VV)</td>
<td>Optic Nerves</td>
</tr>
<tr>
<td>Bone and Joint (distal femur)</td>
<td>Ovaries (SFV)</td>
</tr>
<tr>
<td>Bone Marrow (rib)</td>
<td>Pancreas</td>
</tr>
<tr>
<td>Brain*</td>
<td>Peripheral Nerve (sciatic)</td>
</tr>
<tr>
<td>Caecum (VV)</td>
<td>Pituitary Gland</td>
</tr>
<tr>
<td>Colon (VV)</td>
<td>Salivary Gland (submandibular)</td>
</tr>
<tr>
<td>Duodenum (VV)</td>
<td>Skeletal Muscle (thigh)</td>
</tr>
<tr>
<td>Oesophagus (VV)</td>
<td>Skeletal Muscle (diaphragm)</td>
</tr>
<tr>
<td>Eyes (VV)</td>
<td>Skin (VV)</td>
</tr>
<tr>
<td>Gallbladder (VV)</td>
<td>Spinal Cord (cervical and lumbar)</td>
</tr>
<tr>
<td>Gut-associated lymphoid tissue</td>
<td>Spleen</td>
</tr>
<tr>
<td>Heart</td>
<td>Stomach (cardiac, fundic and pyloric) (VV)</td>
</tr>
<tr>
<td>Ileum (VV)</td>
<td>Testes (VV)</td>
</tr>
<tr>
<td>Injection Site (dosing sites only) (VV)</td>
<td>Thymus</td>
</tr>
<tr>
<td>Jejunum</td>
<td>Thyroid/Parathyroid Glands</td>
</tr>
<tr>
<td>Kidneys</td>
<td>Tongue (VV)</td>
</tr>
<tr>
<td>Liver</td>
<td>Trachea (VV)</td>
</tr>
<tr>
<td>Lungs</td>
<td>Urinary Bladder (VV)</td>
</tr>
<tr>
<td>Lymph Nodes, mandibular</td>
<td>Uterus (SFV)</td>
</tr>
<tr>
<td>Lymph Nodes, mesenteric</td>
<td>Vagina (SFV)</td>
</tr>
</tbody>
</table>

* For SFV: hippocampus, cortex, corpus callosum, cerebellum and pons
Materials and methods

### Table 14  Tissue collected for Semliki Forest virus immunohistochemistry (IV).

<table>
<thead>
<tr>
<th>Brain: hippocampus</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain: cortex</td>
<td>Lymph Nodes, mandibular</td>
</tr>
<tr>
<td>Brain: corpus callosum</td>
<td>Lymph Nodes, mesenteric</td>
</tr>
<tr>
<td>Brain: cerebellum</td>
<td>Skeletal Muscle (thigh)</td>
</tr>
<tr>
<td>Brain: pons</td>
<td>Spinal Cord, cervical</td>
</tr>
<tr>
<td>Kidneys</td>
<td>Spleen</td>
</tr>
</tbody>
</table>

## 4.5 STATISTICS

Statistical analysis for virus transduction, oncolytic potency and the xenograft model were performed with Microsoft Office Excel 2010 (Microsoft, Redmond, WA, USA), GraphPrism software (GraphPad Software Inc., La Jolla, CA, USA) and SAS 9.2 (SAS Institute Inc., Cary, NC, USA), and the results are shown as means and standard deviation (I). The oncolytic potency of the viruses in each cell line was analysed separately with analysis of variance (ANOVA) for repeated measurements.

The analysis of SCCF1 virus production was performed with GraphPrism software and is presented as means and standard error of means (II). Differences in the viability of cells were calculated using Student’s t-test for two independent samples with GraphPrism software (GraphPad Software Inc., La Jolla, CA, USA) (II).

Viability of SFV-infected cells was analysed with GraphPad Prism software, and results are shown as means and standard deviation (IV). Statistical analysis was performed using unpaired, two-tailed t-test.

Tumour volume was calculated as follows: \(0.52 \times \text{longest diameter} \times \text{shortest diameter}^2\). The mean volume of both tumours of each mouse was used for calculations (I). ANOVA for repeated measurements was used, and the baseline value was considered as a covariate and day, treatment and their interaction as fixed effects. Log-transformation of the outcome variable was used to reach normality.

For all statistical tests, \(P < 0.05\) was considered significant. For dog experiments, only descriptive statistics were used (III, IV).
5 RESULTS

5.1 IN VITRO STUDIES

5.1.1 TRANSDUCTION AND CYTOTOXICITY (I, II, IV)

Transduction of luciferace was detected in all cell lines, including the canine normal kidney cell line, even with low dosages, confirming that vvdd-luc was able to enter, infect and induce early gene expression in all examined cell lines (Figure 8). In addition, transduction of tdTomato by vvdd-tdTomato was also detected in all cell lines (Figure 9).

Vvdd-tdTomato and vvdd-CD40L-tdTomato both effectively killed all canine cell lines (Figure 10), but at lower viral doses oncolytic potency of vvdd-tdTomato was significantly better in D17 (0.01 and 0.1 pfu/cell: \( P<0.001 \)), ACE-1 (0.01 pfu/cell: \( P=0.0001 \); 0.1 pfu/cell: \( P<0.001 \)) and MDCK cells (0.01 pfu/cell: \( P<0.001 \)). No difference was detected in Abrams cells (\( P=0.1128 \)). Both viruses killed more cells over time, although vvdd-tdTomato-induced oncolysis was significantly more effective than vvdd-hCD40L-tdTomato-induced oncolysis in D17 (0.1 pfu/cell at 72, \( P<0.0001 \) and 96 h, \( P<0.0001 \); 1 pfu/cell at 24 h, \( P=0.0022 \)) and ACE-1 cells (0.1 pfu/cell at 94 h, \( P=0.0007 \) and 1 pfu/cell at 24 h, \( P=0.0014 \)).

![Image of graph showing luciferase transduction of vvdd-luc in canine and feline cell lines.](image)

**Figure 8**  Luciferase transduction of vvdd-luc in canine and feline cell lines. Cells were infected with vvdd-luc with different virus doses in triplicate and luminance of luciferase was measured 4 h after infection in relative light units (RLUs). Values are presented as means. Abrams and D17, canine osteosarcoma cell lines; ACE-1, canine prostatic carcinoma cell line; MDCK, canine kidney cell line; SCCF1, feline squamous cell carcinoma cell line; pfu, plaque-forming units; error bars, standard deviation.
Results

Interestingly, vvdd was unable to effectively lyse SCCF1 cells when 0.01 or 0.1 pfu/cell was used, and cytotoxicity was significantly more effective ($P=0.0073$) in A549 cells than in SCCF1 cells at the dose of 1 pfu/cell and when viability was measured on day 3. When SCCF1 was growing as an intact monolayer, the cells stayed alive up to 10 days and only a high dose (100 pfu/cell) of vvdd-luc was able to effectively kill the cells. UV-inactivated vvdd had no effect on the viability of SCCF1 cells (Study II: Figure 1).

