**Faculty of Medicine**
Doctoral Programme in Clinical Research
University of Helsinki

# MECHANISTIC INSIGHTS INTO ONCOLYTIC ADENOVIRUS THERAPY

**Camilla Heiniö**

DOCTORAL DISSERTATION

To be presented for public discussion with the permission of the Faculty of Medicine of the University of Helsinki, in Lecture Hall 1, Haartman Institute on the 20th of January, 2023 at 12 o'clock.

Helsinki 2023

6/2023

ISBN 978-951-51-8835-9 (softcover)
ISBN 978-951-51-8836-6 (PDF)
ISSN 2342-3161 (print)
ISSN 2342-317X (online)
Unigrafia
Helsinki 2023

**Supervisor**
Akseli Hemminki, MD., PhD
Professor of Oncology
Department of Medicine
Programme of Translational Immunology,
Research programe unit
University of Helsinki, Finland
And
Helsinki University Hospital
Comprehensive Cancer Center, Finland

**Thesis committee**
Maria Södelund-Venermo
Adjunct professor, University Lecturer
Department of Virology
University of Helsinki

Seppo Meri
Professor of Immunology
Department of Bacteriology and Immunology
University of Helsinki

**Opponent**
Nadine van Montfoort
Group leader, Adjunct professor
Oncode Institute, Leiden University Medical Center, Netherlands

**Pre-examiners**
Leena Latonen
Group leader, Deputy director
Institute of Biomedicine, University of Eastern Finland

Kirsi Rautajoki
PI, University lecturer
Faculty of Medicine and Health Technology, Tampere University

# 1 ABSTRACT

The effects of cancer immunotherapy are based on activating and utilizing the immune system to fight cancer cells. In this thesis, a form of immunotherapy, specifically oncolytic adenoviruses, were studied. Promising results have been achieved in the clinic with oncolytic viruses. Therefore, this cancer modality should be further explored and developed.
Immunotherapies are costly to produce and different oncolytic viruses are used in relatively high concentrations and amounts when cancer patients are treated. Thus, it is important to consider factors that affect the production and infection efficacy of the final product. In this study we wanted to optimize adenovirus vector design, enhance the delivery of adenoviruses to the tumor as well as define and show the immunological response to the adenovirus vector we have developed. This information can be used to enhance treatment outcome. It was shown, that if adenoviral vectors were constructed within 105% of original genome size, the physical and functional titers of the produced vectors were relatively stable. However, production was negatively affected if very large constructs (such as antibodies) were inserted into the virus genome. Similarly, more than five mutations in the genome also negatively affected the outcome.
Adenoviruses are attractive vectors due to their good safety profile and high production concentrations (titers up to $10^{12-13}$ VP/ml). Additionally, the adenovirus genome and capsid size allows for insertion of several genes, thus providing several opportunities to modify the vector as desired.
Due to the positive features of oncolytic adenoviruses, an oncolytic adenovirus was created specifically for treatment of solid tumors in combination with T cell therapy. This oncolytic adenovirus is called A5/3-E2F-d24-hTNFa-IRES-hIL2 (aka. TILT-123). The virus has a chimeric fiber protein, thus increasing affinity to receptors that are often expressed on cancer cells (desmoglein 2), a E2F promotor, a 24 base pair deletion for cancer-cell specificity, and two cytokine-coding genes for immune stimulation and microenvironment modulation. Studies have shown that this virus is very effective in reduction of tumor burden and has a 100% cure rate *in vivo* when combined with T cell therapy.
When oncolytic virotherapy was originally conceptualized and the first studies were conducted, it was believed that the lytic effect of the virus was driving the benefits for patients and tumor burden reduction. Later, it was shown that other signaling events, such as danger- and pathogen-associated molecular pattern signaling, also play important roles, maybe even more so than virus-induced lysis of cancer cells. Therefore, pattern recognition receptor(PRR) signaling during TILT-123 infection was studied. We showed that TILT-123 induces alarmin release through PRRs (such as AIM2) that trigger the activation of immunostimulating signaling cascades, leading to immune-cell activation and tumor clearance. The knowledge of which signaling cascades are activated during infection can be used to further

enhance effects of TILT-123 and be extended to enhance the efficacy of other virotherapeutics as well.
Since adenoviruses are frequently the causative agent of the common cold, it is likely that most humans have encountered the virus and thus have immune memory towards it. Therefore, when adenovirus vectors are administered intravenously for treatment purposes, pre-existing immunity among other virus clearing reactions, leads to unwanted early clearance of the virions from the blood, before the vectors can reach their target. Consequently, methods to avoid early clearance were studied in this thesis. Some previous studies indicated that viruses may be able use cells in the blood, such as erythrocytes and lymphocytes, to "hitchhike" and hide from immune clearance.
Simultaneously, the benefits of T cells and TIL therapy to cancer patients have been proved. Combining these pieces of information, we hypothesized in this thesis that if a mixture of both TILT-123 and tumor infiltrating lymphocytes TILs are administered intravenously, we may achieve higher efficacy and eliminate the need for intratumoral injections. Intratumoral injections can be difficult to perform and require specialized personnel and equipment. Indeed, in our studies we observed that TILT-123 did bind to the surface of T cells. When injected intravenously together, this appeared to cause a positive tumor-clearing effect *in vivo* and showed that hard-to-performe intratumoral injections can be possibly avoided through utilization of carrier cells
Even though better cancer treatment methods have been developed, unfortunately, some cancer types are still hard to treat. For example, ovarian cancers are often diagnosed at late stages when the tumor size and metastases may hinder effective treatment of the disease. Furthermore, the tumor microenvironment in ovarian cancers is often immunosuppressive. To solve these issues, the effects of TILT-123 on ovarian cancer was studied. It was shown that TILT-123 can be employed to treat ovarian cancer. TILT-123 treatment modified the immunosuppressive microenvironment and enabled a more successful combinatorial treatment with T cell therapy.

## 1.1 TIIVISTELMÄ (FINNISH ABSTRACT)

Immunoterapioiden vaikutus perustuu immuunipuolustuksen aktivoimiseen ja valjastamiseen syövän vastaiseen taisteluun. Tässä väitöskirjassa on tutkittu immunoterapian yhtä muotoa, viroterapiaa, ja tarkemmin sanottuna onkolyyttisiä adenoviruksia. Adenoviruksia on valjastettu moneen käyttöön bio- ja lääketieteessä. Tästä syystä virusten tuottoa, kuten tuotetun virusten määrää, laatua ja infektiivisyyttä, tutkittiin tässä työssä. Halusimme optimoida adenovirusvektoreiden tuottoa ja toimivuutta, edistää virusten pääsyä kasvaimiin, sekä tutkia immunologisia reaktioita kehittämäämme TILT-123 virukseen. Tutkimuksessa huomattiin, että adenovirukselle tehdyt modifikaatiot vaikuttavat viruksen tuottoon ja tuotettujen virioneiden

infektiivisyyteen hyvin vähän. Aikaisemmat tutkimukset ovat osoittaneet, että viruksen muokkaukset eivät vaikuta viruspartikkelien tuotantoon tai infektiivisyyteen mikäli muokkauksen jälkeen genomin koko on edelleen noin viisi prosenttia alkuperäisestä koosta. Tutkimuksessamme todistimme, että tämä pitää paikkansa. Adenovirusten 5/3-kimeerisyyden todettiin laskevan tuotettujen viruspartikkelien määrää, muttei vaikuttavan merkittävästi tuotetuttujen virusten infektiivisyyteen. Tämän lisäksi tutkimuksessa ilmeni, että virustuotannon määrä ei muuttunut, mikäli immuunipuolustusta stimuloivia tai vasta-aineita koodaavia geenejä lisättiin adenoviruksen genomiin. Vaikka virustuotannon määrä ei muuttunut, yllä mainituilla geeneillä oli kuitenkin negatiivinen vaikutus tuotettujen viruspartikkelien infektiivisyyteen.

Viroterapiasta on saatu eläinmalleissa ja kliinisissä kokeissa parempia vasteita, kun adenovirusten genomiin on lisätty immuunipuolustusvastetta muokkaavien molekyylien koodaavia geenejä. Esimerkiksi TILT-123 on muunneltu kimeerinen adenovirus (Ad5/3-E2F-d24-hTNFa-IRES-hIL2) joka koodaa kahta sytokiinia: TNFa ja IL2. Eläinmallissa huomattiin kyseisen TILT-123-viruksen yhdistettynä T-soluterapiaan parantaneen kaikki hoidetut eläimet.

Kun onkolyyttisiä viruksia alettiin käyttämään syövän hoitoon, ajateltiin, että virusten aiheuttama syöpäsolujen lyysis olisi virusten tärkein ominaisuus syövän hoidossa. Myöhemmin kuitenkin huomattiin, että immunologisilla reaktioilla virusta vastaan on myös tärkeä rooli viroterapiassa. Soluilla on monta tapaa tunnistaa viruksia ja esimerkiksi pattern recognition receptors (PRR)-reseptorit ovat oleellisessa roolissa taistelussa infektioita vastaan. Näitä samoja reseptoreita, sekä reseptoreista aiheutuvia vasteita, voidaan käyttää viroterapiassa vahvistamaan syövänvastaisia immuunireaktioita. Tämän takia tutkittiin tässä työssä mitkä PRR signalointikaskadit aktivoituvat TILT-123 infektion aikana. Tällöin todistettiin, että TILT-123 aktivoi tutkitusta PRR-reseptoreista AIM2-reseptoria, muttei oletettua toll- like receptor 9 (TLR9). Tätä tietoa voidaan käyttää syöpähoidon tehostamista varten, ja tieto mahdollistaa paremman hoitovasteen luomisen.

Viroterapiaa käytettäessä syöpähoitona virusta voidaan annostella eri tavoilla kuten kasvaimensisäisesti (i.t.) tai suonensisäisesti (i.v.). Potilaan kannalta i.v. annostelua pidetään i.t:tä mielekkäämpänä hoitotapana muun muassa helpon toteutettavuuden takia. I.v.- injektio myös edesauttaa virusten leviämistä tehokkaasti koko kehoon ja mahdollisiin metastaaseihin. Tutkimuksessamme huomattiin hoidon tehostuvan kun viroterapia annosteltiin i.v. injektiona yhdessä T-solujen kanssa. Todennäköisesti adenovirukset pystyivät kulkeutumaan T-solujen pinnalla tai solunsisäisesti välttäen vasta-aineneutralisaatiota ja eliminaatiota esimerkiksi Kupffer-solujen myötä.

Vaikka syövän hoitomuodot ovat kehittyneet huimasti, monet syövät ovat yhä vaikeasti hoidettavia. Esimerkiksi munasarjasyöpäpotilailla on monesti huono ennuste, koska syöpä huomataan usein myöhäisessä vaiheessa. Tuolloin kasvaimen mikroympäristö on kehittynyt immuunipuolustusta hiljentäväksi

ja kasvaimen koko ja levinneisyys vaikeuttavat hoitojen toimivuutta entisestään. Tutkimuksessa todettiin, että TILT-123-terapia muokkasi munasarjakasvainten mikroympäristöä ja mahdollisti T-soluterapian toimivuuden.

# LIST OF CONTENTS

# 2 LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

I Heiniö C, Sorsa S, Siurala M, Grönberg-Vähä-Koskela S, Havunen R, Haavisto E, Koski A, Hemminki O, Zafar S, Cervera-Carrascon V, Munaro E, Kanerva A, Hemminki A. Effect of Genetic Modifications on Physical and Functional Titers of Adenoviral Cancer Gene Therapy Constructs. Hum Gene Ther. 2019 Jun;30(6):740-752.

II Santos JM, Heiniö C, Cervera-Carrascon V, Quixabeira DCA, Siurala M, Havunen R, Butzow R, Zafar S, de Gruijl T, Lassus H, Kanerva A, Hemminki A. Oncolytic adenovirus shapes the ovarian tumor microenvironment for potent tumor-infiltrating lymphocyte tumor reactivity. J Immunother Cancer. 2020 Jan;8(1):e000188.*

III Heiniö C, Havunen R, Santos J, de Lint K, Cervera-Carrascon V, Kanerva A, Hemminki A. TNFa and IL2 Encoding Oncolytic Adenovirus Activates Pathogen and Danger-Associated Immunological Signaling. Cells. 2020 Mar 26;9(4):798.

IV Santos J**, Heiniö C**, Quixabeira D, Zafar S, Clubb J, Pakola S, Cervera-Carrascon V, Havunen R, Kanerva A, Hemminki A. Systemic Delivery of Oncolytic Adenovirus to Tumors Using Tumor-Infiltrating Lymphocytes as Carriers. Cells. 2021 Apr 22;10(5):978.

The publications are referred to in the text by their roman numerals.
* This article has been used in João Santos dissertation 2020
** Shared first authorship

# 3 ABBREVIATIONS

| | |
|---|---|
| ACK | Ammonium-Chloride-Potassium |
| ACT | Adoptive cell therapy |
| Ad5 | Adenovirus type 5 |
| Ad5/3 | Adenovirus type 5 with type 3 fiber knob |
| ANOVA | Analysis of variance |
| APC | Antigen presenting cell |
| bp | Base pair |
| CAR-T | Chimeric antigen receptor T cells |
| CAR | Coxackie-adenovirus receptor |
| CBA | Cytometric bead array |
| CMV | Cytomegalovirus |
| CTLA-4 | Cytotoxic T-Lymphocyte Associated Protein 4 |
| DC | Dendritic cell |
| DMEM | Dulbecco's Modified Eagle medium |
| DMSO | Dimethyl sulfoxide |
| ELISA | Enzyme-linked immunosorbent assay |
| EMA | European Medicine agency |
| FBS | Fetal bovine serum |
| FDA | Food and Drug Administration |
| GM-CSF | Granulocyte-macrophage colony-stimulating factor |
| HLA | Human leukocyte antigen |
| hTERT | Human telomerase reverse transcriptase |
| HSV | Herpes simplex virus |
| IDO | Indoleamine 2,3 -dioxygenase |
| IFN | Interferon |
| IL | Interleukin |
| IRES | Internal ribosome entry site |
| MDSC | Myeloid-derived suppressor cell |
| MTS | 3-(4,5-dimethylthiazol-2-yl)-5-(3- carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt |
| NK | Natural killer |
| OVA | Chicken ovalbumin |
| PBMC | Periferal blood monuclear cell |
| PBS | Phosphate-buffered saline |
| PCR | Polymerase chain reaction |
| PD-1 | Programmed death 1 |

| | |
|---|---|
| qPCR | Quantitative polymerase chain reaction |
| PRR | Pattern recognition receptor |
| Rb | Retinoblastoma |
| RPMI | Roswell Park Memorial Institute medium |
| T-VEC | Talimogene laherparepvec |
| TCR | Tcell receptor |
| TDO | Tryptophan-2,3-dioxygenase 2 |
| TGFβ | Transforming growth factor beta |
| TIL | Tumor infiltrating lymphocyte |
| TNF-α | Tumor necrosis factor alpha |
| TNFR | Tumor necrosis factor receptor |
| Treg | Regulatory T cell |
| VEGF | Vascular endothelial growth factor |
| VP | Viral particles |

# 4 INTRODUCTION

Science has, since the beginning of time, been interested in finding ways to help cancer patients. Already in the times of the pharaohs in Egypt, Egyptian physicians noted the power of immunotherapy. Even if they did not have the right terms, there are records describing infections that seemed to cause regression of tumor growth (Kucerova and Cervinkova, 2016). Hence, this can be seen as the first recorded observations of immune-mediated reactions towards tumors. Since then, humanity has learned more about cancer and the immune system and subsequently developed more effective treatment methods. As historical data pointed to a connection between infection and tumor regression, one of the more recent treatment approaches has focused on viruses as cancer therapeutics. Virions could activate the immune system while simultaneously lysing cancer cells. Initially, low doses of wild-type viruses were tested as the first generation of virotherapy. However, the nonspecificity of infection and the inevitable symptoms caused by the viruses discouraged this approach, until new laboratory tools (such as gene-modification methods) were developed. This allowed scientists to modify the effect and outcome of the infection. By modifying wild-type viruses, scientists could then create safer and more cancer cell specific viruses, add immune-stimulating genes, or employ tumor extracellular matrix destroying or lysis-enhancing strategies (Heiniö et al., 2019;Hemminki, Santos and Hemminki, 2020). As more promising clinical results were observed as more viruses for cancer virotherapy were created, some oncolytic viruses were approved for clinical use. The first oncolytic virus approved for use in Europe and US is a modified Herpes simplex virus (HSV) called Talimogene laherparepvec (T-VEC). Another, somewhat controversial, oncolytic virus (Rigvir) was used in Latvia; however, marketing rights were suspended in 2019. In Asia, oncolytic viruses such as H101 and delytact have been approved for use in China and Japan, respectively. However, even if the results for the approved oncolytic viruses were convincing, the viruses created to date are not a 100 % curative and thus new modalities are needed.