Attenuated SFV VA7-EGFP was also able to infect both tested canine osteosarcoma cell lines, and fluorescence of EGFP was detected in both cell lines (Figure 11). The virus replicated at a slightly higher rate in Abrams cells than in D17 cells and induced a more rapid and pronounced cell kill within 48 h (Abrams: 0 vs. 0.01, 0.1 and 1 pfu, $P<0.001$ in all and D17: $P=0.0053$, 0.008 and 0.007, respectively).
Results

Cell killing efficacy of vvdd-tdTomato (A) and vvdd-hCD40L-tdTomato (B) in canine and feline cell lines 94 h after virus infection with different virus doses. Cells were infected in triplicate, and values are presented as means and standard deviation. Abrams and D17, canine osteosarcoma cell lines; ACE-1, canine prostatic carcinoma cell line; MDCK, canine kidney cell line; SCCF1, feline squamous cell carcinoma cell line; pfu, plaque-forming units; error bars, standard deviation.

Figure 10
Results

Transduction and oncolysis of VA7-EGFP in canine osteosarcoma cell lines, Abrams and D17. Cells were infected with MOI 1, MOI 0.1 or MOI 0.01 in triplicate (one replica per sample type presented) and infection was monitored by fluorescence microscopy at 24 and 48 h after infection. Green colour indicates gene transfer and virus infection. Cytopathic effect was confirmed by crystal violet staining at 48 h after infection and is seen as loss of the stain. MOI, multiplicity of infection; p.i., post-infection. Scale bar 200 μm.

Figure 11

Transduction and oncolysis of VA7-EGFP in canine osteosarcoma cell lines, Abrams and D17. Cells were infected with MOI 1, MOI 0.1 or MOI 0.01 in triplicate (one replica per sample type presented) and infection was monitored by fluorescence microscopy at 24 and 48 h after infection. Green colour indicates gene transfer and virus infection. Cytopathic effect was confirmed by crystal violet staining at 48 h after infection and is seen as loss of the stain. MOI, multiplicity of infection; p.i., post-infection. Scale bar 200 μm.
Results

Figure 12  Virus production of vvdd-tdTomato (A) and vvdd-hCD40L-tdTomato (B) over time in canine and feline cell lines. Two hundred thousand cells were infected in triplicate with the virus at 0.01 pfu/cell, and plaque forming test was performed to determine the amount of infectious virus. Values are presented as means and standard deviation. Abrams and D17, canine osteosarcoma cell lines; ACE-1, canine prostatic carcinoma cell line; MDCK, canine kidney cell line; SCCF1, feline squamous cell carcinoma cell line; pfu, plaque-forming units.
5.1.2 VIRAL REPLICATION AND CLONAL ASSAY (I, II)

Viral replication varied significantly in different cell lines over time (Figure 12). The highest amount of infectious virus was recovered from ACE-1 cells at 96 h (1,420,000 pfu), whereas the lowest amount was found in SCCF1 cells and it was approximately the same at all time-points (∼300–400 pfu). Virus recovery decreased in osteosarcoma cell lines after 48 h.

Clonal assay was performed in ACE-1 and SCCF1 cell lines since they were more resistant to oncolysis caused by vvdd (Table 15, Figure 13). Infected ACE-1 cells were able to form cell colonies one day after infection when 10,000 or 1000 cells were plated, but only a few colonies were detected when 10,000 cells were plated 3 days after infection.

SCCF1 was more resistant to the killing effect of vvdd. Cell colonies were detected in all samples when cells were collected one day after infection, except when 10 infected cells were plated. Relative to the negative control, approximately 50% fewer cell colonies were identified when 1000 cells were plated 3 days after infection. The difference was smaller when less cells were used.

| Table 15 Clonality assay. ACE-1 and SCCF1 cells were infected with vvdd-tdTomato or vvdd-hCD40L-tdTomato 10 pfu/cell in duplicate and 10,000, 1000, 100 or 10 infected cells were grown 1 or 3 days to evaluate whether cells would form colonies. Values are shown as means. |
|---|---|---|---|---|
| **Cell line** | **Virus** | **Number of cells plated** | **Time** | **Number of cell colonies** |
| | | 10 | 100 | 1000 | 10,000 |
| **ACE-1** | | | | | |
| Day 1 | Mock | 1.9 | 7.9 | 34.9 | NE |
| | vvdd-tdTomato | 0 | 0 | 2.1 | 8.2 |
| | vvdd-CD40L-tdTomato | 0 | 0 | 0.3 | 17.8 |
| Day 3 | Mock | 8.2 | 67.4 | NE | NE |
| | vvdd-tdTomato | 0 | 0 | 0 | 0.5 |
| | vvdd-CD40L-tdTomato | 0.1 | 3.3 | 43 | NE |
| | | | | | |
| **SCCF1** | | | | | |
| Day 1 | Mock | 4.0 | 29 | 159 | NE |
| | vvdd-tdTomato | 0 | 3.3 | 43.3 | NE |
| | vvdd-CD40L-tdTomato | 1.4 | 5 | 131.5 | NE |
| Day 3 | Mock | 2.1 | 10.6 | 187 | NE |
| | vvdd-tdTomato | 1.5 | 7.5 | 102 | NE |
| | vvdd-CD40L-tdTomato | 1.0 | 7.0 | 107.9 | NE |
Cell colony formation of vvdd-infected feline squamous carcinoma cells, (SCCF1). A. placebo wells. B. vvdd-infected cells. SCCF1 cells were infected in duplicate with vvdd-tdTomato at 10 pfu/cell, after which infected cells were collected, plated on 6-well plates (10, 100, 1000 or 10 000 cells/well; the amount of cells is on the left side of the wells) and cultured for 24 hours to evaluate whether they would form colonies. Colonies were visualized by crystal violet staining before counting.

5.1.3 QUANTIFICATION AND INFECTIVITY OF THE PRODUCED VIRIONS IN VITRO (II)

Titrating of supernatants from chronically infected confluent SCCF1 cell cultures showed low but persistent production of the virus for up to 12 days after infection. The produced virions were able to re-infect both SCCF1 and A549 cells, suggesting that the secreted virions retain their infectious capacity. Neutralization assay used to further evaluate the characteristics of the virions confirmed that anti-vaccinia antibody recognized and neutralized almost all of the particles (Study II: Figure 2).

5.1.4 ABNORMAL MORPHOLOGY OF VACCINIA VIRIONS SECRETED BY SCCF1 CELLS (II)

Virions produced by SCCF1 and A549 cells differed from each other when examined under EM. A549 cells produced brick-shaped virions surrounded by an outer envelope, which are typical characteristics for a mature, enveloped VV. Contrary to this, the virions manufactured in SCCF1 cells were immature and misshapen and lacked the outer envelope. In addition, the particles had irregular surface and staining intensity, and they were loosely packed (Study II: Figure 3).
5.1.5 ULTRASTRUCTURE OF VIRAL PARTICLES WITHIN INFECTED CELLS (II)

EM results from thin sections of vaccinia-infected cells were in line with those of negative-stained whole-mount viral particles.

In A549 cells, numerous viral particles of different maturation stages were visible at low magnification. Mature virus (MV) was the most common virion type, the majority being either wrapped virions (WVs) or intracellular enveloped virions (IEVs) enfolded by trans-Golgi cistern membrane. Extracellular enveloped virions (EEVs) were found outside the cells and were surrounded by two membranes, one of which was constructed from the cell plasma membrane. EEVs, which were still in contact with cells, were also detected and recognized as cell-associated extracellular enveloped virus (CEV). At higher magnification, clear membrane layers were visualized. The viral factory, where early replication takes place, was also recognized as a low-to-middle electron density zone in the juxta-nuclear area, where other cellular organelles were missing. IEVs and WVs were detected around the viral factory (Study II: Figure 4).