To further boost the lytic and immunostimulating effects of virotherapeutics, combinations with for example chemotherapeutics, radiation, immune-checkpoint inhibitors, and adoptive cell therapies have been considered. Although emerging data show that combinatorial treatments may be a promising approach to boost the efficacy of these modalities, the biological mechanisms behind these treatments should be further understood to achieve their full potential. In this thesis, we studied the mechanisms of action of oncolytic adenoviruses to gain insight in how virotherapeutics react to and affect the complex disease that is cancer. These insights may inform the development of improved cancer therapeutics.

# 5 LITERATURE REVIEW

There are approximately 10 million cancer-related deaths annually worldwide (Ferlay et al., 2021). This statistic indicates just how much still needs to be done in the field of cancer to improve outcomes. Mattiuzzi and Lippi estimated the lifetime risk of developing cancer to be 20% for those aged 0-74 years, with women having a slightly lower risk. The risk varies depending on the sources and which countries are included. For example, cancer research UK estimated the risk to be higher and stated that approximately 1 in 2 men and 1 in 3 women will develop cancer in their lifetime for persons born after 1960s (UK, Accessed 3/2022).
Of all cancer types, lung, breast, colorectum, stomach, and liver are the deadliest cancer types in the population overall. For women, breast, colorectum, lung, and cervix uteri cancers are the most common types; lung, prostate, colorectum, and stomach cancers are the most common types for men (WHO, 2022).

## 5.1 TREATMENT OPTIONS FOR CANCER PATIENTS

### 5.1.1 CLASSICAL TREATMENT OPTIONS

The most common treatment modalities for cancer are surgery, chemotherapy, and radiotherapy. In Finland, a combination of several different treatments are often personalized for each patient and consider patient age, relevant medical history, tumor type, location, and stage (THL, 2022).
Surgery is one of the earliest forms of cancer treatment. Surgery is a first-line treatment option in most countries as it is relatively reliable, safe, and affordable. Cancer surgery methods have developed from dangerous and radical removal of tissue, with little regard to patient safety and welfare and often without any analgesia, anesthesia, or knowledge of aseptic surgery techniques, to highly specialized surgery performed with minimal damage to surrounding tissues (Wyld, Audisio and Poston, 2015). Radical removal of tissues was based on the notion that the patients that did indeed survive the surgery generally had a better prognosis if larger tissue sections were removed, plausibly due to complete removal of the tumor and possible metastases. However, excess tissue removal sometimes resulted in permanent disabilities in the patient. Now, modern tumor examinations, such as pre- and post-operation histological examination and deeper knowledge of cancer types, provide surgeons with detailed information and assist in surgery planning and outcome assessment (Ceelen et al., 2007;Wyld, Audisio and Poston, 2015). Debulking surgeries are also gaining attention, with positive outcomes reported in several cancer types, such as

renal cell carcinoma and ovarian, colorectal, and breast cancer (Hishida et al., 2021).

Other common treatment methods include radiotherapy, and chemotherapy. Chemotherapeutics are medicines that stop cell growth and therefore may have severe side effects, as they affect all (replicating) cell types. There are several different chemotherapy types, such as alkylating agents, nitrosoureas, anti-metabolites, and anti- tumor antibiotics. These agents induce DNA damage (by forming damaging covalent bonds in DNA), inhibit DNA synthesis, or stop DNA repair mechanisms (Corrie, 2008;Malhotra and Perry, 2003). Chemotherapeutics are often preferred treatment options as they can be used as a single-agent therapy, as adjuvant therapies, or in combination with other therapies. For example, chemotherapy can be used to pretreat patients to minimize tumor mass to achieve a more accessible and resectable tumor for surgery. They are highly effective drugs that reach tumors that are difficult to operate on in addition to still undetectable micrometastases. However, patients developing a resistance to the drugs are a reoccurent problem (DeVita and Chu, 2008;Knezevic and Clarke, 2020).

Radiotherapy is another common cancer treatment modality and can also be used as a single-agent therapy, as an adjuvant, or in combination with other therapies. Local radiotherapy generally causes fewer systemic side effects than chemotherapy, although systemic immunological reactions after radiotherapy have been reported (Sia, Neeson and Haynes, 2022). Radiotherapy is used in the treatment of many types of cancers and as a complementary therapy to prevent recurrence (for example, irradiation of the surgical cavity after tumor excision). It is also routinely used in palliative treatment to reduce symptoms and increase quality of life of patients with advanced cancers. Although there is always some collateral damage to nearby tissues, radiotherapy is relatively specific and has a high success rate in some cancer types. However, as it only targets the area it is directed to, distant micrometastases and circulating cancer cells are not killed (Gerber and Chan, 2008).

Sometimes patients are pre-treated non-curatively before surgery. This is called neoadjuvant therapy and can be done by utilizing for example chemotherapy or radiotherapy to increase operability (Imyanitov and Yanus, 2018). These are also two commonly used mono-, dual-, or triple-therapy modalities. Since these classical modalities are not always curative, especially for metastatic, non-local disease, new curative cancer treatments are being developed. Subsequently, in 2018 a Nobel prize for immunotherapeutic advances was awarded and major advances in the field were made.

Once it became known that activation of the immune system can reduce tumor burden, different microbes or microbe extracts have been tested

experimentally. One of the most famous experiments was designed and executed by Coley. In these experiments, Coley developed different potions of *Serratia* and *Streptococcus* species that he called Coley's toxins to treat cancer (Kucerova and Cervinkova, 2016). The results were inconclusive but laid the foundation for immunotherapy and showed the potential of the immune system. Later, immunotherapy has made large leaps forward and is currently an intensively studied field. Immunotherapy can be described as a way of activating and/or using the immune system, or components of it, for disease control (Becht et al., 2016).
Cancer immunotherapy can be passive or active. In passive immunotherapy, an immunotherapeutic agent (such as an antibody or cytokine) is administered that as such can compensate for or enhance the patient's own immune reactions. In contrast, active cancer immunotherapy attempts to train the immune system against cancer. For example, this can be achieved by administering a cancer vaccine or virotherapy. Thus, the active methods target more the effector component of the immune system (Bechtet al., 2016;Galli et al., 2020;Papaioannou et al., 2016).

### 5.1.2 ACTIVATING THE IMMUNE SYSTEM TOWARDS CANCER -THE ADAPTIVE AND INNATE IMMUNE SYSTEM

The immune system can be divided into the innate- and adaptive immune system. The latter is also known as the acquired immune system.
The innate immune system is built from various cell types, typically of myeloid lineage, such as monocytes, DCs, macrophages, mast cells, polymorphonuclear cells, and innate lymphoid cells, such as NK cells. But the complement system and passive barriers such as the skin are also considered to be a part of the innate immune system.
In contrast to the adaptive immune system, the innate immunity does not form an immunological memory. These cells of the innate immunity detect mostly conserved molecules that are usually absent in humans and present on pathogens. After antigen recognition, the innate immune system usually tries to eliminate the pathogen through phagocytosis. Alternatively, the recognized antigen can cause the release of cytokines and chemokines which attract or activate cells from the adaptive immune system (Liu and Zeng, 2012).
The adaptive immune system consists of lymphocytes: T- and B cells. The adaptive immune response is specific to the antigen presented. The adaptive system can recognize a broad range of antigens specifically, due to VDJ recombination.
The innate and adaptive immune system works together. A part of the innate immune system functions as professional antigen presenters (APC). After they have processed a possible danger, such as a pathogen, they present the antigen to the adaptive immune system on the cell surface on their major histocompability complex. This complex is then recognized by T cell's T cell

receptors (TCRs). T cells become activated if they also receive a costimulatory signal. Usually this costimulatory signal is achieved through T cell expressed CD28 interaction with CD80 (B7.1) and CD86 (B7.2) on the membrane of an APC. Another well known costimulatory receptor expressed by T cells is called Inducible T cell Costimulator or CD278 (Anderson, Stromnes and Greenberg, 2017;Waldman, Fritz and Lenardo, 2020).
In development of new cancer modalities, this knowledge of immune cells and their activation have been harnessed to achieve better results. For example in adoptive cell therapies.

### 5.1.3 ADOPTIVE CELL THERAPY

Adoptive cell therapy (ACT) is a form of immunotherapy that can generally be classified into three different types, namely tumor-infiltrating lymphocytes (TIL), ACT using T-cell receptor (TCR) modified T cells, and ACT with chimeric antigen receptor (CAR) modified T cells. Additionally, therapies with other cell types, such as dendritic cells and natural killer cells, have also been explored (Rosenberg and Restifo, 2015). To date, several different ACT types have advanced to clinical trials and some have also been approved by the Food and Drug Administration (FDA). At the time of this writing, 128 clinical trials (labeled as “active” or “active, not recruiting”) were reported in clinicaltrials.gov (July 2021) when “adoptive cell therapy” was used as a search term. This suggests that there is considerable promise in this type of treatment. However, further research is needed before ACT strategies can achieve their full potential. This is particularly the case for solid tumors. Future research areas include development and identification of effective, (sufficiently) specific target antigens, optimization of TCR selection, human leukocyte antigen (HLA) restriction, T-cell persistence, repression of the tumor microenvironment, and improving T-cell infiltration into solid tumors (Manfredi et al., 2020).

### 5.1.4 TILS, TCR-MODIFIED T CELLS, AND CAR-T

The first version of ACT was developed by Rosenberg and colleagues in late 1980’s. They hypothesized that administration of T cells could eliminate the tumor. Hence, they did a series of studies, where T cells cultured from murine tumors, were administered to tumor-bearing mice. These studies showed promising results for tumor growth control. This protocol has subsequently been adapted for humans, modernized, and optimized (Rosenberg and Restifo, 2015;Spiess, Yang and Rosenberg, 1987). Now, TILs are harvested, cultured and expanded in the presence of T cell proliferation and differentiation stimulating cytokine: IL-2. Then they are re-administered to patients who often have already received a lymphodepleting regimen

(Dudley et al., 2002;Morgan et al., 2006). However, as lymphodepleting treatments can cause severe adverse events and leaves patients vulnerable to infections, TILs are not always an optimal treatment (Zacharakis et al., 2022). Additionally, some argue that the removal of lymphocytes is counterproductive. Hence, the need of lymphodepletion has been questioned, and even some evidence against it has been produced (Santos et al., 2018).
Despite of some difficulties faced by the field, adoptive TIL therapy has shown good response rates. For example, an objective response rate of 50% was observed in ACT-treated melanoma patients (Besser et al., 2010). Additionally, an objective response rate (ORR) of 50% was observed when autologous mutation-specific TILs to breast-cancer metastasis were administered to six patients in combination with pembrolizumab (Zacharakiset al., 2022). An ORR of 44% was observed when cervical carcinoma patients were treated with autologous TILs (Jazaeri et al., 2019) . Collectively, these results indicate that TIL therapy may be efficacious in several different cancer types.

As TILs are not always abundant in the tumor or the tumor is not accessible, other ACT strategies have been developed. Two well studied alternative methods have been developed: adoptive therapy with TCR-modified T cells or CAR T cells. Here, specialized T cells are engineered from peripheral-blood T cells. In TCR-modified T cells, T cells are isolated from the blood of the patient and genetically modified (often by lentiviral transduction) *in vitro* to express TCRs that target specific tumor antigens (Manfrediet al., 2020;Zhao and Cao, 2019). This approach allows for generation of T cells that are specifically active against the patient's tumor antigens. TCR adoptive cell therapy has shown some promise in clinical trials. For example, when melanoma patients were treated with TCR ACT targeted against MART-1 antigen (an antigen present in approximately 80-90% of melanoma patients), objective partial tumor responses were observed in 2/17 treated patients (13 %) with progressive melanoma, although some on-target off-tumor autoimmune responses occurred (Morganet al., 2006). In another trial, objective clinical response for NY-ESO-1$^+$ patients with synovial cell sarcomas was 61% (11/18 patients) and 55% (11/20) for NY-ESO-1$^+$ melanoma patients. These results indicate the TCR therapy may be effective for selected patients (Robbins et al., 2015).

Unfortunately, TILS and TCR therapies require the T cells to react to the antigens presented by MHC complexes. As many cancers downregulate MHC presentation to avoid immune recognition, such treatment approaches would be ineffective (Zhao and Cao, 2019).
Therefore, a third type of ACT has been developed, called CAR T cells. CAR T cells bear artificial receptors that have an internal activation module that circumvents the need of MHC stimulation of T cells. Impressive results with

CAR T cells in treatment for hematological malignances (such as CD19-specific CAR T cells) have been observed (Locke et al., 2019) and thus CAR-T therapies have been approved for clinical use. This led to exploration of CAR-T treatments for patients with solid tumors, which have not yet gained approval for use. In a retrospective study pooling results encompassing 372 patients treated with different CAR T cells, only 52 responded to treatment. Some hurdles that CAR T therapy have to overcome for treatment of solid tumors is the lack of suitable target epitopes and prevention of adverse events due to on-target off-tumor toxicity (Schaft, 2020;Zhao and Cao, 2019).

### 5.1.5 VIRUSES FOR TREATMENT OF CANCER

When the inherent cancer tropism and (cancer) cell-killing lytic action were described for some viruses, cancer scientists hypothesized that these viruses could be used to destroy cancer cells. Later, when gene technologies evolved to current standards, the genomes of viruses could be modified to only infect cancer cells and genes that further stimulate the immune system against the cancer, induce infiltration, and enhance infection in the tumor microenvironment could be inserted (Zhang and Rabkin, 2021). In pre-clinical settings, many viruses have been investigated or modified for cancer therapies. In 2020, Macedo et al estimated that adenovirus is the most studied oncolytic virus in clinical trials (Macedo et al., 2020). Other viruses that have been investigated for oncolytic applications include (but are not limited to) HSV, Reovirus, New Castle Disease virus, and Vaccinia virus. Vaccinia viruses are relatively large (approximately 270 x 350 nm), enveloped, brick-shaped linear dsDNA viruses with an approximately 190 kbp genome. A genetically modified Vaccinia virus, Pexastimogene devacirepvec (Pexa-Vec), has been studied in clinical trials. This virus was designed for solid tumors and contains a GM-CSF encoding gene (Breitbach et al., 2011). Clinical trials for treatment of solid tumors with Pexa-Vec or Pexa-Vec in combinatorial treatments are ongoing. Pexa-Vec gained orphan drug status from both the EMA and FDA for hepatocellular carcinoma. However, no significant difference between standard treatment and Pexa-Vec was observed in a clinical trial for advanced hepatocellular carcinoma (Moehler et al., 2019).
OncolyticsBiotech's Reovirus, Reovirus type 3 Dearing (RT3D) is a naturally occurring, nonpathogenic, double-stranded RNA virus. It was originally isolated from the respiratory and gastrointestinal tracts of human patients (Rosen, Evans and Spickard, 1963). Almost all humans have experienced a Reovirus infection, with close to 100% seropositivity in the adult population. Although RT3D has shown promising results in preclinical studies, responses to RT3D monotherapy in clinical settings were modest. A clinical study examined the effect of dose escalation intravenously and then established the

efficacy of RTR3D in heavily pretreated patients. The treatment was well tolerated, with no serious toxicities. When treated with at least two cycles of RT3D (raging from TCID50 3x109 to 3x 1010, 2 to 8 treatment cycles), 3.8% of patients had a complete response, 23.1% had a partial response, 7.6% had major clinical responses, 34.6% had stable disease, and 30.8% had disease progression (Karapanagiotou et al., 2012).
The only FDA-approved virus for cancer treatment is a Herpes simplex virus (HSV-1) called Talimogen Laheprarepvec (T-VEC). HSV-1 is a relatively large, icosahedral, double-stranded, enveloped, linear DNA virus. To create T-VEC, HSV-1 was modified by removing both copies of the ICP34.5 genes to prevent replication in healthy cells, and in its place a gene encoding granulocyte-macrophage colony-stimulating factor (GM-CSF) was inserted for immune cell stimulation. Additionally, the ICP47 gene was removed due to its immune evasive effect (Harrington et al., 2015;Rehman et al., 2016). In an open-label phase III trial, T-VEC was compared with GM-CSF in patients with unresected stage IIIB to IV melanoma. Four-hundred thirty-six patients were followed. The durable response rate was significantly higher with T-VEC (16.3%) than with GM-CSF (2.1%). Median overall survival was higher with T-VEC (23.3 months) than with GM-CSF (18.9 months) (Andtbacka et al., 2015).