In SCCF1 cells, the replication factory was not clearly visible and contained abnormal granular virosome. Only a few immature viruses (IVs) of different size and some immature virion with nucleoids (IVNs) were recognized. Enlarged IVs without a membranous envelope were visualized in the peripheral area, where viruses are normally released outside the cell, and EEVs were not seen at all (Study II: Figure 5).

5.1.6 CELL DEATH TYPE ASSESSMENT

When D17 osteosarcoma cells were infected with vvdd, multiple dying cells were detected in the samples 24 and 48 h after infection. Dying cells had changes characteristic for apoptosis, as recently reported in cells killed by oncolytic viruses (Whilding et al. 2013). Expression of the activated executor caspase-3 by immunohistology and TEM confirmed the findings (Study III, Figure 4).
5.2 IN VIVO STUDIES

5.2.1 MOUSE XENOGRAFT MODEL
The oncolytic effect of vvdd-luc was confirmed by a significant delay ($P=0.046$) in tumour progression in the ACE-1 xenograft model in nude mice (Figure 14).

In virus-treated tumors, histological evaluation revealed multiple regions suggestive of necrosis since no viable cells were seen. A few poxvirus-like cytoplasmic inclusion bodies were identified in degenerating cells around the necrotic areas. Pronounced stroma formation was also visualized in tumours. Other organs examined in necropsy did not reveal any pathological changes.

The presence of vaccinia virus in the tumours was shown by immunohistology; anti-vaccinia and anti-β-galactosidase antibodies were detected in the cytoplasm of both dead and viable tumour cells in the necrotic areas and their surrounding regions (Study III, Figure 6). Staining with the anti-vaccinia antibody was more pronounced than with the anti-β-galactosidase antibody, and in one tumour (left tumour in mouse 1 in the virus-treated group) it was not present. Virus was not found in any examined organs by immunohistology.

![Figure 14](image_url)

**Figure 14  Oncolytic potency of vvdd-luc in canine ACE-1 prostate cancer tumours in mice.** Six mice were injected with $1\times10^7$ ACE-1 cells subcutaneously. Vvdd-luc was injected intratumourally twice (arrows) at a dose of $1\times10^5$ pfu (dashed line) in three mice, and three other mice received the same volume of phosphate-buffered saline. The anti-tumoural response was evaluated by measuring tumour size. The tumour size differed significantly ($P=0.046$) between the virus and control groups (asterisk). Values are presented as means and standard deviation.
Production of interleukin 8 by canine PBMCs after stimulation with supernatant collected from cells infected with adenovirus Ad5/3-hTERT and Ad5/3-hTERT expressing human CD40L. A549 cells were infected with Ad5/3-hTERT or Ad5/3-hTERT-CD40L, and the supernatant was collected, purified by filtering and used to stimulate canine PBMCs. Non-infected cells were used as controls. IL-8 production was measured by ELISA at 24, 48, 72 and 96 h after virus infection.

With the anti-caspase antibody, identifying apoptotic cells or cells going into apoptosis, scattered positive staining was seen within the same areas as with the anti-vaccinia and anti-β-galactosidase antibodies, i.e. within and around the necrotic areas. Both groups showed some degree of staining, although it was more intensive in the tumours of the virus-treated mice.

5.2.2 FUNCTIONALITY OF HUMAN CD40L IN DOGS
Canine PBMCs produced IL-8 after exposure to the supernatant collected from A549 cells infected with Ad5/3-hTERT-CD40L (Figure 15). Longer exposure increased production of IL-8. This supports our hypothesis that human CD40 ligand can stimulate canine PMBCs and induce pronounced immunostimulation in dogs.

5.2.3 BIOPSY TRASDUCTION ASSAY
Transduction of vvdd-hCD40L-tdTomato was evidenced in six canine biopsy samples, including one canine osteosarcoma, one grade 2 mast cell tumour, one mammary carcinoma in a benign mixed mammary tumour (Figure 16), two hyperplastic splenic nodules and one ossifying epulis. In mammary carcinoma, transduction was detected at all time-points, whereas in osteosarcoma transduction was only monitored on days 2 and 4, but not on day 1 after infection. In the rest of biopsies, transduction was identified only one day after infection and not at later time-points. Transduction of tdTomato
Results

Fluorescence of tdTomato confirming transduction oncolytic vaccinia virus infection in canine tumour biopsies. Biopsies were infected with vvdd-hCD40L-tdTomato at 5 x 10⁶ pfu/well, and the infection was followed at 1, 2 and 4 days after infection. Grade II mast cell tumour at day 1 (A) and osteosarcoma (B) and mammary carcinoma at day 4 (C).

was not detected in three samples, including tubulopapillary mammary carcinoma and two grade 3 mast cell tumours from the same dog.

5.2.4 VACCINIA VIRUS IN A DOG MODEL (III)
The dogs enjoyed a good quality of life during the study period, and only a few adverse events associated with virus administration occurred.

5.2.4.1 Adverse events
Overall, the dogs did well and experienced only short-term adverse events. Dog 1 had grade 1 fever (39.5°C) and lethargy after the virus administration, but no other adverse events. Dog 2 had a mild increase in rectal temperature, although not fulfilling grade 1 elevation. Rectal temperature of both dogs is shown in Figure 17.

Unexpectedly, dog 2 had a possible grade 3 seizure 5.5 h after the first virus administration. The incident was not evidenced by anyone, but barking was heard from the kennel, and dog 1 was attacking dog 2, who was situated in lateral recumbency on the floor when the investigator arrived less than one minute after the barking started. Dog 2 did not stand up immediately when dog 1 was moved away, although no abnormalities were found in his physical and neurological examination after he was picked up and carried immediately to an examination table.

No other adverse events were observed. Subsequent virus doses were reduced to 0.8 x 10⁸ TCID₅₀/kg, and the dogs were monitored by video cameras for 24 h after each virus administration.

A grade 1 increase in ALP was detected in laboratory tests in dog 1 (Table 16). Dog 2 had low albumin (grade 1) already in the baseline samples, and it decreased further during the study, but the values did not exceed grade 1. In addition, both dogs had low bilirubin and cholesterol already before virus
administration. Haematology and urinalysis showed no significant changes.

**Figure 17** Rectal temperature of the dogs receiving oncolytic vaccinia virus. The number after dog number shows the infusion number. The horizontal dashed lines highlights the upper and lower limit of normal rectal temperature of the study dogs.

**Table 16** Clinical chemistry values of the dogs receiving oncolytic vaccinia virus. Results outside the reference values appear in boldface.

<table>
<thead>
<tr>
<th>Day*</th>
<th>Dog 1</th>
<th>Dog 2</th>
<th>Reference value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>ALP</td>
<td>111</td>
<td>373</td>
<td>63</td>
</tr>
<tr>
<td>ALT</td>
<td>43</td>
<td>53</td>
<td>64</td>
</tr>
<tr>
<td>Albumin</td>
<td>31.5</td>
<td>30.2</td>
<td>29.7</td>
</tr>
<tr>
<td>Bilirubin</td>
<td>1.4</td>
<td>1.4</td>
<td>2</td>
</tr>
<tr>
<td>Phosphate</td>
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<td>1.12</td>
<td>0.97</td>
</tr>
<tr>
<td>Glucose</td>
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<td>4.3</td>
</tr>
<tr>
<td>Potassium</td>
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<td>4.9</td>
<td>4.8</td>
</tr>
<tr>
<td>Sodium</td>
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<td>152</td>
<td>147</td>
</tr>
<tr>
<td>Calcium</td>
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<td>2.47</td>
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</tr>
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<td>3.2</td>
</tr>
<tr>
<td>Creatinine</td>
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<td>60</td>
</tr>
<tr>
<td>Urea</td>
<td>6.2</td>
<td>4.6</td>
<td>3.4</td>
</tr>
</tbody>
</table>

*From 1st virus infusion, Day 0 is baseline before virus infusion
ALP, alkaline phosphatase; ALT, alanine aminotransferase
5.2.4.2 Detection of the virus

Viral DNA was identified in the blood of both dogs in all samples by qPCR (Figure 18). The highest amount of viral DNA was present in the samples taken immediately after virus infusion. Although viral genomes decreased quickly during the first 4 h in blood, viral DNA was still measurable one week later. Transduction test and plaque assay detected infectious virus only in samples taken immediately after virus administration, but the amount of virus was too low to be quantified (Figure 19).

Detectable but not quantifiable amounts of viral DNA were discovered in three saliva and two urinary samples, but not in faeces (Table 17). Infectious virus was not identified in the transduction test or in the plaque assay.

In autopsy samples, virus DNA was mainly recovered from the spleen, and small amounts of viral DNA were discovered in most organs of dog 1 and in the lungs in dog 2 (Table 18).

---

**Figure 18**  Vaccinia genomes in the blood of dogs receiving intravenous infusion of oncolytic vaccinia virus measured by qPCR. The number after dog number shows the infusion number.

**Table 17**  Positive secretion samples for vaccinia virus genomes from the dogs receiving intravenous infusion of oncolytic vaccinia virus measured by qPCR. Number of DNA copies was <100/10 μl of purified DNA in all samples. Negative samples are not shown.
## Results

![Image](image_url)

**Figure 19** Vaccinia plaque on A549 cells confirming infectious virus in the blood sample taken immediately after intravenous administration of vvdd-hCd40L-tdTomato. The red colour detected with the fluorescence microscope shows transduction of tdTomato (A) and lysis of cell monolayer detected with light microscopy (B) indicates oncolysis of cells. Sample taken from dog 1 after first virus dose. Scale bar = 200 μm.

### Table 18 Positive secretion samples for vaccinia virus genomes from the dogs receiving intravenous infusion of oncolytic vaccinia virus measured by qPCR.

<table>
<thead>
<tr>
<th>Dog</th>
<th>Tissue</th>
<th>Copies/mg of genomic DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Spleen</td>
<td>9.9 x 10⁵</td>
</tr>
<tr>
<td></td>
<td>Adrenal glands, aorta, bone and joint, epididymis, oesophagus, gallbladder, gingiva, heart, kidneys, pancreas, sciatic nerve, pituitary gland, prostate, salivary gland, muscle, testes, thyroid, tongue, trachea</td>
<td>&lt; 100</td>
</tr>
<tr>
<td>2</td>
<td>Spleen</td>
<td>3.9 x 10⁵</td>
</tr>
<tr>
<td></td>
<td>Lungs</td>
<td>&lt; 100</td>
</tr>
</tbody>
</table>

### 5.2.4.3 Neutralizing antibodies and cytokines

At baseline, no vaccinia antibodies were monitored, but they increased after each virus infusion. At 2 weeks, the serum dilution that was able to block >50% of gene expression was 1:16, while at 4 weeks a 1:4096 fold dilution was required (Figure 20).

Cytokines showed no obvious changes. Indeed, we were only able to detect measurable levels of IL-2 in the baseline sample of dog 1, but no other cytokines. In dog 2, measurable levels of IL-2, -6 and -12p40 were detected, but the rest were under detection limits.
Serum neutralizing antibodies measured by transduction inhibition in dog 2 receiving vaccinia virus. Serum was heat inactivated, diluted and incubated with vvdd-luc, after which A549 cells were challenged in triplicate for the serum-virus mixture for 20 h until luciferase expression was measured. Values are presented as means and standard deviation.

5.2.4.4 Post-mortem examination, histology and immunohistology

No specific findings associated with virus administration emerged in the post-mortem examination. An incidental finding was that dog 1 suffered from aspermatogenesis.

Since vaccinia DNA was mainly found in the spleen, immunohistochemistry for vaccinia virions and β-galactosidase was performed only in that organ. Viral antigen was detected with the anti-vaccinia antibody in the cytoplasm of a few macrophages in the red pulp, consistent with phagocytosis of the virus (Figure 21). As expected, the anti-β-galactosidase antibody did not result in any staining since the virus cannot replicate in normal tissues and β-galactosidase is expressed only after viral replication.

5.2.5 SEMLIKI FOREST VIRUS IN A DOG MODEL (IV)

The dogs enjoyed a good quality of life throughout the study period, and no adverse events were monitored.

5.2.5.1 Adverse events

The dogs showed no clinical signs of adverse events. Haematology did not reveal any abnormalities, and only a minor decrease in serum albumin in addition to incidental findings of mild hypoglycaemia and hyponatraemia were observed. In addition, both dogs had low serum bilirubin, protein and
cholesterol already at baseline. The clinical chemistry results are shown in Table 19.

![Image of immunocytochemistry results]

**Table 19**  
**Serum clinical chemistry of the Beagles receiving SFV.** Results outside the reference values appear in boldface.

<table>
<thead>
<tr>
<th>Day*</th>
<th>Dog 1</th>
<th>Dog 2</th>
<th>Reference value</th>
</tr>
</thead>
<tbody>
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<td></td>
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<td>7</td>
<td>14</td>
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<tr>
<td>ALAT</td>
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<td>41</td>
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</tr>
<tr>
<td>Albumin</td>
<td>30.6</td>
<td>34.9</td>
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<tr>
<td>Bilirubin</td>
<td>1.3</td>
<td>1.3</td>
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<tr>
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</tbody>
</table>

*From 1st virus infusion, Day 0 is baseline before virus infusion  
ALP, alkaline phosphatase; ALT, alanine aminotransferase
5.2.5.2 Detection of the virus

Virus could not be recovered in any of the collected serum, urine or faecal samples. When the negative baseline faecal samples were spiked with $10^6$ pfu of SFV, recoveries after the freeze-thaw cycle were $6.25 \times 10^4$ pfu/0.1 g and $3.75 \times 10^5$ pfu/ml in dogs 1 and 2, respectively, and the corresponding recovery proportions were 6.25% and 37.5%. With the lower recovery proportion of 6.25%, the calculated detection limit for virus titration from faecal samples was 400 pfu/g.

5.2.5.3 Neutralizing antibodies

NAbs were not detected in baseline samples, but increased after virus administration; on day 9, the titres were 1250 and on day 21, 250 for both dogs. Our results thus suggest that both dogs had contracted the virus and developed a humoral neutralizing response to the virus.