### 5.1.6 ADENOVIRUSES

Wild-type adenoviruses belong to the human Adenoviridae family and are divided into several serotypes. There are currently more than 50 recorded human adenovirus serotypes (Lynch and Kajon, 2016). These serotypes are grouped into seven species groups (A to G). Serotypes are determined by neutralization assays, while species are grouped by erythrocyte agglutination (hemagglutination properties), oncogenicity in rodents, DNA homology, and genome organization (Dhingra et al., 2019).
Adenoviruses are icosahedral non-enveloped viruses with a linear double-stranded DNA genome. They are approximately 90-100 nm in diameter and can contain approximately 36 kilodaltons of DNA. Adenoviruses can cause common cold like symptoms, conjunctivitis, and gastroenteritis. A role for adenovirus infections even in obesity has been proposed (Ponterio and Gnessi, 2015). However, in laboratory and clinical settings, adenoviruses are one of the most widely used vectors for gene transfer. As symptoms of infection with wild-type virus are relatively mild (Lynch and Kajon, 2016), and adenovirus vectors have rarely caused severe adverse events in numerous clinical trials, adenoviruses are considered to be a relatively safe platform for use in humans (Buijs et al., 2015;Lang et al., 2018;Ranki et al., 2016). The adenovirus is extensively studied, quite flexible in terms of genome modification, replicates to relatively high titers in both academic and commercial laboratory settings, rarely integrates into host genomes, and is a

stable construct (Heiniöet al., 2019). Thus, adenoviruses are considered an appealing vector. Adenovirus virions are stable constructs until the genome size of modified virions grew up to approximately 105% of the original size (Kennedy and Parks, 2009). Additionally, in our comparative study, we showed that of approximately 50 virus constructs designed in CGTG laboratory, most modifications did not significantly affect the production and infectivity of adenovirus constructs, thus demonstrating the feasibility of generating modified adenoviruses for research and clinical settings (Heiniöet al., 2019).

Among many the viruses studied and designed for cancer therapy, modified oncolytic adenoviruses (OAd) are among the most promising anti-cancer agents (Cervera-Carrascon et al., 2020a). OAds have strong anti-tumor abilities and replicate effectively intratumorally. Additionally, the immunological responses towards the virus and the effects mediated by cytokines inserted into the virus lead to favorable immune-activating effects through positive modifications of the often immunesuppressive tumor microenvironment (Cervera-Carrascon et al., 2020b;Havunen et al., 2017;Heiniö et al., 2020). However, adenoviruses as cancer therapies have limitations. The infectivity of serotypes that use Coxsackie adenovirus receptor (CAR) (for example Ad5) may be low in several cancers, as the expression of this main receptor is often downregulated in many cancer types. Although toxicity and shedding of virus products are of concern, these have not been observed in the clinic. Additionally, pre-existing neutralizing antibodies in the patient may neutralize and eliminate the virus before it has an effect. There is also some evidence of unstable transgene expression. However, anti-viral immunity may also contribute to antitumor immunity (Buijset al., 2015;Kanerva et al., 2013;Lemos de Matos, Franco and McFadden, 2020).

To address these issues, attempts have been made to make the vectors more infective. For example, proteins have been inserted into the highly variable regions of the hexon protein and the AB loop or HI loop of virion fibers to retarget the virion to use a new host-cell receptor for attachment by modification of the knob protein (Coughlan et al., 2010).
An alternative approach involved comparing different adenovirus species receptors and determining their transducing efficiency of breast-cancer cells. Ad3, Ad35, Ad37, and Ad52 were identified as potential candidates for treatment of breast cancer, partially because they use alternative (not CAR) receptors for attachment and subsequent infection (Mach et al., 2020).

It is not enough to have a virus that multiplies to high titers and exhibits efficient host-cell infection; the vector must replicate specifically in cancer cells to avoid severe adverse events. There are two main types of conditionally replicative adenoviruses, specifically mutation-based and adenoviruses that

depend on cancer specific promoters (Jounaidi, Doloff and Waxman, 2007). The adenovirus genome consists of early (E1-4) and late (L1-5) transcription units. Mutations or deletions in the E1 region are used to create replication deficiency and E1 mutants replicate only in retinoblastoma compensating cancers (Sauthoff et al., 2004). In a non-replicating cell, the retinoblastoma protein (pRB) is a negative regulator of the E2F transcription factor family. E2F is needed for cell cycle progression and thus when pRB and E2F interact it hinders cell cycle progression. This pathway is often genetically disrupted in tumour cells. The E1A region of the adenoviral genome also interacts with pRB and this results in release of E2F and enables viral replication and cell cycle continuation(Mantwill et al., 2021). In TILT-123, a deletion of 24 base pairs in the adenovirus E1A prevents the binding of E1A to pRB (Havunen et al., 2017). Therefore, in healthy cells, TILT-123 can't replicate due to ongoing E2F and pRB interaction and cell cycle halt. However, in tumor cells with mutated or dysregulated pRB, E2F is no longer negatively regulated by pRB and it can activate viral gene transcription and thus cell replication. Unfortunately, of E1 also stabilizes p53. P53 is a tumor suppressor protein. The stabilization of p53 leads to apoptosis and is unfavorable for viral replication. To prevent this, E1B55K proteins form a complex with p53, causing its degradation through ubiquitin mediated proteolysis. E1B19K additionally prevents E1A-induced apoptosis by interfering with the actions of the pro-apoptotic proteins Bak and Bax. It has been reported that oncolytic adenoviruses with E1B19K, as well as E1B55K-deleted viruses, replicated efficiently in a variety of tumor cell lines independent of their p53 status. E1B19K deletion, however, has been indicated to generate more rapid viral release from apoptotic cells, resulting in enhancement to viral delivery across tumor tissues. (Mantwillet al., 2021). Therefore, these deletions have also been introduced to TILT-123s genome

The second approach to promote viral replication is focused on promoters that induce replication specificity in cancer cells. Such promoters include MUC1/DF3, the prostate-specific antigen promoter, the α-fetoprotein promoter, the human telomerase reverse transcriptase promoter, and the E2F promoter (Jounaidi, Doloff and Waxman, 2007).

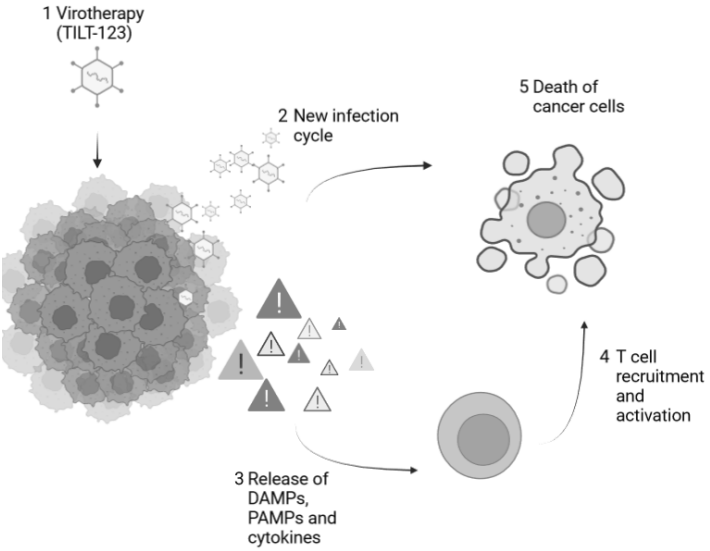


Figure 1. Antitumor mechanisms mediated by virotherapy. The virus infects tumor cells. This leads to the release and expression of danger- and pathogen associated molecular patterns (DAMP and PAMP) and cytokines, which alert the immune system towards cancer. When new progeny virions exit the tumor cells, they are lysed and the infection spreads to the neighboring cells.

### 5.1.7 ONCOLYTIC TILT-123 VIRUS

The virus used in these studies is a modified adenovirus called Ad5/3-E2F-d24-hTNFa-IRES-hIL2 (or TILT-123) (Figure 2). Tähtinen et al. provided the rationale behind this virus construct. In their study, a panel of cytokines relevant for cancer therapy and their combinatorial effects on the adoptive immune system (with a focus on T cells) was studied (Tähtinen et al., 2015). They showed that IL-2 and TNF-α combined had the greatest immune-activating and tumor-destroying effect. Based on these results, Havunen and Siurala et al. further developed the virus (Havunenet al., 2017;Siurala et al., 2016a). To avoid inefficient transduction due to inaccessible or low expression of the Ad5 serotype receptor CAR on cancer cells, the adenovirus knob was exchanged from 5 to a serotype 3 knob, with desmoglein 2 adhesiveness (Wang et al., 2011). E2F functions as a cancer-specific (cell cycle) promoter. Furthermore, a 24 base pair deletion was made to the E1 region for cancer-cell specificity (explained above). This virus kills several cancer cell types efficiently, is highly specific, and causes minimal adverse effects in animal models (Havunen et al., 2021;Yamamoto et al., 2017). In a Syrian hamster model, TILT-123 cured 80% of all tumors as a single-agent

therapy; in combination with TILs TILT-123 showed a 100% cure rate (Havunenet al., 2017). Thus, with these encouraging results, clinical trials with TILT-123 have begun (NCT04217473 and NCT04695327).

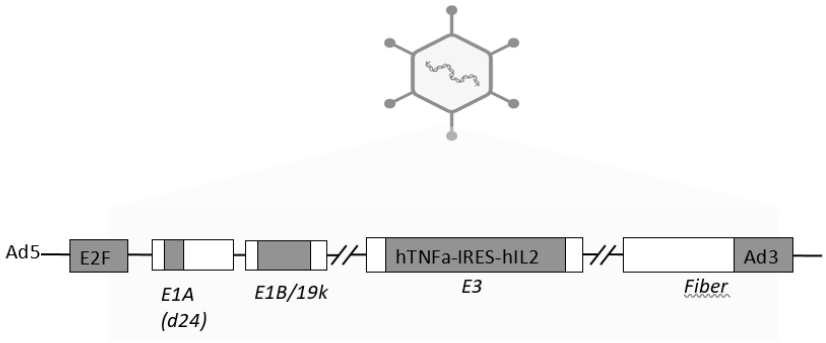


Figure 2. Schematic visualization of of TILT-123.

### 5.1.8 CYTOKINES IN CANCER THERAPIES

Cytokines are proteins that function as signaling agents between cells and tissues. As cytokines can promote immune reactions and immune cell maturation and mediate other functions as chemokines, cytokines have been used as a cancer therapy. Interleukins (IL) are a type of cytokine. As ILs do not share high sequence similarity, they have been divided into four groups based on distinguishing structural features (Brocker et al., 2010).
Cytokines play major roles as regulators of innate and adaptive immunity, ensuring that cells signal and react accordingly to local conditions. Clinical trials with IL- 2, GM-CSF, Interferon (IFN)γ, IL-7, IL-12, and IL-21 are active or have been completed. Cytokines as monotherapy often exhibit promising efficacy in preclinical experiments. However, cytokine concentration needed in the tumor often lead to dose-limiting off- tumor effects systematically. Although this could be managed with lower and appropriate dosing, this would also reduce efficacy and the immune response (Berraondo et al., 2019;Chulpanova et al., 2020). Additionally, it is now known that cytokines can also induce immune-suppressive humoral factors, suppressive cells, and cellular checkpoints without consistently inducing a tumor-specific response. To address these issues, cytokines are added into delivery vehicles (raging

from nanoparticles to viruses) and new cytokine variants and mutants are engineered (Havunenet al., 2017;Quixabeira et al., 2021;Riley et al., 2019).

IFNα is FDA approved for resected high-risk melanoma patients and for several refractory malignancies. There are 17 different IFN genes, divided into IFN types I–II. Type I includes the α and β forms. IFN-γ is the only IFN considered to belong in type II. This division is based on the type of cell that produces the IFN and the functional characteristics of the protein and genome sequences of the IFNs (Dunn, Koebel and Schreiber, 2006). IFNs can be produced by almost any cell upon stimulation by a virus after toll-like receptor (TLR) stimulus; their primary function is to induce viral resistance in cells. Furthermore, IFNs reduce cancer growth and activate dendritic cells. Type II IFN is mainly secreted by natural killer (NK) cells and T lymphocytes; its main purpose is to warn the immune system of potential cancer cell growth or different pathogens. However, its role in cancer is manyfold, as it can trigger cell growth but also activate the immune system. The complexity of reactions towards IFNs is still under research.

IL-2 (High dose IL-2) is approved for treatment of metastatic renal cell cancer and melanoma. IL-2 is a 15 000-kDa α-helical cytokine and is mostly secreted by activated CD8+ and CD4+T cells. Besides T cells, dendritic cells (DCs), NK cells, and NKT cells can also produce IL-2. IL-2 is rapidly and transiently produced upon TCR engagement for T-cell expansion. IL-2 was originally discovered in 1976 as a T-cell growth factor and plays a role in T-cell differentiation by supporting Th1/Th2 differentiation and inhibiting the development of T helper 17 lineage. However, it was subseqently discovered that IL-2 receptor deficient mice did not develop autoimmune diseases, thus indicating that IL-2 has a more multifaceted role in cancer as well (Jiang, Zhou and Ren, 2016).

The IL-1 family consist of 11 interleukins, of which IL-1α and IL-1β are the best characterized. These are produced as propeptides as a reaction to alarmins, which are then proteolytically cleaved to their active form by proteases such as caspase-1. The signaling cascade of IL-1α and IL-1β consists of two parts. An initial priming step is needed, which consists of alarmins and activating receptors, such as TLRs, IL-1 receptor, or TNF-α receptor and their signaling, in the case of IL-1R MYD88 IRAK proteins to activate translation of pro-IL-1 through NF-κB. In the second part of the signaling cascade, sensors such as NOD-like receptors and AIM2 activate caspase-1 to process IL-1 to its active form for release. While IL-1 has several functions, as a proinflammatory cytokine it has an activating effect on many immune cells (Gelfo et al., 2020).

## 5.2 RECEPTORS INVOLVED IN IMMUNE RECOGNITION AND THEIR CONTRIBUTION IN CANCER TREATMENTS

The innate response can be triggered through activation of Fc receptors (FcRs) by antibodies, causing innate immune activation of effector responses; antibody-dependent cellular phagocytosis (ADCP), or antibody-dependent cellular cytotoxicity (ADCC). Another important sensing mechanism exists, it is generally called pattern recognition receptors (PRR). PRRs are germline-encoded host sensors, specialized in detecting pathogen derived molecules. They are most often expressed by cells of the innate immune system, such as macrophages, dendritic cells, monocytes and neutrophils. They can also be found in non-immune cells such as epithelial cells. PRR can identify two different classes of molecules, namely pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). TLRs are the best known receptors in this group.

### 5.2.1 TOLL LIKE RECEPTORS

TLRs sense and bind to evolutionarily conserved motifs that are shared by many infectious agents,for example lipopolysaccharides, peptidoglycan, nonmethylated CpG, and double-stranded RNA(PAMPs. However, some TLRs recognize molecules that are “normal” and endogenous but are only misplaced, such as dsDNA in the cytosol (DAMPs).TLRs are germline encoded and although their expression may be restricted to particular cell types, they are not clonally distributed. Within a given species, the genes encoding innate receptors show various degrees of polymorphism.
There are 13 different TLRs, of which TLR11, 12, and 13 are not found in humans. TLR10 is only found in mice. TLRs are single-pass membrane receptors, of which TLR3, 7, 8, and 9 are located intracellularly on endosomes. Several cell lines express TLRs, such as antigen-presenting cells, fibroblasts, and epithelial cells. The main role of TLRs is host protection from microbial infection. However, cancer cells also express TLRs. Although TLR expression is associated with cancer prognosis, this is dependent on TLR type and signal strength, and contradictory results have also been observed. When the immune-stimulating reactions of TLRs were recognized, different innate immunity stimulating drugs were tested for cancer treatments. For example, a study by Cerullo et. al. used an adenovirus coding for a TLR9 agonist, which improved the anti-tumor efficacy of adenoviral therapy compared to mock treatment.

After recognition of PAMPs, TLRs activate signaling cascades which results in downstream signaling cascades and activation of transcription factor nuclear factor-kappaB (NF-κB). Alternatively cytokine receptors and their

downstream signaling can activate NF-κB. As a transcription factor, NF-κB controls the transcription of proinflammatory cytokines such as TNF-α, IL6, BCLXL, BCL2, BCLXS, XIAP, and VEGF, and chemokines. It can also induce the upregulation of co-stimulatory molecules on DCs that are essential for T-cell activation. NF-κB is found in almost all animal cell types. Incorrect regulation of NF-κB has been linked to cancer. Also other diseases such as inflammatory and autoimmune diseases, septic shock, viral infection, and improper immune development has been linked to defective NF-κB signaling.

### 5.2.2 AIM2

In a study of innate immune reactions towards TILT-123, it was found that AIM2 was activated during infection (Heiniöet al., 2020).
AIM2 is a sensor of double-stranded DNA. Activation occurs via a two-step reaction. First, a signal pathway activates production of AIM2 proteins, either through exposure to cytokines or through PRR activation. A second molecule, an inflammasome-activating molecule (DNA in the case of AIM2) is needed for assembly of the sensor. When the HIN-200 domain of AIM2 binds it multimerizes around the DNA, PYHIN is simultaneously released and attracts ASC, which modifies caspase-1 to its mature form. The mature capase-1 proceeds to cleave and mature IL-1β, IL-18, and gasdermin. Gasdermin can create pores in the cell membrane, from which IL-1β can be released. The pores can eventually lead to pyroptosis of the cell. IL-1β also has many effects on cells and the tumor microenvironment (Wang and Yin, 2017).
Reduced AIM2 expression was observed in colon, liver, prostate, and breast cancer, suggesting that low AIM2 expression leads to tumorigenesis and is associated with poor prognosis. However, at the same time results show that in some models overexpression of AIM2 in tumor cells can lead to cell growth arrest and cell apoptosis. For example, in oral squamous cell carcinoma, squamous cell carcinoma, and lung cancer, where AIM2 expression is high, inhibition or knockout of AIM2 resulted in inhibited cell growth, induction of apoptosis, and reduced cell migration. Thus, although the role of AIM2 in the innate immune response has been well documented, its function in cancer initiation and development should be further explored (Chen et al., 2006;Chen, Wang and Yu, 2017;Man, Karki and Kanneganti, 2016).
Active caspase-1 has clear links to adaptive immunity through the release of the IL-1 family cytokines, which are important for T-cell activation and memory cell formation. In addition to cleavage of IL-1β and IL-18 to their bioactive forms, inflammasomes also facilitate secretion of inflammatory proteins HMGB-1 and IL-1α, which do not require processing for biological activity. IL-1β can function as a survival signal for naïve T cells by causing transient release of IL-2 from T cells.