5.2.5.4 Post-mortem examination, histology and immunohistology

No specific changes were found in the macroscopic or histological evaluation of the major organs and specific tissues. Virus antigen was not detected in any of the examined organs.
6 DISCUSSION

6.1 ANTI-TUMOUR ACTIVITY OF ONCOLYTIC VACCINIA AND SEMLIKI FOREST VIRUS IN CANINE AND FELINE CANCER CELLS, XENOGRAFTS AND TUMOUR BIOPSIES (I, II, IV)

Vvdd of Western Reserve and attenuated SFV were able infect and kill all cell lines examined (I, II, IV). Vvdd also delayed the growth of prostatic carcinoma xenografts in a subcutaneous nude mouse model and was able to infect fresh tumour biopsies (I). However, when feline squamous cell line SCCF1 was growing as an intact monolayer, oncolytic efficacy of vvdd was lost (II).

The oncolytic VV has been tested in canine cancer cell lines and xenograft tumour models (Gentschev et al. 2009, Gentschev et al. 2010, Gentschev et al. 2012, Patil et al. 2012, Gentschev et al. 2013) and one VV is currently being assessed in a clinical trial in dogs with cancer (Gentschev et al. 2013).

We started our studies with vvdd-luc because it has already been evaluated in murine and human cell lines (Guse et al. 2010). The other reason was that viruses expressing tdTomato and hCD40L were constructed later (Parviainen et al. 2014). Vvdd-luc was used for gene transfer analysis because the bioluminance of virus-expressed luciferase can be quantified numerically, allowing more exact evaluation of gene transfer. In addition, vvdd-luc was used as a reference virus in some other experiments.

Efficacy of transduction and oncolytic potency of viruses differed to some degree between cell lines, suggesting different permissiveness of cell lines to VV and SFV infections. At lower virus doses, vvdd-tdTomato killed more cells than hCD40L-expressing virus, which could be explained by slower replication of the virus containing more transgenes. Oncolytic efficacy of SFV in canine osteosarcoma cells was comparable to sensitive human or mouse cell (IV) (Vähä-Koskela et al. 2006, Ketola et al. 2008, Määttä et al. 2008, Heikkilä et al. 2010, Ruotsalainen et al. 2012, Ruotsalainen et al. 2014). Feline squamous cell line SCCF1 was resistant to vvdd-induced oncolysis when growing on a monolayer, but despite this the virus was still able to infect and transduce the cells (II). This cell line was also the only one that was able to form cell colonies after vvdd infection, suggesting marked oncolytic potency of our viruses (II).

Unfortunately, tumour-specific vvdd also efficiently transduced and killed canine non-tumourous kidney MDCK cells (I). Since MDCK is a quickly dividing cell line, VGF is not needed to stimulate cell division and numerous nucleotides are available for viral DNA synthesis without TK. Increased permissiveness of the cell line could also affect the results, but it is likely not characteristic of all kidney cells because mice or dogs in our studies (I, III) or in previous studies (Guse et al. 2010, Parviainen et al. 2014) did not have any pathological changes in kidneys.
Quick replication and production of new virions is an important feature of oncolytic virus. Tested cell lines produced very different amounts of new virions, which again could be explained by cell line permissiveness for VV infection. Quick oncolysis could also decrease the amount of virions produced due to the lack of virus-producing cells, but our results do not support this because prostatic carcinoma and kidney cells produced the highest amounts of infectious virus but were also the most sensitive to oncolysis in the MTS test (I).

In a subcutaneous xenograft tumour model, VV slowed the progression of canine prostatic carcinoma in nude mice, although the difference was only just statistically significant and none of the tumours regressed completely after virus injection (I). Vvdd-luc was chosen in that study because CD40L is efficient in immunodeficient mice and human CD40L is not active in mice, and we also wanted to evaluate VV-induced oncolysis with as little interference from transgenes as possible. CD40L-expressing virus should be more powerful in the treatment of cancer patients due to its immunostimulatory properties compared with vvdd-luc.

Apoptosis has been thought to be the main route of cell death in vaccinia-infected cells (Guo et al. 2005, Greiner et al. 2006, Liskova et al. 2011). Recently, programmed necrosis was reported to be the primary mode of cell death in ovarian cancer cells (Whilding et al. 2013). This is in line with our results, as we detected both apoptosis and necrosis in VV-treated xenograft tumours (I).

Administration of vvdd did not induce any detectable adverse events in mice, and necropsy showed no pathological changes associated with virus administration, as also reported previously (Guse et al. 2010, Parviainen et al. 2014). The virus was only found in the tumours and not in any organs, confirming tumour specificity of the virus.

von Euler et al. (2008) showed the activity of human CD40L in dogs by stimulating canine PBMCs with supernatant collected from canine prostatic carcinoma ACE-1 cells transduced with hCD40L-expressing adenovirus. We also confirmed the activity of hCD40L in canine PBMCs and showed increased levels of IL-8, suggesting monocyte activation following the protocol used in humans (Pesonen et al. 2012) (I). IL-8 was also measured in canine cancer patients treated with hCD40L gene therapy (Westberg et al. 2013).

Immortalized cell lines and xenograft tumours differ from clinical tumours in many ways: spontaneous tumours are more heterogenic both intratumourally and between individuals than transplanted tumours, cancer cell kinetics differ from naturally occurring tumours, tumour development is usually very quick in laboratory rodents compared with pets or humans, xenograft tumours are commonly grown in immunodeficient rodents lacking a normal intact immune system (Vail & MacEwen 2000, Vail & Thamm 2011, Ranieri et al. 2013) and the tumour microenvironment is different (Julien et al. 2012, Williams et al. 2013). Considering these differences, we infected biopsies of suspected canine naturally occurring tumours with our virus. We
did not have a diagnosis of all biopsies beforehand, and thus, benign lesions and hyperplastic tissues were also included. VV was able to transduce tumour cells and form plaques, although transduction of tdTomato was not detected in three malignant tumours.

Transient transduction was detected on day 1 in hyperplastic tissue, benign tumours and one grade II mast cell tumour, suggesting either that the virus can infect the cells but is unable to replicate in the tissues or that the cells died. Viability of naturally occurring tumour tissues in the laboratory is a problem, and to avoid it cancer cell lines are commonly used. Production of tdTomato in hyperplastic and benign lesions could be due to the high proliferative rate of cells and an activated EGRF pathway. Rudin et al. (2003) used oncolytic adenovirus to treat premalignant human oral dysplasia, and therefore, the thought of using VV in hyperplastic and benign lesions is appealing.

6.2 PRODUCTION OF VACCINIA VIRIONS IN FELINE
SCCF1 CELLS IN THE ABSENCE OF ONCOLYSIS (II)

Although feline squamous cell carcinoma cell line SCCF1 was resistant to vvdd-induced oncolysis when growing on an intact monolayer, the virus was able to replicate in the cells, but the produced virions were morphologically abnormal.

Two poxviruses, myxoma virus (MacNeill et al. 2012) and VV (Adelfinger et al. 2014), are the only oncolytic viruses investigated in feline cancer cell lines. The SCCF1 cell line used here was also used in the myxoma virus study (MacNeill et al. 2012). In their study, SCCF1 cells were found to be more resistant to virus-induced oncolysis and the number of viable cells after virus infection was not significant relative to the control group, suggesting resistance to myxoma virus-induced oncolysis. Also, SCCF1 showed less apoptotic cells than mammary carcinoma cells. These results are partly in line with our findings.

Poxviruses are long-known human and animal pathogens and some of them are regarded as zoonotic. Sequencing of the poxvirus genome has offered more specific knowledge about host cell infections, virus replication and virulence factors of VV, but there is still a lack of information on the transmission cycle in nature, and the origin and reservoir of VV-inducing pox disease in milkers in South America remain unknown (Essbauer et al. 2010, Kroon et al. 2011). Recently, molecular and biological characterization has revealed differences between isolates, indicating that multiple different strains are involved in outbreaks (Kroon et al. 2011).

In contrast, the life cycle of VV in the host cell is well characterized (Moss 2006). VV produces four different types of virions: intracellular mature virus (IMV), intracellular enveloped virus (IEV), cell-associated enveloped virus (CEV) and extracellular enveloped virus (EEV) (Ward & Moss 2001). Since we wanted to emphasize the identification of different mid-stage immature particles, we chose to use the old nomenclature in our study. Virions remaining
inside the cell lack the outer envelope found in the virions released outside the cell.

IMVs are stable virions that enable efficient transmission between hosts, whereas IEVs are formed by wrapping IMV with intracellular membranes and facilitate the transfer of the virus to the cell surface via microtubes before converting to CEV (Ward & Moss 2001). CEVs utilize actin tail to move and infect nearby cells (Ward & Moss 2001), while EEVs, containing an additional host cell-derived envelope, mediate virus dissemination in the circulation (Payne 1980). The production of different VV particles promotes efficient virus spread and aids escape from complement and antibodies (Vanderplasschen et al. 1998, Hollinshead et al. 1999, Smith et al. 2002).

Several virus-encoded proteins are involved in VV morphogenesis (Liu et al. 2014). One of them, viral protease I7, is essential in cleavage of VV membrane and core components in both early and late stages of virus assembly and is critical for transition from immature virus with nucleoids (IVN) to mature virus (MV) (Ansarah-Sobrinho & Moss 2004). Lack of I7 causes arrest of VV morphogenesis, with similar features as those observed in SCC1 cells infected with vvdd, i.e. formation of immature viruses (IVs) and IVNs, but no MVs.

Other identified virus-encoded proteins include F10, A14, A17, A11 and A6, among others (Traktman et al. 1995, Meng et al. 2012, Unger et al. 2013, Maruri-Avidal et al. 2013). Defect in F10 kinase induces arrest in VV morphogenesis at the earliest stage of virion morphogenesis, before membrane crescents or IMs are formed (Traktman et al. 1995). Crescent membrane formation is further prevented by the repression of A14 and A17 transmembrane proteins (Unger et al. 2013), and empty immature virion-like structures and masses of viroplasm are formed when the synthesis of A11 is prevented (Maruri-Avidal et al. 2013). In addition, lack of A6 prevents synthesis of viral membranes and results in the accumulation of large viroplasm inclusions containing virion core proteins (Meng et al. 2012).

As discussed above, VV maturation and morphogenesis may be affected by defects in viral proteins. Our results suggest that host cells could similarly be impacted in virus production. The lack of MVs seems to result from an inability of VV particles to form the core unit from viral DNA within SCCF1 cells. Although the crescent can close to form IV, the viral genome DNA is not packed or condensed to form the core unit. The loose nucleosome threads need some core nucleoproteins to further condense and encapsidate. Since no MV was detected by electron microscope, the transition of IVN to MV was completely arrested in SCCF1 cells. As viral genome encodes all of the core and membrane proteins necessary for virion maturation, our results imply that the SCCF1 cell line is not expressing some of the cellular co-factor target proteins crucial for the virus or that they are defective. Recognizing the mechanism behind this phenomenon would offer valuable information regarding virus and cell interaction.
6.3 SAFETY AND BIODISTRIBUTION OF ONCOLYTIC VACCINIA AND SEMLIKI FOREST VIRUS IN LABORATORY BEAGLES (III, IV)

Intravenous infusion of both VV and SFV was well tolerated, and only mild adverse events were monitored, with the exception of a potential unconfirmed seizure in dog 2 after receiving VV (III, IV). Infectious VV was detected only in the blood samples taken immediately after virus administration, and infectious SFV was not found in any of the samples. Necropsy revealed no changes associated with either virus, including no abnormalities in the central nervous system in dogs receiving SFV.

Clinical adverse events
SVF administration did not cause any clinical signs in our study dogs, consistent with oncolytic adenovirus administered intravenously to healthy dogs (Smith et al. 2006) and intratumourally to tumour-bearing pet dogs (Laborda et al. 2014). In contrast, oncolytic VV induced mild fever and a possible seizure, which goes in line with the mild to moderate fever and self-limiting nausea and vomiting reported in a dose escalation study of vesicular stomatitis virus (VSV) (LeBlanc et al. 2013). VV is more immunogenic than SFV, which could at least partly explain the lack of adverse events in dogs receiving SFV. In addition, VV dose was higher than SFV, but since infectious dose of neither of these viruses in dogs is known, comparison is difficult.

Onset of the suspected seizure in dog 2 receiving VV was not observed by staff (III). We found dog 2 lying on the floor in lateral recumbency and dog 1 was attacking him. The dogs had fought previously, but signs inconsistent with a fight included that dog 2 did not stand up immediately when dog 1 was removed and his limbs were extended, but not stiff, when he was picked up. After dose reduction, no further adverse events were monitored. We classified the episode as a grade 3 seizure following VCOG-CTCAE guidelines (Veterinary cooperative oncology group 2004). VCOG-CTCAE grade 2 seizure is a brief generalized seizure, as in dog 2, and grade 3 is a seizure in which consciousness is altered. Contradictorily, in generalized epileptic seizures dogs’ consciousness is always decreased (Platt & Olby 2004).

Idiopathic epilepsy is common in dogs, and although no signs typical of a grand mal seizure, such as unconsciousness, tonic-clonic motor activity, drooling, urinating or defecating, were witnessed in dog 2, idiopathic epilepsy cannot be ruled out. About 0.1% of the dogs in the Harlan colonies have epileptic seizures, but dog 2 or his relatives did not have a history of epilepsy (Stephen Hillen, Harlan Laboratories, personal communication January 2012).

The most likely reason for the suspected seizure is a hypersensitivity reaction associated with cytokine production induced by the virus or other components in the virus solution. Pro-inflammatory cytokines modulate brain excitability and predispose to seizures, but do not usually induce seizures...
alone, instead, they decrease the threshold for seizures from other causes (Galic et al. 2012). TNF-α has been shown to increase neuronal excitability (Galic et al. 2012), but we did not find increased TNF-α or other cytokine levels in dog 2. In addition, hypersensitivity reactions usually worsen in the next contact with the foreign antibody, but dog 2 did not have any symptoms after the next three virus infusions.

The risk and extent of hypersensitivity reaction are difficult to evaluate, but the more homologous the foreign and host protein are, the less likely a hypersensitivity reaction is (Kaneko et al. 2008). Our virus preparation contained 25.7 μg/ml of host cell protein and 3.4 μg/ml of bovine serum albumin, corresponding to 52.4 μg of foreign protein in the first dose received by dog 2. Human serum albumin is used to treat critically ill dogs with hypoalbuminaemia, and the homology between human and canine albumin is 91.7% (Kaneko et al. 2008). No severe hypersensitivity reactions or seizures were monitored in any of 418 dogs receiving 1 g of human albumin/kg/d (Vigano et al. 2010); however, type III hypersensitivity reaction was reported in two dogs (Powell et al. 2013). Thus, if dog 2 experienced a seizure, we think it was due to the high dose of immunogenic VV rather than the small amount of human or bovine protein residues in the virus solution.