## 5.3 TUMOR VASCULATURE AND ADENOVIRUS INTRAVENOUS DELIVERY

Like any tissue, tumors require oxygen, nutrients, and a waste disposal system to survive. These functions are mediated by blood vessels and the circulatory system. Therefore, for tumors to grow over an estimated 2-3 $mm^2$, it requires vascularization. Otherwise, the tumors will suffer from hypoxia and metabolite accumulation.

There are several ways that new blood vessels can be formed in a tumor. For example, old vessels can branch into the tumor and form two separate vessels, tumor cells can start to mimic endothelial cells and form vessel-like structures, and new vessels can branch out from old surrounding vessels. These vessels are usually abnormal, highly branched, and leaky with high intravenous pressure.

Accordingly, different approaches to target and destroy tumor vasculature have been developed. One of the best known is bevacizumab (Avastin), an anti-vascular endothelial growth factor (VEGF) antibody. It was believed that through blocking a vessel-inducing receptor (VEGFR), the growth of tumor vessels would be inhibited and the tumor will self-destruct. Although not a 100%- curing results were observed in clinical trials as a single-agent therapy, in 2004 Avastin became the first anti-angiogenic drug approved in the United States for the treatment of metastatic colon cancer in combination with chemotherapy (Ganss, 2020;Kabbinavar et al., 2008). It is believed that the anti-angiogenic medication induces killing of the most central part of the tumor, while the outer edges are still maintained through peripheral blood vessels. Therefore, the tumor can regrow later when the medication is withdrawn. Other approaches to inhibit tumor vasculature have subsequently been employed. Instead of killing the endothelial cells, vessels are normalized through alternative treatments. By normalizing the vessels, the immune system can use the vessels to reach all parts of the tumor. Once pressure has been normalized, the cells can travel better and endothelial cells can express the molecules required for movement through the vessel wall (diapedesis) into the tumor (Ganss, 2020).

### 5.3.1 T CELL INFILTRATION THROUGH VASCULATURE

To mediate their effects, activated T cells in lymph nodes have to reach their target tissue. First, dendritic cells or other antigen presenting cells must present antigens of interest to T cells. This activates T cells, and the primed T cells travel to their target. Triggering of T cells is specific for different targets of the body to ensure the that T cell infiltrates to the area where it is needed. Lymphocytes exposed to antigens can be further subdivided into different subsets based on their expression of characteristic sets of trafficking receptors that favor their accumulation in certain target organs, including the

skin and gut. Differences in cytokine and chemokine contents of tumors modulates the recruitment of T cells and also affect the entire cellular composition of the tumor microenvironment. Success in preclinical and clinical models has been shown when the cytokine or chemokine environment is modified, such as with IL-2 treatments(Bright et al., 2017;Rosenberg, 2014). However, several factors, such as the concentration of a single cytokine, the presence and amount of other cytokines, and the timing of cytokine expression and "release schedule" can modulate the effects of another or the same cytokine or chemokine. Thus, the same cytokines can have either antitumor or protumor activities and induce unwanted side effects (Berraondoet al., 2019). When a non-immune cell is in distress, infected, or abruptly killed, it secretes danger signals to the environment, such as cytokines and chemokines. The T cell is attracted to the area in a concentration-specific manner; in other words, the concentration of the "alarm molecule" is greater closer to the area of disruption. The T cell that traveled from the lymph node via the circulatory system reacts to secreted chemokines and cytokines in another way as well, as they can prime the T cells to interact with the endothelial wall by modulating expression of selectins and integrins on both the immune cell and endothelial cell wall. These also affecting gap junctions in the vessel walls. For example, the chemokine CCL25 and the CAM MAdCAM-1 are involved in recruitment of T cells that express the chemokine receptor CCR9 and the integrin $\alpha 4\beta 7$ to the small intestinal mucosa, which induces the T cells to express molecules such as selectins and integrins on their cell surface (such as LFA-1 and CD31). These molecules then attach to their complementary molecule on the endothelial vessel wall and slow down to roll on top of the endothelial surface. This is rolling further slows to a halt, which is called adhesion. At this stage, the expression of molecules on the T-cell wall must match the composition of receptor molecules expressed on the endothelial cells. This activates a signaling system that triggers and enables T cell infiltration through the gap junction or through the endothelial wall.

### 5.3.2 INTRAVENOUS DELIVERY OF ONCOLYTIC VIRUSES UTILIZING CELL CARRIERS

Oncolytic viruses have been delivered through both intravenous (i.v.), and intratumoral (i.t.) injections. It is thought that i.v. delivery of oncolytic viruses has benefits compared with other delivery routes, due to rapid systemic delivery and ease of administration. Then also, tumors that are not yet detected or cannot be injected could be reached and possibly cured by a oncolytic virus. However, this approach is limited by the fact that there are several highly efficient mechanisms to remove unwanted entities from the circulation, and unfortunately they cannot distinguish between true pathogens or oncolytic viruses A theoretical impediment to systemic

application of adenoviruses is pre-existing antiviral immunity (see figure 3). Almost all individuals have circulating anti-adenovirus antibodies and T lymphocytes developed as a result of immunization or natural infection. Also, adenoviruses can non-specifically be neutralized by proteins in the blood serum. Accordingly, carrier cells have been investigated to overcome this limitation. Several preclinical studies have shown that viruses can be loaded into, or onto, different types of cells without losing the biological activity of either virus or cell carrier. Cell carriers can protect viruses from neutralization and elimination through "hiding" in the in the grooves of their carrier. Further benefits of carrier cells also include inherent cell type or tumor tropism of chosen carrier cells, which can function as a delivery guide for the oncolytic virus (Roy and Bell, 2013). Naturally, viruses have already utilized this. For example HIV hides within T cells to avoid destruction and some viruses use TCRs or desmogleins to spread from cell to cell without the need to be exposed to the factors neutralizing viruses outside host cells. For carrier cells to be used in the clinic, the cells should be easy and cheap to manufacture and handle, preferably have a target specificity and be safe. Inherent cancer tropism and cancer cell toxicity would also be preferable. To date, mostly progenitor cells and immune cells have been used as carrier cells. Their downside is that these cells are sometimes hard to obtain and handle. Additionally, both cell types are not cheap to obtain and grow(Roy and Bell, 2013;Zafar et al., 2020).

It has been shown that Mesenchymal stem cells (MSCs) can improve delivery of oncolytic adenoviruses into tumors in preclinical animal models and in humans (Rincón et al., 2017). Consistent with these results, T cells have been shown to be able to deliver oncolytic viruses into tumors. Additionally, T cells can have the added benefit of tumor-specific cytotoxicity (antigen-specific T-cells, CAR-T cells, or TILs) (Dieci et al., 2015;Havunenet al., 2017;Santos et al., 2021;van den Berg et al., 2020).

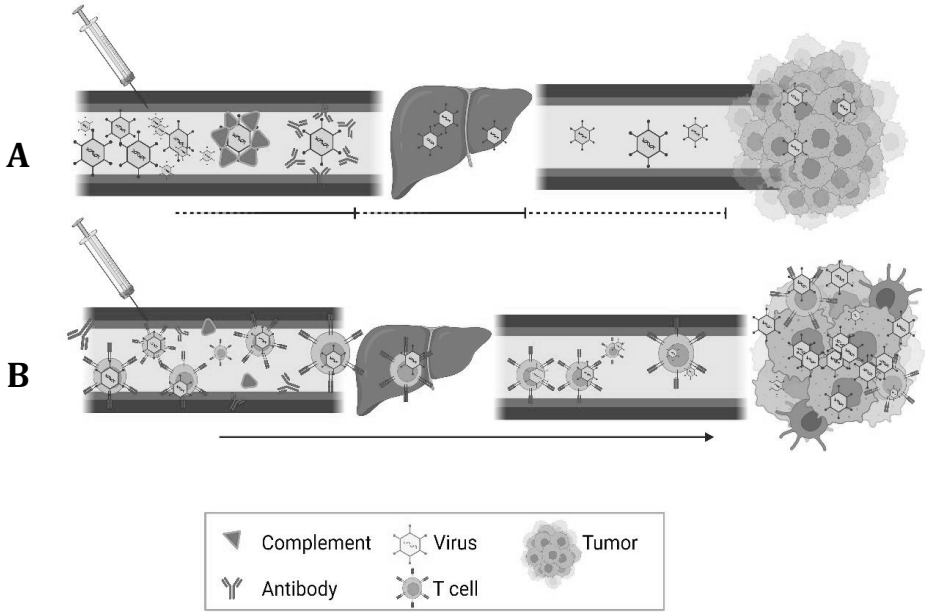


Figure 3. Cells as virus carriers. When using cell carriers for virotherapy, viruses are more likely to reach the tumor. A) Viruses are injected i.v. Before they can reach the tumor, many of them are neutralized by the immune system for example by the complement system or antibodies. Also phagocytotic cells eliminate free virions in the blood stream, like for example Kupffer cells in the liver. This limits the therapeutic effect. B) If viruses are loaded on carrier cells, such as T cells, they can avoid neutralization and elimination by hiding on or in their carrier cell. Therefore, more viruses will reach the tumor and consequently elicit a stronger reaction at the tumor.

# 6 MATERIALS AND METHODS

## 6.1 IN VITRO AND EX VIVO-METHODS

### 6.1.1 VIRUSES (I-IV)

Details of adenovirus constructs used in this study are described in (Havunenet al., 2017;Heiniöet al., 2019) and are listed in Table 1. Non-replicative viruses were usually generated by insertion of a transgene into a cloning plasmid that was consecutively co-transfected with a "rescue plasmid" (containing the rest of the virus genome) into bacteria containing eukaryotic recombination enzymes. Then, the linearized plasmids were propagated in HEK293 cells, which have the needed transcomplementary E1 region.
Oncolytic, replication-competent, chimeric adenoviruses were mostly constructed by an adapted BAC-recombineering method. Viruses were then propagated in non-transcomplementing A549 cells to minimize back-recombination risk, generating a modified but non-tumor selective virus. Virions were collected from cells by incubating in trypsin or scraping, followed by two consecutive CsCl density centrifugations. CsCl was removed by dialysis.

Table 1. Viruses used in this study

| Virus name | Transgene | Replication competence | Modifications | Reference |
|---|---|---|---|---|
| **Ad5-based viruses** | | | | |
| **Ad300wt** | No transgenes | Yes | None | ATCC |
| **Ad5-D24** | No transgenes | Oncolytic [b] | 24-bp deletion in E1A | Suzuki 2002 |
| **Ad5Luc1** | Luciferase | No | Insertion of CMV/Luc into deleted E1; deletion of E3 | Krasnykh 2001 |
| **Ad5LucRGD** | Luciferase | No | Insertion of CMV/Luc into deleted E1 region; insertion of RGD; deletion of E3 | Dmitriev 1998 |
| **Ad5LacZ** | LacZ | No | Insertion of CMV/LacZ into deleted E1; deletion of E3 | Yotnda 2004 |

| | | | | |
|---|---|---|---|---|
| **Ad5pK21LacZ** | LacZ | No | Insertion of CMV/LacZ into deleted E1; insertion of 21 polylysines; deletion of E3 | Yotnda 2004 |
| **Ad5RGD.pK7** | Luciferase + Green fluorescent protein | No | Insertion of CMV/Luc-GFP into deleted E1; insertion of 7 polylysines; insertion of RGD; deletion of E3 | Wu 2002 |
| **Ad5-GL** | Luciferase + Green fluorescent protein | No | Insertion of CMV/Luc-GFP into deleted E1; deletion of E3 | Wu 2002 |
| **Ad5.pK7** | Luciferase + Green fluorescent protein | No | Insertion of CMV/Luc-GFP into deleted E1; insertion of 7 polylysines; deletion of E3 | Wu 2002 |
| **Ad5-D24-hGMCSF** | Human granulocyte macrophage colony stimulating factor | Oncolytic | 24 bp deletion in E1A; insertion of hGMCSF into deleted 6.7K/gp19k region of E3 | Cerullo 2010 |
| **Ad5-D24-RGD-hGMCSF** | Human granulocyte macrophage colony stimulating factor | Oncolytic | 24-bp deletion in E1A; insertion of hGMCSF into deleted 6.7K/gp19k region in E3; insertion of RGD | Pesonen 2011 |
| **Ad5-D24-CpG** | 18 CpG islands | Oncolytic | 24-bp deletion in E1A; insertion of 18 CpG islands into E3 | Cerullo 2012 |
| **Ad5-D24-mC5a** | Murine complement component 5a | Oncolytic | 24-bp deletion in E1A; insertion of mC5a into E3 | Unpublished |
| **Ad5-CMV-mIL2** | Murine interleukin 2 | No | Insertion of CMV/mIL2 into deleted E1; deletion of E3 | Siurala 2016 |
| **Ad5-CMV-mIFNg** | Murine interferon gamma | No | Insertion of CMV/mIFNg into deleted E1; deletion of E3 | Siurala 2016 |
| **Ad5-CMV-mIFNb1** | Murine interferon beta 1 | No | Insertion of CMV/mIFNb1 into deleted E1; deletion of E3 | Siurala 2016 |
| **Ad5-CMV-mTNFa** | Murine tumor necrosis factor alpha | No | Insertion of CMV/mTNFa into deleted E1; deletion of E3 | Siurala 2016 |
| **Ad5-CMV-mGMCSF** | Murine granulocyte macrophage colony stimulating factor | No | Insertion of CMV/mGMCSF into deleted E1; deletion of E3 | Unpublished |
| **Ad5-CMV-tras** | anti-HER2 antibody | No | Insertion of CMV/tras into deleted E1; deletion of 6.7K/gp19k in E3 | Liikanen 2016 |

| Capsid chimeric viruses | | | | |
|---|---|---|---|---|
| **Ad5/3-D24** | No transgenes | Oncolytic | 24-bp deletion in E1A; replacement of knob 5 with knob 3 | Kanerva 2003 |
| **Ad5/3-E2F-D24** | No transgenes | Oncolytic | E2F promoter; 24-bp deletion in E1A; replacement of knob 5 with knob 3; deletion in E1B | Havunen 2017 |
| **Ad5/3-Cox2L-E1** | No transgenes | Oncolytic | Replacement of E1 region with long Cox2 promoter and wtE1A region; replacement of knob 5 with knob 3 | Bauerschmitz 2006 |
| **Ad5/3-Cox2L-D24** | No transgenes | Oncolytic | Replacement of E1 region with long Cox2 promoter and wtE1A; a 24-bp deletion in E1A; replacement of knob 5 with knob 3 | Bauerschmitz 2006 |
| **Ad5/3-Cox2L-D2-D24** | No transgenes | Oncolytic | Replacement of E1 region with long Cox2 promoter; introduction of a 24-bp deletion into inserted wtE1A; additional 2-bp deletion in E1A; replacement of knob 5 with knob 3 | Bauerschmitz 2006 |
| **Ad5/3-Cox2M-E1** | No transgenes | Oncolytic | Insertion of medium-length cox2 promoter into deleted E1; replacement of knob 5 with knob 3 | Bauerschmitz 2006 |
| **Ad5/3-Cox2M-D24** | No transgenes | Oncolytic | Insertion of medium-length cox2 promoter in 24-bp deletion of E1A; replacement of knob 5 with knob 3 | Bauerschmitz 2006 |
| **Ad5/3-Cox2M-D2-D24** | No transgenes | Oncolytic | Insertion of medium-length cox2 promoter in 24-bp deletion in E1A; 2-bp deletion in E1A; replacement of knob 5 with knob 3 | Bauerschmitz 2006 |
| **Ad5/3Luc1** | Luciferase | No | Insertion of CMV/Luc into deleted E1 region; replacement of knob 5 with knob 3 | Kanerva 2002 |
| **Ad5/3lucS*** | Luciferase | No | Insertion of CMV/LucS gene into deleted E1 region; replacement of knob 5 with knob 3; fiber KKTK mutation | Koski 2013 |
| **Ad5/3-9HIF-D24-sFlt** | Soluble VEGF receptor 1 (sFlt) | Oncolytic | Insertion of 9HIF; 24-bp deletion in E1A; insertion of sFlt into deleted 6.7K/gp19k region of E3; replacement of knob 5 with knob 3 | Guse 2009 |
| **Ad5/3-9HIF-D24** | No transgenes | Oncolytic | Insertion of 9HIF; 24-bp deletion in E1A; replacement of knob 5 with knob 3 | Guse 2009 |

| | | | | |
|---|---|---|---|---|
| **Ad5/3-D24-hCG** | Human chorionic gonadotropin | Oncolytic | 24-bp deletion in E1A; insertion of hCG into deleted 6.7K/gp19k in E3; replacement of knob 5 with knob 3 | Rajecki 2007 |
| **Ad5/3-D24-hGMCSF** | Human granulocyte macrophage colony stimulating factor | Oncolytic | 24-bp deletion in E1A; insertion of hGMCSF into deleted 6.7K/gp19k in E3; replacement of knob 5 with knob 3 | Koski 2010 |
| **Ad5/3-E2F-D24-hGMCSF** | Human granulocyte macrophage colony stimulating factor | Oncolytic | Insertion of E2F; 24-bp deletion in E1A; replacement of knob 5 with knob 3; insertion of hGMCSF into deleted 6.7K/gp19k in E3 | Hemminki 2015 |
| **Ad5/3-E2F-D24-hTNFa** | Human tumor necrosis factor alpha | Oncolytic | Insertion of E2F promoter; 24-bp deletion in E1A; insertion of hTNFa into deleted 6.7K/gp19k region of E3; deletion in E1B; replacement of knob 5 with knob 3 | Havunen 2017 |
| **Ad5/3-E2F-D24-hIL2** | Human interleukin 2 | Oncolytic | Insertion of E2F promoter; 24-bp deletion in E1A; deletion in E1B; insertion of hIL2 into deleted 6.7K/gp19k region of E3; replacement of knob 5 with knob 3 | Havunen 2017 |
| **Ad5/3-E2F-D24-hTNFa-IRES-hIL2** | Human tumor necrosis factor alpha + human interleukin 2 | Oncolytic | Insertion of E2F promoter; 24-bp deletion in E1A; insertion of TNFa; insertion of IRES-hIL2 into deleted 6.7K/gp19k region of E3; replacement of knob 5 with knob 3 | Havunen 2017 |
| **Ad5/3-FCU1** | Fusion of yeast cytosine deaminase and uracil phosphoribosyltransferase | No | Insertion of CMV/FCU1 into deleted E1; replacement of knob 5 with knob; deletion of E3 | Dias 2010 |
| **Ad5/3-D24-FCU1** | Fusion of yeast cytosine deaminase and uracil phosphoribosyltransferase | Oncolytic | 24-bp deletion in E1A; insertion of FCU1 into deleted 6.7K/gp19k region of E3; replacement of knob 5 with knob 3 | Dias 2010 |
| **Ad5/3-D24-hTNFa** | Human tumor necrosis factor alpha | Oncolytic | 24-bp deletion in E1A; insertion of hTNFa into deleted 6.7K/gp19k region of E3; replacement of knob 5 with knob 3 | Hirvinen 2015 |

| | | | | |
|---|---|---|---|---|
| **Ad5/3-hTERT-E1A-hCD40L** | Human CD40 ligand | Oncolytic | Insertion of hTERT into deleted E1 region; Insertion of E1A; Insertion of hCD40 into deleted 6.7K/gp19k region of E3 | Diaconu 2012 |
| **Ad5/3-CMV-hCD40L** | Human CD40 ligand | No | Insertion of CMV/hCD40L into deleted E1; replacement of knob 5 with knob 3 | Diaconu 2012 |
| **Ad5/3-CMV-mCD40L** | Murine CD40 ligand | No | Insertion of CMV/mCD40L into deleted E1; replacement of knob 5 with knob 3 | Diaconu 2012 |
| **Ad5/3-D24aCTLA4** | Anti-CTLA-4 antibody | Oncolytic | 24-bp deletion in E1A; insertion of aCTLA4 into deleted 6.7K/gp19k region of E3; replacement of knob 5 with knob 3 | Dias 2012 |
| **Ad5/3-aCTLA4** | Anti-CTLA-4 antibody | No | Insertion of CMV/aCTLA4 into deleted E1; deletion of E3; replacement of knob 5 with knob 3 | Dias 2012 |
| **Ad5/3-CMV-tras** | Anti-HER2 antibody | No | insertion of CMV/tras into deleted E1 region; deletion of E3; replacement of knob 5 with knob 3 | Liikanen 2016 |
| **Ad5/3-D24-tras** | Anti-HER2 antibody | Oncolytic | 24-bp deletion in E1A; insertion of tras into 6.7K/gp19k region of E3; replacement of knob 5 with knob 3 | Liikanen 2016 |
| **Ad5/3-TK-GFP** | Thymidine kinase (TK) fused to GFP | No | insertion of CMV/TK-GFP into deleted E1; deletion of E3; replacement of knob 5 with knob 3 | Raki 2007 |
| **Ad5/3-D24-TK-GFP** | Thymidine kinase (TK) fused to GFP | No | 24-bp deletion in E1A; insertion of TK-GFP into deleted 6.7K/gp19k region of E3; replacement of knob 5 with knob 3 | Raki 2007 |
| **Ad5/3-D24-hNIS** | Human sodium iodide symporter | Oncolytic | 24-bp deletion in E1A; insertion of hNIS into deleted 6.7K/gp19k of E3; replacement of knob 5 with knob 3 | Hakkaraine n 2009 |

### 6.1.2 CELL LINES (I-IV)

Cells lines used in this study are listed in Table 2. Cells were grown in recommended media under standard conditions in a humidified incubator at 37°C and 5% $CO_2$ unless specified otherwise.

Table2. Cell lines used in publications included in this dissertation.

| **Name** | Cell type | Used in publication | Growth media | Source |
|---|---|---|---|---|
| **A549** | Human adenocarcinoma | I,II | DMEM, 10% FBS, 1% P/S+ 1% L-glut | ATCC |
| **HEK-Blue hTLR9** | Human embryonic kidney cells | II | DMEM, 10% FBS, 1% P/S+ 1% L-glut, 1% Normocin | Invivogen (San Diego, CA, USA |
| **OVCAR-3** | Human ovarian cancer | II | RPMI 1640, 20% FBS, 1% P/S+ 1% L-glut | ATCC |
| **SK-MEL-28** | Human melanoma | II | DMEM, 10% FBS, 1% P/S+ 1% L-glut | ATCC |
| **SK-OV-3-LUC** | Human ovarian adenocarcinoma | III | 10% FBS, 1% L-glut and 1% Pen/strep | Dr Negrin, Stanford Medical School |
| **HaPT1** | Hamster pancreatic cancer | I, II, III, IV | RPMI 1640, 10% FBS, 1% P/S+ 1% L-glut | Dr Hernandez-Alcoceba (Pamplona, Spain) or DSMZ |
| **HaPT1 AIM2 KO** | Hamster pancreatic cancer | II | RPMI 1640, 10% FBS, 1% P/S+ 1% L-glut | Klaas de Lint (Netherlands) |

### 6.1.3 DC ISOLATION AND CULTURE (II)

Immature DCs were produced from PBMCs isolated from blood of healthy donors (Finnish Red Cross blood service, approved by the ethical board 12.03.2019, 14/2019). Density centrifugation (Lymphoprep solution, Stemcell technologies, Vancouver, Canada) was used for PBMC extraction according to the manufacturer's instructions. Erythrocytes were then removed by ACK lysis buffer (Thermo Fisher, Waltham, MA, USA). The collected cells were then washed and CD14+ cells (DCs, and macrophages) were isolated from the PBMC fraction using CD14+ magnetic beads (MACS Miltenyi Biotech, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Subsequently, DCs were grown for 6 days in RPMI supplemented with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, 1% L-glutamate, 20 ng/mL IL-4, and GM-CSF.

### 6.1.4 CRISPR/CAS9 KO (II)

The AIM2 gene was knocked out in the HAPT1 cell line. Ribonucleoprotein complexes (RNPs, Integrated DNA Technologies, IA) were prepared according to the manufacturer's instructions. The Neon Transfection System was used to deliver RNPs into HAPT1 cells using the with the Neon Electroporation Transfection system (ThermoFisher, MA) (settings 1200 Volt/20 ms/2 pulses). One day after RNP delivery, cells were seeded in 96-well plates (30 cells/plate) and clonal populations were assayed for successful editing of the target site by PCR.

### 6.1.5 TIL PRODUCTION (III, IV)

TILs for adenovirus delivery experiments (V) and for analysis of the effects of oncolytic viruses and TILS combined on ovarian cancer (III) were produced from human ovarian cancer patient samples and from Syrian hamster grown tumors.
Human patient tumor samples were cut into small fragments and placed into a G-Rex 6-well plate with TIL medium (TM). TIL medium contains RPMI 1640 supplemented with 20% FBS, 1% L-glutamine, 1% Pen/strep, 15 mM HEPES, 1 mM Na-pyruvate, 50 μm β-mercaptoethanol (Sigma-Aldrich), and 3000 IU/mL recombinant human IL-2 (Peprotech, Stockholm, Sweden). The fragments were kept in media for 7 days. Before use, TILs were activated with irradiated allogeneic PBMCs (40 Gy; ratio 1:200 TIL:PBMCs) and anti-CD3 antibody (30 ng/mL; ebioscience™, Thermo Fisher Scientific, Waltham, MA, USA) or OKT3 antibody (30 ng⁄mL; eBioscience, Thermo Fisher Scientific) and expanded in a mixture of activation and rapid expansion (RM) medium containing RPMI supplemented with 20% FBS, 1% L-glut, 1% Pen/strep, and 3000 IU/mL recombinant human IL-2.
Hamster TILs were generated similarly using HapT1 tumor fragments cultured in TM and activated by ConcavalinA (Sigma-Aldrich) on day 5 by a protocol developed in our laboratory (Siurala et al., 2016b)

### 6.1.6 EFFECT OF TILS AND ONCOLYTIC ADENOVIRUSES ON CANCER (III)

Ovarian single-cell samples from human patients were depleted from CD3+ cells using CD3 MicroBeads (Miltenyi Biotec, Germany). Lymphocyte-depleted patient samples were incubated with MOI 100 of Ad5/3-E2F-D24, TILT-123, or medium. Fresh autologous, expanded TILs were added after the washing steps. After 6 days of incubation, the supernatant and cells were collected for detection of IFN-γ and analysis of T-cell markers, respectively.

To investigate the binding of adenovirus to human and hamster TILs, TILS were incubated with 10 virus particles (VP)/cell in TILT-123 or control conditions. After incubation, the samples were centrifuged and cell pellets and supernatants were analysed separately to quantify bound and unbound viruses. Alternatively, a mixture of TILT-123 (100 VP/cell) and hamster TILs were used similarly as above to determine if there are differences between binding of TILT-123 to human or Syrian hamster TILs. After incubation, DNA was extracted using QIamp DNA kits (Qiagen, Germany) according to the manufacturer's instructions to quantify the viral genomes through quantitative PCR (qPCR).

## 6.2 INVESTIGATING DANGER- AND PATHOGEN ASSOCIATED MOLECULAR PATTERN SIGNALING (II)

SK-MEL-28, OVCAR-3, or A549 cells were infected with unarmed, single-armed, or double-armed adenoviruses. The supernatants or cells were collected at three time points to determine extracellular ATP levels or to measure HMGB1. Extracellular ATP was measured using ATP Determination kit (Molecular probes, Invitrogen, Carlsbad, CA, USA) according to a standard protocol. HMGB1 was measured by HMGB1 ELISA kit (IBL) according to the manufacturer's instructions. Calreticulin cell-surface expression was measured by flow cytometry.

For NF-κB signaling pathway analysis, SK-MEL-28 cells were grown and infected with 100 VP/cell or left uninfected (mock control). The cells were collected and proteins were isolated using Qproteome Mammalian Protein Prep Kit (Qiagen) according to a standard protocol. Protein concentrations were determined with Bradford assay and proteins were analysed by Proteome Profiler Human NFκB pathway Array (R&D systems, Minneapolis, MN, USA) according to standard procedures. ImageJ software was used to determine the intensity of membrane protein signals.

AIM2 antagonist and agonist effects on DCs were analysed by antagonist/agonist interaction studies. SK-MEL-28 cells (100 000 cells/well), were seeded on 6-well plates. The next day, immature DCs were added (1:1 ratio) with or without antagonist Ac-YVAD-cmk (10 μg/ml) or Poly Lyo Vec-complex (1 μg Poly dA:dT per 100 μl of LyoVec, 50 μl of complex/ml) (both from InvivoGen) for 24 h and analyzed by flow cytometry (BD Accuri). LPS (100 ng/ml, from Sigma) was used as a positive control. HEK-Blue hTLR9 activation tests were performed according to the manufacturer's protocol. A549 cells were cultured and infected (100 VP/cell),

and the supernatant used for TLR9 activation was collected 48 h post infection and filtered (100-kDa filter unit, Amicon Ultra 4, Merk Millipore Burlington, MA, USA) for virus removal according to manufacturer's protocol. Alternatively, HEK-Blue hTLR9 cells were directly infected and measured according to the manufacturers' protocol (100 VP/mL).

### 6.2.1 PCR (II-IV)

To analyze the intercation of ovarian cancer cells with oncolytic adenoviruses, SK-OV-3-LUC cells were challenged with several MOIs of Ad5/3-E2F-D24-TNFa-IRES-IL-2. After infection, cells were collected and DNA was extracted with QIAmp DNA Mini Kit (Qiagen, the Netherlands) according to the manufacturer's instructions. Viral presence was confirmed by quantitative PCR targeting the d24 deletion of the E1A region using forward primer (5′-TCCGGTTTCTATGCCAAACCT-3′), reverse primer (5′-TCCTCCGGTGATAATGACAAGA-3′) and probe (5′FAM-TGATCGATCCACCCAGTGA-3′MGBNFQ). The results were normalized against the content of human β-actin housekeeping gene DNA.
The success of AIM2 KO (GAACAACTGATTGAGAGTGCAGG, 150–172 bp downstream of the AIM2 start codon) was confirmed with PCR by amplifying the region between 256 bp upstream and 252 bp downstream of the target site in the genomic DNA (primers AAGACAATGGGTGGCAGTCC and TTCTGTGGAGCACTCACCTT, Kapa HiFi HotStart 2x Readymix PCR kit, Roche, Switzerland) and Sanger-sequencing (primer GCGGAGGAATTTCTAACACTCCTTTTA, Eurofins Mix2Seq kit, Eurofins, Luxembourg). The selected clone was found to have a homozygous 5-bp deletion directly upstream of the PAM sequence of the target site leading to an out-of-frame deletion mutation (GAACAACTGATTGAG-----AGG).
For cytokines and maturation marker microenvironmental analysis in an AIM2 KO and oncolytic adenovirus setting, qPCR probes and oligos (forward and reverse) were purchased from Metabion and designed by (Zivcec et al., 2011), PCR reactions were performed using LightCycler® 480 Probes Master (Roche) according to the following program: denaturation at 95°C for 30 sec, annealing at 50°C for 30 sec, and extension at 72°C for 30 sec for 40 cycles.

### 6.2.2 RNA SEQUENCING (II)

SK-MEL-28 infected with TILT-123 (100 VP/cell), DCs, or both were added to achieve a cell ratio of 1:2 (2 000 000 DCs/well). Cells were collected and suspended into RNAlater (Qiagen, Valencia,CA, USA). RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Finally, RNA quality and quantity were

measured using a Nanodrop 1000 (Waltham, MA, USA). Sequencing was performed by BGISEQ-500RS (BGI Europe Genome Center, Amsterdam, The Netherlands).

### 6.2.3 PRODUCTION OF TIL/VIRUS MIXTURE FOR TRAFFICKING EXPERIMENTS (III)

For TILs for delivery of adenovirus animal experiments, washed hamster TILs and TILT-123 (ratio 1:100 or 1:500 TIL:VP) were combined in a 24-well plate. After a 30-min incubation, the mixture was collected and injected in mice. Separately, for TIL tracing, Qtracker™ 525 Cell Labeling Kit (Thermo Fisher Scientific) was used according to the manufacturer's instructions for hamster TILs before virus incubation (as stated above) to determine TIL trafficking.

## 6.3 ANIMAL MODELS (II, III, IV)

### 6.3.1 ASSESMENT OF ONCOLYTIC ADENOVIRUS EFFECTS ON TUMOR MODELS IN VIVO (II-IV)

Established Syrian Hamster HapT1 subcutaneous tumors were treated by intratumoral (i.t.) injections of PBS or $1 \times 10^9$ VP of TILT-123 only. A third treatment group received virus i.t. ($1 \times 10^8$ VPs) injections simultaneously with intraperitoneal (i.p.) injection of $8 \times 10^6$ hamster TILs. A fourth group was treated with TILs pre- loaded with TILT-123 with $4 \times 10^9$ VPs (ratio 1:500 TIL:TILT-123) injected i.p.. After treatments, tumors were analysed for tumor growth and collected for virus particles determination.
A higher carrier to virus ratio and longer incubation time was also investigated in hamsters. Animals received i.p. injections of $8 \times 10^6$ hamster TILs loaded with TILT-123 with $0.8 \times 10^9$ VPs (ratio 1:100 TIL:TILT-123) or $0.8 \times 10^9$ VPs of TILT-123 (or same controls as above). Samples were collected 5 days after the start of treatment.
Subcutaneous HapT1 tumors were implanted in immunocompromised NOD.Cg-Prkdcscid-Il2rgtm1Sug/JicTac (Taconic, Silkeborg, Denmark) mice in both left and right flanks. Mice were treated by either a combination of a i.t. injection of PBS or 1 × 108 VPs/50 µL and
i.v. injection of $5 \times 10^6$ hamster TILs, i.v. of $5 \times 10^6$ TILs pre-loaded with $2.5 \times 10^9$ VPs of TILT-123 (ratio 1:500 TIL:TILT-123), or i.t. $2.5 \times 10^9$ VP/100 µL of TILT-123 for analysis of the presence of adenoviruses by PCR genome determination.