High fever may induce seizures, especially in children, and they are associated with increased levels of IL-6 (Hu et al. 2014). Rectal temperature of dog 2 was only mildly increased, and we did not detect an increase in IL-6, making a fever seizure highly unlikely. Another differential diagnosis for a seizure is collapse due to hypotensive shock or cardiovascular or respiratory disease, but the dog’s normal blood pressure immediately after the event, the lack of other clinical signs and the lack of abnormalities in necropsy do not support this.

Seizures or collapses have not been monitored in any of the human patients treated with oncolytic VV, but fever and lethargy are reported as the most common adverse events (Mastrangelo et al. 1999, Park et al. 2008, Breitbach et al. 2011, Hwang et al. 2011, Jaime et al. 2012, Heo et al. 2013, Zeh et al. 2015). Fever and lethargy are also common symptoms in inflammation and viral infections and are most likely due to an innate immune response to the virus (Cerullo et al. 2012a, Cerullo et al. 2012b). The body temperature of dog 1 receiving VV was just at the lower limit of grade 1 fever and he also had grade 1 lethargy (III). Dog 2 receiving VV had a mild increase in rectal temperature after the first virus administration, immediately after the potential seizure, but no lethargy (III).

Adverse events in haematology and clinical chemistry
Haematology and differential blood count did not show any marked changes in any of the study dogs and was supported by histology of the bone marrow collected at the time of necropsy (III, IV). In humans, decreases in lymphocyte, platelet and red blood cell counts and neutrophilia have been reported (Park et al. 2008, Heo et al. 2013, Zeh et al. 2015). Lack of haematological changes
Discussion

in our dogs may be due to absence of virus replication since they did not have tumours.

Lymphopenia was found in healthy Beagle dogs receiving oncolytic VSV (LeBlanc et al. 2013), but lymphocyte count increased in 5 of 6 healthy dogs receiving oncolytic canine adenovirus and 3 of them had lymphocytosis (Smith et al. 2006). In addition, neutrophil count decreased in all dogs, but half of the dogs were immunosuppressed with cyclophosphamide. Viral infections are often associated with lymphopenia, although post-vaccinal lymphocytosis due to chronic immunostimulation is reported in dogs (Nelson & Couto 2009). It has been suggested that lymphopenia is regulated by type 1 interferons, which are the principal cytokines involved in viral infection, and that lymphocytes remain attach to the endothelium and do not migrate to tissues (Kamphuis et al. 2006).

Virus detection

The copy number of the VV genome was highest in the blood samples taken immediately after virus infusion, quickly declining thereafter (III). In human cancer patients, a secondary peak of viraemia may be detected, suggestive of viral replication in the tumours (Park et al. 2008, Hwang et al. 2011, Heo et al. 2013, Zeh et al. 2015). The long stability of VV genomes in the circulation of dogs with no tumours was not anticipated, but suggests slow clearance of viral genomes from the blood.

We only found infectious virus from the blood samples collected immediately after virus infusion (III). The amount of infectious virus was too low to be reliably quantified. Complement severely inhibited VV delivery in a recent study (Sampath et al. 2013); this may have affected our results as well, in addition to innate immune response.

Infectious SFV was not detected in the blood of the dogs (IV), agreeing with an earlier VSV study (LeBlanc et al. 2013). However, since the virus was inactivated during the administration protocol, the detection limit for infectious virus may have been too low. In adult mice, attenuated SFV induces a transient viraemia lasting up to 4 days (Jagelman et al. 1978, Fazakerley et al. 1993). Susceptibility to infection and virus replication may be different between species and may account for the absent virus recovery in the present study and could also explain the differences between VV and other viruses tested in dogs. We did not measure viral genome copy number in the SFV experiment by PCR since our interest was in the risk of possible spreading of the virus (IV). PCR results may have offered interesting information on how quickly the virus clears from the circulation. Genomes of oncolytic adenovirus were detected in all canine blood samples until euthanasia at 4 days post-administration (Smith et al. 2006).

At necropsy, small amounts on viral genomes were detected in multiple organs in dog 1 euthanized 24 h after virus infusion. In dog 2, the virus was mainly found in the spleen, suggesting wide spreading of the virus via circulation soon after virus administration and then gathering to the organ
consisting of immune tissue (III). Genomes of oncolytic VSV were detectable in the adrenal gland, kidney, spleen and testes one month after virus administration, and genomes of oncolytic adenovirus were mainly in the liver and spleen, but low numbers were detected also in other organs 4 days after virus administration (Smith et al. 2006).

**Histopathological findings**
Aspermatogenesis seen in dog 1 receiving VV (III) was the only significant pathological finding in our study dogs. In dogs, each spermatogenic cycle lasts approximately 2 weeks and spermatogenesis 2 months (Soares et al. 2009). Since the dog was euthanized 24 h after virus administration, aspermatogenesis had to be present before our study. We also discovered that dog 1 did not have any offspring (Stephen Hillen, Harlan Laboratories, personal communication January 2012) despite the fact that he was a breeding male.

Importantly, dogs receiving SFV had no pathological changes or cellular infiltrates in the central nervous system (IV). Mild CNS inflammation and demyelination are commonly noted in immunocompetent mice after SFV A7(74) infection (Suckling et al. 1978, Kelly et al. 1982). CD8+ T cells mediate demyelination by destruction of infected oligodendrocytes (Subak-Sharpe et al. 1993). Demyelination is not seen in athymic nude mice lacking T cells, but instead SFV A7(74) administration results in persistent infection (Jagelman et al. 1978). Antibodies are not associated with demyelination, although they are necessary to clear the infection from the brain (Fragkoudis et al. 2008). Signs of remyelination were observed 15 days post SFV infection (Mokhtarian et al. 2003). Since we collected histological samples only at necropsy 21 days after virus administration, we may have missed mild lesions due to the ongoing healing process. Other possibility is that the virus never entered the CNS since we did not detect viraemia in our dogs.

**Biosafety of viral vectors**
Infectious SFV was not found in the blood of the study dogs (IV), and thus, transmission of SFV by mosquito bites is unlikely. Neither infectious VV nor SFV was detected in any of the secretion samples collected, but small amounts of VV genomes were present in a few saliva and urine samples (III). This agrees with the results of dogs receiving VSV, where low numbers of viral genome copies were detected in urine and buccal swabs up to 3 weeks after virus administration. Further, low viral genome numbers were detected in some faecal and urine samples from the dogs receiving oncolytic adenovirus (Smith et al. 2006).

The dogs in our study did not have tumours to allow viral replication, and thus, the viral load may be higher in dogs bearing cancers. Pet dogs treated with oncolytic VV should not lick humans. Urine may also pose a biosafety risk for spreading the VV into the environment.
In theory, recombination of the study virus with wild-type orthopoxvirus might occur if the cell is co-infected with both the study virus and another orthopoxvirus, although double-strand DNA viruses, such as VV, are usually stable (Brochier et al., 1996). Since the vvdd-tomato-hCD40L virus does not contain any genes increasing pathogenicity and transgenes make it more immunogenic, in a rare case of recombination the new virus would be less likely to induce infection than wild-type orthopoxviruses.