The antitumor efficacy of TILs loaded with TILT-123 was examined. HapT1 tumors were injected subcutaneously in both the left and right flanks of immunocompromised NOD.Cg-Prkdc$^{scid}$-Il2rg$^{tm1Sug}$/JicTac (Taconic). Mice were treated twice; left tumors received i.t. injections of PBS or TILT-123 ($1 \times 10^9$ VP) and i.v. injections of $5 \times 10^6$ hamster TILs, $5 \times 10^6$ hamster TILs loaded with TILT-123 with $2.5 \times 10^9$ VPs (ratio 1:500 TIL:TILT-123), i.t. $2.5 \times 10^9$ VPs of TILT-123, or PBS. Tumor size was evaluated by electronic caliper measurements. The normalized tumor volume was determined by normalizing tumor volumes from each day to the corresponding days from the PBS tumor group.
For AIM2 studies, two subcutaneous tumors (HapT1 or HapT1 AIM2 KO, $4 \times 10^6$ cells/tumor) were implanted into the flanks of Syrian hamsters. When tumor size reached approximately 5 mm in diameter, tumors were treated by i.t. injections with either PBS, unarmed oncolytic adenovirus (OAd), or TILT-123 ($1 \times 10^9$ VPs/ Cells /tumor/treatment time). The tumors were collected into RNAlater (Qiagen, Valencia, CA, USA) and analyzed by RTqPCR.

## 6.4 TUMOR SAMPLE PROCESSING AND FLOW CYTOMETRIC ANALYSIS (II-IV)

For DAMP and PAMP analysis with flow cytometry, the primary antibody was incubated for 30-45 min in dark. After washes the secondary antibody was incubated similarly according to the manufacturer's suggested concentrations. After staining, cells were analyzed using a BD Accuri C6 flow cytometer with BD Accuri C6 software (BD biosciences, Version 1.0264.15, BD Biosciences, San Jose, CA, USA).
For project III, data analysis was performed with a FACS Aria II cell sorter (BD Biosciences). Data analysis was performed using FlowJo software V.10 (FlowJo, BD Biosciences). Geometric mean data from activation markers were normalized to the vehicle group.
The antibodies used in these studies are listed below in Table 3

Table 3. Antibodies for flow cytometrical analysis

| Antibody | Company |
|---|---|
| Anti-Rat CD8b PE | ebioscience™, Thermo Fisher Scientific |
| Anti-mouse CD4 APC | ebioscience™, Thermo Fisher Scientific |
| Anti-human CD1 | Biolegend, |

| | |
|---|---|
| Anti-human CD3 | Biolegend |
| Anti-human CD11c | Biolegend |
| Anti-human CD80 | Biolegend |
| Anti-human CD86 | Biolegend, |
| Ab2907 anti-calreticulin antibody as primary antibody | IBL |
| Ab 15007 Goat Anti-Rabbit IgG H&L as secondary antibody (Alexa Fluor®488) (Abcam, Cambridge, United Kingdom). | Biolegend |
| anti-EpCAM, Alexa Fluor 700-labeled | Biolegend |
| anti-CD45 | Biolegend |
| PE/Dazzle 594-anti-CD69 | Biolegend |
| PerCP-Cy5.5-anti-CD4 | Biolegend |
| FITC-anti-CD8 | Biolegend |
| APC-anti-CD25 | eBioscience, Thermo Fisher Scientific |
| PE-Cy7-anti-programmed death ligand 1 (PD-L1) | Biolegend |
| PE/Dazzle-594-anti-FoxP3 | Biolegend |
| PE/Dazzle 594-anti-CD3 | Biolegend |
| APC/Cy7-anti-CD16 | Biolegend |
| APC/Cy7-anti-PD-1 | Biolegend |
| APC-anti-CD56 | Biolegend |
| APC-anti-CD25 | eBioscience, Thermo Fisher Scientific |
| PerCP-Cy5.5-anti-CD4 | eBioscience, Thermo Fisher Scientific |
| FITC-anti-CD8 | eBioscience, Thermo Fisher Scientific |
| PE-Cy7-anti-CD27 | BD Biosciences |

### 6.4.1 PATIENT MATERIAL PROCESSING, ESTABLISHMENT OF EX VIVO AND IN VITRO TUMOR CULTURES

Fresh single-cell tumor digests were prepared from OVCA tumors. Small OVCA tumor fragments were placed in RPMI 1640 (Sigma-Aldrich, Missouri, USA) supplemented with 1% L-glutamine, 1% Pen/strep (Gibco, Thermo Fisher Scientific, Massachusetts, USA), collagenase type I (170 mg/L), collagenase type IV (170 mg/L), DNase I (25 mg/mL), and elastase (25 mg/mL) (all enzymes from Worthington Biochemical) for overnight enzymatic digestion with rocking at 37°C. The digested cell suspension was filtered (100-µm filter) and treated with Ammonium-Chloride-Potassium lysis buffer (Sigma-Aldrich) to lyse and remove red cells. The treated tumor cells were used to establish *ex vivo* tumor cultures used for ovarian cancer and oncolytic virus assessments.

### 6.4.2 CYTOTOXICITY ASSAY

The cell viability of OVCA ex vivo tumor cultures was verified by incubating 50 µL of CellTiter 96 AQueous One Solution Proliferation Assay reagent (Promega, Wisconsin, USA) for 2–3 hours. Absorbance was determined at 492 nm by a Fluostar OPTIMA analyzer (BMG Labtech, Offenburg, Germany). Each data timepoint was normalized to the vehicle control group.

### 6.4.3 CYTOKINE EXPRESSION AND VIRUS PROPAGATION ASSAY

SK-OV-3-LUC cells were seeded and incubated overnight in a 96-well plate. The following day, several MOIs of Ad5/3-E2F-D24-TNFa-IRES-IL-2 were added to the culture. After 72 hours, supernatant from cultures was collected in protease inhibitor and stored at -20°C until analysis. Cells were also collected in phosphate-buffered saline and DNA was extracted with QIAmp DNA Mini Kit (Qiagen, the Netherlands) according to the manufacturer's instructions. Virus presence was confirmed by quantitative PCR.

### 6.4.4 CYTOKINE ANALYSIS

To analyse the effect of viruses on the cytokine environment, supernatants from the treated OVCA ex vivo tumor cultures were collected and the presence of IFN-γ, TNF-α , IL-2, IFNβ, C-X-C motif chemokine 10 (CXCL10), interleukin 6 (IL-6), arginase, and transforming growth factor β-1 (TGF-β1) was determined using a LEGENDplex panel and a free active/total TGF-β1 detection kit (Biolegend, California, USA) according to the manufacturer's

instructions. The levels of TNF-α and IL-2 in supernatants from SK-OV-3-LUC cells were determined using the human IL-2 and TNF-α flex sets (BD Biosciences, California, USA). Samples were measured using Accuri C6 flow cytometer (BD Biosciences) and analyzed with LEGENDplex V.8.0 software (VigeneTech, Massachusetts, USA) or FCAP Array software (BD Biosciences). When appropriate, data obtained were normalized to vehicle control group and data points outside of the standard curves were extrapolated according to the software predictions and only considered when at least four values inside the standard curve could be retrieved in one of the analyzed days. The normalized levels of pooled proinflammatory and suppressive cytokines were derived from a three-step procedure: (1) each patient's individual cytokine average concentration value of the different therapeutic groups was normalized against the vehicle control; (2) each patient's individual normalized cytokine values were summed according to their category (proinflammatory or suppressive) and the average of each therapeutic group was calculated; and (3) the sum of each patient's grouped cytokine normalized values we renormalized against the vehicle control group. The values shown in the graph are the averages of each therapeutic group.
OVCA TIL-tumor cocultures were collected and IFN-γ detection was performed using ELISA MAX Deluxe Set Human IFN-γ (Biolegend) following the manufacturer's instructions. Absorbance was measured at 450 nm using a Fluostar OPTIMA analyzer (BMG Labtech, Offenburg, Germany) and values were interpolated using a line as standard curve with GraphPad Prism V.8 software (GraphPad, La Jolla, CA, USA).
For further proof of AIM2 activation, SK-MEL-28 and A549 cells were infected with OAd. TNF-α and IL-2 (1000 VP/cell) and cell-culture supernatants were analysed by ELISA (Invitrogen IL1Beta kit) 24 h p.i. indicating active, plausibly AIM2 dependent, cleavage and release of IL-1β.

### 6.4.5 HISTOPATHOLOGY

After resection, tissues were fixed, paraffin embedded, and cut into thin sections (4-5 μm) for slides. Slides were stained for immunohistochemistry with H&E staining. Anti-PD-L1 (Abcam, UK) staining was performed using Lab Vision Autostainer (Thermo Fisher Scientific). Anti-CD8 (Novocastra, Leica Biosystems, Illinois, USA) and anti-CD4 (Cell Marque, Merck, California, USA) staining were performed using a VENTANA Autostainer (Roche, Switzerland).

## 6.5 STATISTICAL ANALYSIS (I- IV)

GraphPad Prism 8 (GraphPad Prism Software Inc., San Diego, CA, USA) was used to generate graphs. Unpaired Student's *t*-test with Welch's correction and correlations between variables were investigated using Spearman's non-parametric correlation. Assessment of differences between experimental groups was performed with regular one-way ANOVA with Tukey's multiple comparison test or Kruskal-Wallis with Dunn's multiple comparisons. In the mouse efficacy experiment, statistical differences between therapeutic groups were assessed on log-transformed absolute tumor volumes or PBS group-normalized tumor volumes using linear mixed models in SPSS version 26 (IBM, North Castle, NY, USA).

## 6.6 ETHICAL STATEMENTS (I- IV)

All animal protocols were approved by the Provincial Government of Southern Finland and the experimental animal committee of the University of Helsinki (ESAVI/28404/2019). The use of healthy human donor blood was approved by the ethical board of the Finnish Red Cross blood service (12.03.2019, 14/2019). Analyses performed on human patient samples were approved by the central hospital operative Ethics committee (permit number 120/13/03/16) and were collected after patients provided informed consent.

# 7 RESULTS AND DISCUSSION

Although adenovirus vectors have shown great promise in several clinical trials, further optimization of this approach is warranted. This thesis examined the factors affecting the use and efficacy of adenovirus vectors, vector production, immune stimulating signaling, and administration route and infiltration in the tumor vasculature. We sought to understand factors that limit the use and efficacy of patients treated with adenovirus vectors.

## 7.1 EFFECTS OF GENE MODIFICATIONS TO ADENOVIRAL VECTORS (I)

Even though many types of oncolytic and vector adenoviruses have been constructed, the effect of these modifications are poorly known. The lack of understanding is a problem in the field, since, it is easy to attempt to construct almost any type of virus but, but, many modifications might easily affect for example virus assembly, stability etc.
When adenovirus vectors were designed, the knob proteins of several adenovirus vectors were exchanged to serotype 3. This switches the virus receptor to desmoglein 2, which is more frequently expressed on many cancer types. The effect of knob protein switch was measured by comparing the physical and functional titers of different adenovirus vector constructs. We found that the production of chimeric 5/3 adenoviruses is not as efficient as non-chimeric viruses ($p = 0.0412$). However, the numbers of functional virions were not lowered by this modification. Interestingly, supporting these results, it has been shown in other studies that the chimera OAd5/3Δ24 led to greater toxicity in 3D spheroids compared to wildtype OAd5 (McKenna, Rosewell-Shaw and Suzuki, 2020). When compared to other capsid modifications (such as RGD, pk7, pk21, RGD + pk7), a similar but non-significant trend of toxicity was observed.
Transgenes are often inserted into virus vectors to modify virus effect on the tumor. For example, GM-CSF is often inserted to enhance the immune reaction towards cancer. Although we observed that the type of inserted transgene significantly affected functional virus titers, it did not significantly affect the number of produced functional virion particles. The functional titer of virus groups with immunostimulatory ($p = 0.0054$), non-immunostimulatory transgenes ($p = 0.0403$), and antibody transgenes ($p = 0.0131$) was significantly lower compared to the control group of viruses with no added transgenes. It can be speculated, that the production of a big construct, such as an antibody, might slow down the production of viruses as the machinery needed for virus protein production is occupied.
If virus vectors were compared according to number of functional modifications, there was a significant drop in both functional and physical virion numbers if more than four modifications were made (from $p = 0.0139$

and p = 0.002, respectively). The vectors were originally constructed according to previously approved size standards (the genome size was moderately similar); a correlation between genome size and physical or functional virus titers was not observed. The drop in both functional and physical titers show how much is still to be learned about adenovirus replication. By several insertions we are more likely to interfere with an important function of the virus. However, this study shows that adenviruses are a great tool as the virus size allows for insertion of bigger or several genes, and many things can be modified. By investigating adenoviruses the knowledge can be used to optimize vector design and can be even used to understand other vectors as well.

## 7.2 DAMP AND PAMP SIGNALING AND TILT-123 (III)

The TILT-123 virus was created for use in combination with T-cell therapies to activate the immune system against cancer. However, the mechanism behind immune activation is not fully understood. Therefore, we studied whether PRRs are activated through virotherapy and lead to release of immune-stimulating DAMP and PAMP molecules in cancer cells. This study revealed that TILT-123 induced the expression of known DAMP signalling markers. An increase in both extracellular ATP and HMGB1 as well as increased cell-surface expression of calreticulin was observed (Chan et al., 2012). Lymphocyte activation and attraction occur when these three molecules are outside the cell.

Chronic high-level expression of alarmins is associated with negative outcomes for patients, whereas dynamic and acute expression may provide a positive immune response. Alarmin measurements vary between cell lines and timepoints and it is plausible that the measurements missed the peak alarmin release or expression, as expression in other cell lines was significant at some timepoints. Alarmin release has been associated with immune cell infiltration and positive treatment outcome in cancer patients and thus, this might indicate a new mode of action for TILT-123. Not only does it lyse cancer cells, but also attracts immune cells to the tumor through alarmin release (Chanet al., 2012;Hernandez, Huebener and Schwabe, 2016).

To complement this data, we used mRNA sequencing to identify the most differentially activated pathways responsible for DAMP and PAMP recognition. This confirmed the effectiveness of virotherapy and the activation of several DAMP- and PAMP-signaling pathways. Interestingly, the inflammasome AIM2 was activated in both DC and SK-MEL28 cells (unfiltered results), which warranted further investigation.

NF-κB signaling pathway analysis revealed upregulation of ASC protein, which is a part of the AIM2 inflammasome. This further supports the RNA sequencing results. With this in vitro data, we studied the reaction in vivo by comparing virus-treated Syrian hamster HaPT1 tumors in either wild-type or

AIM2 knockout genetic background. The knockout tumors grew faster and virotherapy was less effective than in wild-type tumors treated similarly. These results indicate that AIM2 plays an important role in TLT-123 recognition and induces danger signaling during TILT-123 infection, thus creating an tumor diminishing environment through activation of pro IL-1β to its active form.
IL-1β expression was high in wild-type HapT1 tumors, while the converse was true for KO tumors. IL-1 is a co-stimulatory cytokine and induces T-cell proliferation, which consequently triggers IL-2 secretion and expression of IL-2Rs by activated T cells. Consistent with this, IL-1β levels were significantly upregulated in TILT-123-treated tumors, and simultaneously, a trend for higher IL2 and IL2R expression could be also be seen in these tumors. This suggests that AIM2 activation plays an important role in TILT-123 virotherpy: the activation of AIM2 inhibits cancer growth during virotherapy administration and causes the secretion of inflammatory cytokines, which attract and activate immune cells against cancer. This result has been shown in other studies as well without the knowledge of the exact mechanisms (Havunenet al., 2017;Quixabeiraet al., 2021).
Additionally, TILT-123 may activate AIM2-dependent secretion of gasdermin, which induces cell killing and is associated with immune modulation of the tumor microenvironment. Additionally, IL-6 expression was abrogated in knockout tumors. Previous studies have shown that IL-6 has several effects on cancer growth. IL-6 can recruit neutrophils and T cells but can also activate several cancer cell growth-inducing signaling pathways. Furthermore, IL-6 can affect IL-2 signaling and thus inhibit T-cell recruitment and activation(Chen et al., 2013;Nish et al., 2014). In virotherapy-treated tumors, a trend of higher IL-2 expression was noted. IL-2 plays an essential role in the immune system, affecting tolerance and immunity primarily via its effects on T cells(Berraondoet al., 2019). IL-2 promotes differentiation of T cells into effector and memory T cells after antigen stimulation and, was thus chosen as one of the two cytokines added into the OAd arming device. The other part of the TILT-123-arming device, TNF-α , is a cytokine that is involved in both local and systemic inflammation. TNF-α is produced chiefly by activated macrophages, although it can be produced by many other cell types as well. While TNF-α has a plethora of effects, it is known for its cell-destroying properties (Tähtinenet al., 2015). Thus, the high expression of both TNF-α and IL-2 during TILT-123 infection in vivo probably enhanced the observed tumor growth limitation. Similar effects of TILT-123 have been seen in Havunen et al's studies in 2017 (Havunenet al., 2017).

When samples were examined, more evidence of the contribution of TILT-123 to antitumor efficacy was revealed. Virotherapy induced expression of the DC maturation marker CD83 in tumors. This work revealed that TILT-123 adenovirus virotherapy modulates the tumor microenvironment through

DAMP and PAMP release and subsequent activation of sensors, such as AIM2. These effects may underlie the efficacy of TILT-123 and may be relevant for many other oncolytic viruses as well.

## 7.3 EFFICACY OF ONCOLYTIC ADENOVIRUS TREATMENTS ON OVARIAN CANCER (II)

The effect of the tumor microenvironment is multifaceted. In this project, human ovarian cancer patient samples were analyzed to evaluate the effect of the tumor microenvironment on virotherapy. By using patient samples, a more versatile and realistic view of the effects of virotherapy could be assesed in this mixed context of several different cell types found in human tumors. We observed that TILT-123 therapy was an effective killer of most ovarian cancer cell samples. By one week, the virotherapy decreased the viability of five out of six ex vivo cultures by 95% to 60% relative to the vehicle control. One sample did not show as marked of a decrese in viability. This could be due to the specificity of the virus, as it only infects and lyses tumor cells. It is possible that cultured tumor piece that remained viable in this sample may have contained high numbers of infiltrated immune cells, fibrocysts, or other cells that were not cancerous and were thus not lysed. When the cultures were further analyzed, we observed that TNF-α and IL-2 expression in tumor cultures were dependent on amount of virus DNA. Ad5/3-E2F-D24-hTNFa-IRES-hIL2 therapy also caused induction of IFN-γ and CXCL10 production which peaked at day 6 and beyond. The proinflammatory cytokines correlated negatively with tumor histoculture viability. This indicates that the virus can cause a change in the tumor towards a proinflammatory microenvironment. It has been shown that this supports tumor infiltration, which in turn is often associated with positive patient outcome (Mita et al., 2018). Furthermore, the virotherapy induced several TNF-α and IL-2 dependent changes in TILs of the ex vivo culture. The activation status of CD8+ T cells were increased overall, as measured by number of CD69+ CD45+ CD4- CD3+ CD8+ cells. As these are T cell activation markers, their activation is expected to lead to cytotoxic activity and death of tumor cells. However, as often in immunology most reactions have an counter reaction. In the case of CD69, it has also been shown that it can indicate T cell exhauston and could be a marker for poorer prognosis (Mitaet al., 2018). A trend for lower numbers of D4+CD69+ TILs on day 6 and beyond was observed during oncolytic adenovirus therapy in comparison with the vehicle control group. When CD69 mean fluorescent intensity (MFI) expression of CD4+ cells was measured, 3 out of 5 samples showed statistically significantly higher expression.

The overall number CD25+ cells out of measured CD4+ EpCam- CD8- cells was greater in virotherapy-treated cells, while expression of CD25 was not statistically significantly changed in CD4+ cells (based on MFI).

To analyze the effect of TIL therapy on patient samples, patient samples were depleted of CD3+ cells and TILs from each sample were cultured and expanded. These products contained mostly CD8+ TILs (65.5%), followed by CD4+ TILs (26.4%) and CD3+CD56+ natural killer (NK) T cells (3.3%). The CD3- fraction included 14.8% NK cells and <2 % of CD4+CD25+FoxP3+ T regs at day 14 when patient ex vivo cultures were treated with this TIL therapy in combination with virotherapy or control treatment. IFN-γ levels were measured on three different days. These measurements showed already enhanced IFN-γ production in two samples on day 2 and peaking at day 7 with high concentrations of IFN-γ in four out of six samples. The differences were statistically significant compared to mock-treated samples.

To determine the mechanism of the tumor-destroying reactions that was boosted by virotherapy, we further characterized the expanded TIL cell subset. By day 7, Ad5/3-E2F-D24-hTNFa-IRES-hIL2 therapy had substantially increased the presence of CD8+ TILs compared to control treatments; mock and Ad5/3-E2F-D24, in sample OVCA #1, OVCA #3 and OVCA #4. In sample OVCA #2P, the same reaction could be measured in the Ad5/3-E2F-D24 treatment group. Interestingly, IFN-γ concentration correlated with decrease in the presence of CD8+ TILs ($p=0.0015$). In contrast, higher IFN-γ concentrations correlated with higher percentage of CD4+ cells, and four out of six cultures had higher percentage of CD4+ cells post culturing in the virotherapy group. Still, the cytokine environment created by TILT-123 seems to support an cytotoxic cell population, also shown to be beneficial in terms of tumor size reduction in other studies (Havunenet al., 2017).
The marked differences in the IFN-γ responses led us to hypothesize that PD-1/PD-L1 status could be driving the reaction. In ex vivo cultures, only one tumor showed significantly fewer PD-L1 positive cells after virotherapy on day 3; the difference diminished by day 6. There were no significant differences in MFI of PD-L1 measured on either time point.
IHC analysis revealed that samples with low PD-L1 expression additionally had lower infiltration of CD4+ and CD8+ cells. A strong correlation between high PD-L1 expression and IFN-γ expression co-occurred with high CD4+ cell count.
Despite the existence of multiple immune targets in the tumor microenvironment of patients with ovarian cancer, usage of immunotherapies have not shown consistent clinical efficacy. This may be due to differences in patient inclusion criteria or the clinical protocol. (Drakes and Stiff, 2021;Son et al., 2022). In this study, TIL-based treatments have demonstrated promising results in patients with ovarian cancer, supporting the use of TILT-123 and T ACT in treatment of ovarian cancer patients.

## 7.4 LYMPHOCYTES AS VIRUS CARRIERS (IV)

Several studies have shown that combining oncolytic virotherapy with cellular therapies generally result in good antitumor efficacy (Havunenet al., 2017). The reason behind the formed synergy can be due to several factors. One of them could be the efficient transportation of viruses by the administered cells to the tumor site. For example, mesenchymal cells have been shown to improve the delivery of oncolytic adenoviruses (Garcia-Carbonero et al., 2017).

The effects of T cells on tumors are numerous. It is known that T cell infiltration and patient outcome correlates and several studies show that TIL therapy can generate great, tumor diminishing results (Dieciet al., 2015).
It is also known that Adenoviruses can bind to T cells, and that the virus used in this study (TILT-123) has been modified to express cytokines that enhance T cell therapy. Building upon this information we formed a hypothesis: maybe TILs could be used as virus carriers when mixed together and injected intravenously. Furthermore, maybe TIL's preferential infiltration into tumors could potentially lead to both TILs and viruses to the tumor, providing additional therapeutic benefits compared with virotherapy alone. Intravenous administration is also relativeliy easy and arguably more safe to perform than intratumoral injections, and it could enhance rapid spread of the virus (to also distant and micro-tumors).

The results gathered in our study support our hypothesis that TILs can support virus transport to the tumor. Firstly, we proved *in vitro* that viruses attach to both human and hamster T cells, thus suggesting that the cells can be used as "virus delivery vessels". This experiment was followed up by an *in vivo* Syrian hamster model, in which lymphocyte + adenovirus treatment enhanced virus presence in tumors and showed better anti- tumor efficacy compared to mock, or only virus treated hamsters on day 5 (not statistically significant). The reduction in tumor size might have been caused by the combined effect of both antitumor activity and the changes in intratumoral immune cell populations, which have also been shown in previous studies, as well (Cervera-Carrasconet al., 2020b;Havunenet al., 2021). The combination of TILT-123 and TILs in tumor-bearing immunocompetent Syrian hamster can change the immune cell composition and induce the infiltrations of TILs (Cervera-Carrasconet al., 2020a;Donia et al., 2012;Havunen et al., 2018), which are indicative of a induction of immune cell mediated clearance of the tumor.

Continuing the investigation, an *in vivo* NOG mouse model was used. Engrafted HAPT1 tumors were treated with vehicle, intratumoral virus + intraperitoneal TILs, virus only (intratumoral), or a mixture of virus and TILs

administered intravenously. This model was chosen because hamsters lack suitable veins to do intravenous injections.
This experiment showed a trend that the premixing enhances virus delivery to the tumor and induces lymphocyte infiltrations compared with (intravenous) virus alone.

The results from the animal experiments give insight into how delicate animal work is. In such a complex setting, timing is of essence. In order for the sampling to show proper results, the virus has to reach the tumor and the sampling has to happen during the right time. This might be the reason for the lack of statistical significance in the results. More time points, re-evaluating the dosing of TILs and virus could be considered in future trials. This being said the results are still showing consistent trends of animals benefitting from treatment with the combinatorial therapies.

This study, like the field in general, has suffered from the lack of suitable models (Rincónet al., 2017). To combat this, some have used patient derived xenografts. Unfortunately these are hard to generate in NOG mice. Furthermore, creating TILs from patient samples for reduction of incompatibility issues is challenging.

Similar studies, performed with VSV and HSV viruses and carrier cells, have shown positive results. Interestingly, VSV, VV and HSV might enter T-cells, while studies indicate that adenoviruses only bind to the surface of T-cells. For example there's indications that PBMCs are resistant to recombinant serotype 5 adenovirus infection (Bauerschmitz et al., 2006;Zafaret al., 2020). Data also suggest that the 5/3 chimeric adenovirus might bind to the lymphocyte membrane, from where it can supposedly more easily be released into tumors (Sengupta et al., 2011;Zafaret al., 2020). Furthermore, it is proposed that chimeric (5/3) adenoviruses use Desmoglein-2 as their receptor, which is minimally expressed by T cells. In another study, transduction of T-cells was achieved when an RGD-fiber was inserted in the HI-loop of the fiber (Senguptaet al., 2011). These modified viruses have been recorded to transduce several cell types, such as DCs and T cells.

One can speculate about the reasons behind the slightly differing results in this study's animal models using TILs compared to other preclinical and clinical studies. For example, it has been shown that expanding TILs from tumor tissue creates mostly an activated population of tumor reactive T cells, but at the same time, there's a population of TILs that are not significantly reactive (Hall et al., 2016). These cells might benefit from activation by mature antigen-presenting cells (APCs), such as dendritic cells (DC). Dendritic cells are not

functional in immunocompromised mice. This might have affected the results in this study, causing a modest effect of TIL therapy.
In conclusion, even though a slight enhancement of antitumor activity was shown, some optimization in sampling and administration, in order to achieve great results, might be needed. However, still in light of the results, T cells as virus carriers remains a promising tool that could show great results in actual clinical therapeutic settings.

# 8 CONCLUSIONS AND FUTURE PROSPECTS

The results gathered in this study show that oncolytic virotherapy is an promising tool for cancer treatments. The uniqueness and benefit from most viruses over more traditional medications are their ability to both directly kill cancer cells by lysis and further more activate several signaling cascades leading to immune activation towards cancer. Currently, several adenovirus vectors are in clinical trials (in June 2022, 49 clinical trials were "actively recruiting" in clinicaltrials.gov, when the search term "adenovirus and cancer" was used), indicating that there's an interest in the field and encouraging pre-clinical results supporting the development of these vectors. Currently, TILT-123 has entered phase 1 clinical trials, with positive interim results that were presented at ASCO (Inge-Marie et al., 2022).

Even though immunotherapies such as virotherapy have shown promising results, it still isn't "one treatment fits all"- solution. Especially since there aren't currently any virotherapy solutions for treating blood cancers on the market in Europe. Therefore, more work needs to be done before all patients can be treated and cured.
The strength of oncolytic adenoviruses lies in the way they can effectively transform the TME and induce immune responses and lyse cancer cells, both through inherent mechanisms and by genetic modification in the laboratory (addition and removal of genes that affect the immune system)(Cervera-Carrasconet al., 2020b;Santos et al., 2020). For example the concentrations of IL-2 that is required for a therapeutic effect is toxic when administered systematically. However, when a virus such as TILT123 is expressing IL-2 at the infection site, the concentration is locally high enough to cause a positive impact on the TME, but not high enough elsewhere to cause systemic effects. Thus, oncolytic viruses show potential to be a method to overcome therapeutic resistance and recurrence (Cervera-Carrasconet al., 2020b). At least theoretically, viruses could be tailor made for each patient, if production, cost and safety related issues can be solved. Research have uncovered the complexity of cancer as a disease. Consequently, as each tumor is unique, there's an interest and probably a benefit from more personalized treatment

plans. However, unfortunately, hurdles such as cost, time issues and safety still limits the tailor-made development of oncolytic viruses.

For a greater results, some factors, such as administration, still limit the efficiency of oncolytic viruses in general and should be resolved. Scientist and doctors generally agree that intravenous injections or pills would be the easiest way to treat patients, but currently, most oncolytic viruses are administered intratumorally. This method has its limitations: some tumors are not injectable and can not be reached by a needle safely. Also, this requires equipment and expertise from the medical professional who is injecting. However, intravenous injections are not without problems either. For example, neutralizing antibodies and clearance of viruses from the blood by Küpffer cells are also considered as issues to be solved before intravenous administration can be given without hesitation (Ahi, Bangari and Mittal, 2011). In our study, we showed that a potential way to avoid neutralization would be to mix oncolytic viruses with lymphocytes prior to administration. Then, the virus can "hitch-hike" on the cells, to the target unnoticed. When the virus reaches the tumor there's also the benefit of local immune activation through TAA spread, production of cytokines or transgene production. Furthermore, some data has been presented that some oncolytic viruses such as TILT-123 is able to induce an abscopal effect on top of being able to travel to distant tumors. This means the immune compartment is trained and activated by the presence in a virus at one site which is then translated into an anti- tumor effect in distant tumors as well (Havunenet al., 2018;Quixabeira et al., 2022). This is an important factor of virotherapy as some commonly used cancer treatments are local and will not treat all tumors in the body. Methods such as surgery or radiation can not be performed if the tumor is too small to be noticed by common detection methods.

In order for new drugs to be available for use in the clinic, rigorous testing, *in vitro*, in vivo and in clinical trials, coupled with detailed reporting to regulatory agencies has to be done prior to acceptance. This requires a lot of time and money, thus favoring the success of privileged few (such as rich companies and scientists in rich countries). This delays the development of new medications and solutions to diseases. Eventually, when the developed drug has finished clinical trial testing, studies show that 90 % of drugs fail and does not reach their endpoint, causing a huge financial burden on the drug developer (Sun et al., 2022). This is then often paid by future customers, in other words; innocent patients. Therefore, solutions should be sought, without compromising patient safety. Development of better pre-clinical testing methods (AI, biomarkers, new machines etc.) and making clinical testing less burdensome, could be ways to address these issues. Also, fast- tracking studies investigating combinatorial treatments with previously approved medicines, could be considered, due to previous safety and efficacy data. For example, there's an excitement about the plausible combination of oncolytic viruses and

immune checkpoint inhibitors. With TILT-123 there's encouraging data, showing that in vitro and in vivo this combination enhances outcome (Cervera-Carrasconet al., 2020b). New combination therapies could be an easier way to provide more personalized therapies and should be even more utilized in patient care in the future.

Virologist work hard to find and characterize viruses that are new to science. Some of these viruses have different characteristics from well known viruses, such as tolerance for extremely hot or saline habitats and some have unique morphology and capsid compositions. In the future, it would be interesting to see the potential of these less well known viruses. Maybe these novel viruses could provide answers to current issues and thus could be utilized for successful cancer therapy.

# 9 SUMMARY

Adenoviruses are versatile tools. They can be relatively easily modified by insertion or deletion of genes and can withhold their infectivity well. Furthermore, many genes can be inserted simultaneously without major loss of function. Adenoviruses are also considered safe, because few adverse events have been recorded and adenovirus treatments are tolerated well both in *in vivo* and in conducted human trials.

The adenovirus TILT-123 is an efficient tool to treat cancer and its effect seems to be enhanced by simultaneous administration of T cells. This effect may be due to the ability of adenoviruses to "hitch hike" on lymphocytes and because the production of TNF-α and IL-2 by the virus has positive effects on the immune compartment and TME of the tumor.

TILT-123 therapy causes both adaptive and innate immune reactions. PRR receptors are in a important role in early detection and reaction towards threats to the body. TILT-12 activates PRR receptors such as AIM2, but surprisingly (in tested conditions) not TLR9. To further augment TILT-123 effects, combinatorial treatments that affect PRR receptors are attractive alternatives that should be studied in more detail.