Cytokine profile and neutralizing antibodies
We were not able to show any specific changes in cytokine levels in dogs receiving oncolytic VV (III). Recently, serum cytokines were measured in dogs receiving CD40L gene therapy (Westberg et al. 2013). Neither TNF-α nor IL-10 was detected in the dogs, but IL-8 decreased in all but one dog after the treatment. In contrast, increased levels of TNF-α, INF-γ and IL-6 have been found in plasma of human patients receiving intravenous infusion of oncolytic VV; the highest concentration was measured 8 h after virus delivery (Breitbach et al. 2011). In addition, IL-10 increased 4-8 days after virus delivery and IL-4 decreased transiently. The method that we used for cytokine detection has not been well characterized in dogs, which could partly explain our inability to detect cytokines.

Both VV and SFV induced high levels of NAbs (III, IV). Also healthy dogs receiving oncolytic VSV had detectable antibodies from day 5 (LeBlanc et al. 2013), and dogs receiving oncolytic adenovirus had increased antibody titres already on day 4 (Smith et al. 2006). The results are also in agreement with human patients receiving oncolytic VV (Mastrangelo et al. 1999, Park et al. 2008, Breitbach et al. 2011, Hwang et al. 2011, Jaime et al. 2012, Heo et al. 2013, Zeh et al. 2015) and mice receiving SFV (Rodriguez-Madoz et al. 2007). Clinical tumour responses do not correlate with vaccinia antibody titres in humans (Mastrangelo et al. 1999, Park et al. 2008) so significance of anti-viral antibodies remains uncertain. Even if the antibodies neutralize VV, the EEV form is not inactivated by antibodies (Sampath et al. 2013) and spread of the virus occurs via the circulation to metastatic lesions.

In immunocompetent mice, IgM antibodies for SFV were detected already 4 days after virus inoculation, peaked on day 5 and then gradually decreased by day 18 and disappeared (Fazakerley et al. 1993). IgG antibodies were detected 6 days after exposure to virus and increased still at the end of study on day 28 (Fazakerley et al. 1993). Mice receiving oncolytic SFV ≥10^6 virus particles (vp) intravenously had detectable NAbs 15 days after virus administration, whereas a smaller dose did not induce NAbs (Rodriguez-Madoz et al. 2007). When the virus was given intratumourally at a dose of 10^7 or 10^8 vp, re-infection with 10^8 vp of luciferace expressing virus was only moderately inhibited, suggesting that SFV can be given intratumourally multiple times despite antibody formation. Also, antibody formation against SFV vector did not prevent anti-LacZ antibody development when given to SFV primed mice, but increased the amount of SFV virions necessary in
booster injections to increase serum IgG levels suggesting that SFV is not very immunogenic and recombinant SFV vectors can be successfully used as a vaccine in mice (Berglund et al. 1999). Patients with clinical disease are likely to have higher antibody titres than unsymptomatic individuals, although our dogs had very high titres after virus administration.

### 6.4 LIMITATIONS OF THE STUDY

We investigated the oncolytic efficacy of VV and SFV in only a few canine cell lines and in only one feline cell line. Including more cell lines from different cancer types would have yielded more information about the efficacy of the viruses in multiple cancer types. We could have also used other xenograft models in addition to the canine prostatic cancer model and more mice in each study to increase statistical significance. In addition, starting the treatment when tumours were smaller might have improved our results since the cancer model was quite aggressive and led to early euthanasia of the mice.

Since we aimed to treat solid tumours with VV, more biopsies and especially from solid tumours including soft tissue sarcomas with an activated EGRF factor pathway should have been included in our experiments. We did not evaluate the EGRF pathway at all in our studies. We could have also tried to quantify the amount of produced virions from the biopsy samples to determine whether VV can replicate in tumour tissues.

Including more dogs in our safety and biodistribution studies would have yielded more reliable results. From an ethical standpoint, however, we did not want to include more dogs since the information received from non-cancer-bearing dogs is not comparable to dogs with tumours since the viruses replicate in tumour cells, and thus, the viral load is likely much higher in tumour-bearing dogs. The dose of VV was very high and since we planned to inject the virus intratumourally into pet dogs, at least the acute adverse events were likely to be less with a smaller intratumour dose.

In contrast, the dose of SFV could have been higher. However, even 3–8 pfu of attenuated SFV strain can induce 50% protection in mice against the lethal SFV strain, but 20 pfu is necessary for 100% protection depending on the inoculation route and also the animal species (Bradish et al. 1971, Bradish & Allner 1972, Bradish et al. 1972). In rabbits, 20 pfu is required for 50% protection in addition to productive replication, although viraemia is not necessary (Barrett & Dimmock 1984, Kraaijeveld et al. 1984). Thus, SFV infection in heathy animals is self-amplifying. Indeed, we are planning to use the same virus dose that the study dogs received in a clinical trial, but will administer it intratumourally, and therefore, it is unlikely that more pronounced viraemia will be caused even if the virus replicates in tumour tissue.
6.5 FUTURE PLANS

We have opened a clinical trial with vvdd-hCD40L-tdTomato for dogs with incurable solid tumours. For SFV, more tumour cell lines, especially glioma cells, will be tested for oncolytic capacity of the virus and also the efficacy could be evaluated in tumour biopsies. If the virus proves effective, we intend to open a clinical trial for dogs with glioma as well.

Due to the similarity between canine and human spontaneous tumours (Ranieri et al., 2013, Vail and Thamm, 2011, Vail and MacEwen, 2000), the information yielded from the clinical trials in dogs is anticipated to also benefit human oncology and to reveal key safety and efficacy aspects not evident in rodents.
7 CONCLUSIONS

Based on these studies, the following conclusions can be drawn:

1. Oncolytic vvdd infected and killed canine osteosarcoma (Abrams and D17) and prostatic carcinoma (ACE-1) cell lines, infected canine tumour biopsies and slowed tumour growth in nude mouse prostatic carcinoma (ACE-1) xenograft. Thus, oncolytic vaccine virus expressing hCD40L has anti-tumoural efficacy against tested canine cell lines in vitro and in vivo.

2. Oncolytic vvdd infected and killed feline squamous cell line SCCF1 cells, but the killing efficacy was lost when the cells grew in an intact monolayer. Infectious virions were still produced, but they were morphologically abnormal probable due to their inability to form core units from viral DNA and to form mature virions. Specific features of the cell line may be responsible for the abnormal morphology.

3. Intravenous administration of a high dose of oncolytic Western Reserve vaccinia virus vvdd-tdTomato-hCD40L was safe in healthy dogs despite the potential seizure in one dog. Infectious virus was only found in the blood samples taken immediately after virus infusion and not in saliva, urine or faeces.

4. Attenuated SFV infected and killed tested canine osteosarcoma cell lines (Abrams and D17), and intravenous administration of the virus caused no adverse events in healthy dogs and infectious virus was not found in blood, urine or faeces. Based on our results, attenuated SFV and its derivatives are potential candidates for further studies in canine cancer cell lines, tumour biopsies and finally tumour-bearing dogs.
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