# 10 ACKNOWLEDGEMENTS

This thesis was conducted and written at the University of Helsinki from 2017–2022 in the Cancer Gene Therapy Group (CGTG), which is affiliated with the Department of Oncology, Faculty of Medicine, Translational Immunology, and Research Program Unit (RPU) University of Helsinki. The university provided excellent facilities and tools to do research. Therefore, I wish to thank the Dean of the Faculty of Medicine, Professor Risto Renkonen, the heads of Medicum and Clinicum units, Professor Eero Mervaala and Professor Klaus Olkkola, the RPU director, professor Henna Tyynismaa, and the Director of the Doctoral School in Health Sciences, Professor Hannu Sariola. I am grateful to be chosen as a salaried position graduate school student by the Board and Directors of the Doctoral Programme in Clinical Research for two and a half years. In addition, this thesis was supported by TILT Biotherapeutics Ltd., and DSHealth. I want to express my gratitude to my Thesis Committee members Professor Seppo Meri and adjunct professor Maria Söderlund-Venermo for providing excellent scientific comments and support throughout my thesis projects. I also wish to thank my pre-examiners, for reviewing my thesis and providing me with critical insights, and thus improving the quality of this work. I also want to thank my supervisor professor Akseli Hemminki for seeing potential in me and letting me be part of his team. I really admire your enthusiasm, how you can streamline complex problems, and your way of getting things done. I would also like to thank you for letting us students become involved in TILT Biotherapeutics, which provided insight into another side of science. Seeing the translational process has been inspiring.

I want to thank my mentors Riikka Havunen and Mikko Siurala. I really appreciate the way you took me under your wings and showed me around the lab when I started to work on my thesis. Riikka, you are one of the most organized persons I know. You have an amazing ability to bring common sense into complex challenges and can help solve several scientific problems in a blink of an eye. Mikko, you are a true scientist in every meaning of the word. I really admire how you can use your engagement in the field, your imagination, and passion for science to come up with brilliant, out-of-the-box projects and push science forward. It was inspiring to work with both of you.

I also want to thank Víctor Cervera-Carrascón and João Santos, my secondary mentors and people I really look up to. Even though your questions during lab meetings scared me at the beginning of my PhD, I truly value your eagerness to discuss, create, and improve the level of science in CGTG. Additionally, I want to thank you for your friendship and crazy sense of humor; it always brightened long days at work.

I also want to thank Riikka Kalliokoski, Susanna Grönberg Vähä-Koskela, and Minna Oksanen for keeping the lab running and the coffee breaks rolling, ensuring that my caffeine intake was high enough to keep preforming in the lab.

Last but not the least I want to thank my friends, old and new members of the CGTG laboratory family: Dafne Quixabeira, James Clubb, Santeri Pakola, Tatiana Kudling, Sadia Zafar, Anna Kanerva, and Saru Basnet. Wine, friendship, help, support, and most of all laughs (!) are words that describe the times spent with you. I am sure that you all will be successful scientists and have a great career once you graduate. My time at CGTG would not have been the same without you. TILT Biotherapeutics and CGTG form a tight bond, and I would also like to thank my hard working friends and colleagues at TILT for all the fun and help I received.
In addition, I have been fortunate to have help from the brilliant scientists listed as co-authors in our publications.
I am also lucky to have the best friends in the world. I could not have done this work without my "chosen family". Mirka Lampi and Laura Knaapi are brilliant scientists in their own fields and had the unfortunate role of being my go-to people for everything, both in science and in life. Thank you, you have a special place in my heart. I also want to thank the "Paratiisi hotellilaiset" for making me laugh and for the support I've received. The fun times we had in my free time gave me energy to work harder in my professional time.
Of course, the support from my family was invaluable. I am thankful to my Dad Lasse, for instilling a love of learning in me, my sister Tessi for teaching me that ambition, hard work, and happiness can go hand in hand, my brother Toni for cheering me up with his witty puns and jokes, and my mum Iris for being there for me when I needed her.

# 11 REFERENCES

Ahi, Y. S., Bangari, D. S., and Mittal, S. K. (2011). Adenoviral vector immunity: its implications and circumvention strategies. Curr Gene Ther 11, 307.

Anderson, K. G., Stromnes, I. M., and Greenberg, P. D. (2017). Obstacles Posed by the Tumor Microenvironment to T cell Activity: A Case for Synergistic Therapies. Cancer Cell 31, 311.

Andtbacka, R. H., Kaufman, H. L., Collichio, F., Amatruda, T., Senzer, N., Chesney, J., Delman, K. A., Spitler, L. E., Puzanov, I., Agarwala, S. S., Milhem, M., Cranmer, L., Curti, B., Lewis, K., Ross, M., Guthrie, T., Linette, G. P., Daniels, G. A., Harrington, K., Middleton, M. R., Miller, W. H., Zager, J. S., Ye, Y., Yao, B., Li, A., Doleman, S., VanderWalde, A., Gansert, J., and Coffin, R. S. (2015). Talimogene Laherparepvec Improves Durable Response Rate in Patients With Advanced Melanoma. J Clin Oncol 33, 2780.

Bauerschmitz, G. J., Guse, K., Kanerva, A., Menzel, A., Herrmann, I., Desmond, R. A., Yamamoto, M., Nettelbeck, D. M., Hakkarainen, T., Dall, P., Curiel, D. T., and Hemminki, A. (2006). Triple-targeted oncolytic adenoviruses featuring the cox2 promoter, E1A transcomplementation, and serotype chimerism for enhanced selectivity for ovarian cancer cells. Mol Ther 14, 164.

Becht, E., Giraldo, N. A., Dieu-Nosjean, M. C., Sautès-Fridman, C., and Fridman, W. H. (2016). Cancer immune contexture and immunotherapy. Curr Opin Immunol 39, 7.

Berraondo, P., Sanmamed, M. F., Ochoa, M. C., Etxeberria, I., Aznar, M. A., Pérez-Gracia, J. L., Rodríguez-Ruiz, M. E., Ponz-Sarvise, M., Castañón, E., and Melero, I. (2019). Cytokines in clinical cancer immunotherapy. Br J Cancer 120, 6.

Besser, M. J., Shapira-Frommer, R., Treves, A. J., Zippel, D., Itzhaki, O., Hershkovitz, L., Levy, D., Kubi, A., Hovav, E., Chermoshniuk, N., Shalmon, B., Hardan, I., Catane, R., Markel, G., Apter, S., Ben-Nun, A., Kuchuk, I., Shimoni, A., Nagler, A., and Schachter, J. (2010). Clinical responses in a phase II study using adoptive transfer of short-term cultured tumor infiltration lymphocytes in metastatic melanoma patients. Clin Cancer Res 16, 2646.

Breitbach, C. J., Burke, J., Jonker, D., Stephenson, J., Haas, A. R., Chow, L. Q., Nieva, J., Hwang, T. H., Moon, A., Patt, R., Pelusio, A., Le Boeuf, F., Burns, J., Evgin, L., De Silva, N., Cvancic, S., Robertson, T., Je, J. E., Lee, Y. S., Parato, K., Diallo, J. S., Fenster, A., Daneshmand, M., Bell, J. C., and Kirn, D. H. (2011). Intravenous delivery of a multi-mechanistic cancer-targeted oncolytic poxvirus in humans. Nature 477, 99.

Bright, R., Coventry, B. J., Eardley-Harris, N., and Briggs, N. (2017). Clinical Response Rates From Interleukin-2 Therapy for Metastatic Melanoma Over 30 Years' Experience: A Meta-Analysis of 3312 Patients. J Immunother 40, 21.

Brocker, C., Thompson, D., Matsumoto, A., Nebert, D. W., and Vasiliou, V. (2010). Evolutionary divergence and functions of the human interleukin (IL) gene family. Hum Genomics 5, 30.

Buijs, P. R., Verhagen, J. H., van Eijck, C. H., and van den Hoogen, B. G. (2015). Oncolytic viruses: From bench to bedside with a focus on safety. Hum Vaccin Immunother 11, 1573.

Ceelen, W. P., Morris, S., Paraskeva, P., and Pattyn, P. (2007). Surgical trauma, minimal residual disease and locoregional cancer recurrence. Cancer Treat Res 134, 51.

Cervera-Carrascon, V., Quixabeira, D. C. A., Havunen, R., Santos, J. M., Kutvonen, E., Clubb, J. H. A., Siurala, M., Heiniö, C., Zafar, S., Koivula, T., Lumen, D., Vaha, M., Garcia-Horsman, A., Airaksinen, A. J., Sorsa, S., Anttila, M., Hukkanen, V., Kanerva, A., and Hemminki, A. (2020a). Comparison of Clinically Relevant Oncolytic Virus Platforms for Enhancing T Cell Therapy of Solid Tumors. Mol Ther Oncolytics 17, 47.

Cervera-Carrascon, V., Quixabeira, D. C. A., Santos, J. M., Havunen, R., Zafar, S., Hemminki, O., Heiniö, C., Munaro, E., Siurala, M., Sorsa, S., Mirtti, T., Järvinen, P., Mildh, M., Nisen, H., Rannikko, A., Anttila, M., Kanerva, A., and Hemminki, A. (2020b). Tumor microenvironment remodeling by an engineered oncolytic adenovirus results in improved outcome from PD-L1 inhibition. Oncoimmunology 9, 1761229.

Chan, J. K., Roth, J., Oppenheim, J. J., Tracey, K. J., Vogl, T., Feldmann, M., Horwood, N., and Nanchahal, J. (2012). Alarmins: awaiting a clinical response. J Clin Invest 122, 2711.

Chen, I. F., Ou-Yang, F., Hung, J. Y., Liu, J. C., Wang, H., Wang, S. C., Hou, M. F., Hortobagyi, G. N., and Hung, M. C. (2006). AIM2 suppresses human breast cancer cell proliferation in vitro and mammary tumor growth in a mouse model. Mol Cancer Ther 5, 1.

Chen, J., Wang, Z., and Yu, S. (2017). AIM2 regulates viability and apoptosis in human colorectal cancer cells via the PI3K/Akt pathway. Onco Targets Ther 10, 811.

Chen, M. F., Chen, P. T., Lu, M. S., Lin, P. Y., Chen, W. C., and Lee, K. D. (2013). IL-6 expression predicts treatment response and outcome in squamous cell carcinoma of the esophagus. Mol Cancer 12, 26.

Chulpanova, D. S., Kitaeva, K. V., Green, A. R., Rizvanov, A. A., and Solovyeva, V. V. (2020). Molecular Aspects and Future Perspectives of Cytokine-Based Anti-cancer Immunotherapy. Front Cell Dev Biol 8, 402.

Corrie, P. (2008). Cytotoxic chemotherapy: clinical aspects. Medicine 36, 24.

Coughlan, L., Alba, R., Parker, A. L., Bradshaw, A. C., McNeish, I. A., Nicklin, S. A., and Baker, A. H. (2010). Tropism-modification strategies for targeted gene delivery using adenoviral vectors. Viruses 2, 2290.

DeVita, V. T., and Chu, E. (2008). A history of cancer chemotherapy. Cancer Res 68, 8643.

Dhingra, A., Hage, E., Ganzenmueller, T., Böttcher, S., Hofmann, J., Hamprecht, K., Obermeier, P., Rath, B., Hausmann, F., Dobner, T., and Heim, A. (2019). Molecular Evolution of Human Adenovirus (HAdV) Species C. Sci Rep 9, 1039.

Dieci, M. V., Mathieu, M. C., Guarneri, V., Conte, P., Delaloge, S., Andre, F., and Goubar, A. (2015). Prognostic and predictive value of tumor-infiltrating lymphocytes in two phase III randomized adjuvant breast cancer trials. Ann Oncol 26, 1698.

Donia, M., Junker, N., Ellebaek, E., Andersen, M. H., Straten, P. T., and Svane, I. M. (2012). Characterization and comparison of 'standard' and 'young' tumour-infiltrating lymphocytes for adoptive cell therapy at a Danish translational research institution. Scand J Immunol 75, 157.

Drakes, M. L., and Stiff, P. J. (2021). Ovarian Cancer: Therapeutic Strategies to Overcome Immune Suppression. Adv Exp Med Biol 1330, 33.

Dudley, M. E., Wunderlich, J. R., Robbins, P. F., Yang, J. C., Hwu, P., Schwartzentruber, D. J., Topalian, S. L., Sherry, R., Restifo, N. P., Hubicki, A. M., Robinson, M. R., Raffeld, M., Duray, P., Seipp, C. A., Rogers-Freezer, L., Morton, K. E., Mavroukakis, S. A., White, D. E., and Rosenberg, S. A. (2002). Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. Science 298, 850.

Dunn, G. P., Koebel, C. M., and Schreiber, R. D. (2006). Interferons, immunity and cancer immunoediting. Nat Rev Immunol 6, 836.

Ferlay, J., Colombet, M., Soerjomataram, I., Parkin, D. M., Piñeros, M., Znaor, A., and Bray, F. (2021). Cancer statistics for the year 2020: An overview. Int J Cancer.

Galli, F., Aguilera, J. V., Palermo, B., Markovic, S. N., Nisticò, P., and Signore, A. (2020). Relevance of immune cell and tumor microenvironment imaging in the new era of immunotherapy. J Exp Clin Cancer Res 39, 89.

Ganss, R. (2020). Tumour vessel remodelling: new opportunities in cancer treatment. Vasc Biol 2, R35.

Garcia-Carbonero, R., Salazar, R., Duran, I., Osman-Garcia, I., Paz-Ares, L., Bozada, J. M., Boni, V., Blanc, C., Seymour, L., Beadle, J., Alvis, S., Champion, B., Calvo, E., and Fisher, K. (2017). Phase 1 study of intravenous administration of the chimeric

adenovirus enadenotucirev in patients undergoing primary tumor resection. J Immunother Cancer 5, 71.
Gelfo, V., Romaniello, D., Mazzeschi, M., Sgarzi, M., Grilli, G., Morselli, A., Manzan, B., Rihawi, K., and Lauriola, M. (2020). Roles of IL-1 in Cancer: From Tumor Progression to Resistance to Targeted Therapies. Int J Mol Sci 21.
Gerber, D. E., and Chan, T. A. (2008). Recent advances in radiation therapy. Am Fam Physician 78, 1254.
Hall, M., Liu, H., Malafa, M., Centeno, B., Hodul, P. J., Pimiento, J., Pilon-Thomas, S., and Sarnaik, A. A. (2016). Expansion of tumor-infiltrating lymphocytes (TIL) from human pancreatic tumors. J Immunother Cancer 4, 61.
Harrington, K. J., Puzanov, I., Hecht, J. R., Hodi, F. S., Szabo, Z., Murugappan, S., and Kaufman, H. L. (2015). Clinical development of talimogene laherparepvec (T-VEC): a modified herpes simplex virus type-1-derived oncolytic immunotherapy. Expert Rev Anticancer Ther 15, 1389.
Havunen, R., Kalliokoski, R., Siurala, M., Sorsa, S., Santos, J. M., Cervera-Carrascon, V., Anttila, M., and Hemminki, A. (2021). Cytokine-Coding Oncolytic Adenovirus TILT-123 Is Safe, Selective, and Effective as a Single Agent and in Combination with Immune Checkpoint Inhibitor Anti-PD-1. Cells 10.
Havunen, R., Santos, J. M., Sorsa, S., Rantapero, T., Lumen, D., Siurala, M., Airaksinen, A. J., Cervera-Carrascon, V., Tahtinen, S., Kanerva, A., and Hemminki, A. (2018). Abscopal Effect in Non-injected Tumors Achieved with Cytokine-Armed Oncolytic Adenovirus. Mol Ther Oncolytics 11, 109.
Havunen, R., Siurala, M., Sorsa, S., Grönberg-Vähä-Koskela, S., Behr, M., Tähtinen, S., Santos, J. M., Karell, P., Rusanen, J., Nettelbeck, D. M., Ehrhardt, A., Kanerva, A., and Hemminki, A. (2017). Oncolytic Adenoviruses Armed with Tumor Necrosis Factor Alpha and Interleukin-2 Enable Successful Adoptive Cell Therapy. Molecular Therapy - Oncolytics 4, 77.
Heiniö, C., Havunen, R., Santos, J., de Lint, K., Cervera-Carrascon, V., Kanerva, A., and Hemminki, A. (2020). TNFa and IL2 Encoding Oncolytic Adenovirus Activates Pathogen and Danger-Associated Immunological Signaling. Cells 9.
Heiniö, C., Sorsa, S., Siurala, M., Grönberg-Vähä-Koskela, S., Havunen, R., Haavisto, E., Koski, A., Hemminki, O., Zafar, S., Cervera-Carrascon, V., Munaro, E., Kanerva, A., and Hemminki, A. (2019). Effect of Genetic Modifications on Physical and Functional Titers of Adenoviral Cancer Gene Therapy Constructs. Hum Gene Ther 30, 740.
Hemminki, O., Santos, J. M., and Hemminki, A. (2020). Oncolytic viruses for cancer immunotherapy. J Hematol Oncol 13, 84.
Hernandez, C., Huebener, P., and Schwabe, R. F. (2016). Damage-associated molecular patterns in cancer: a double-edged sword. Oncogene 35, 5931.
Hishida, T., Masai, K., Kaseda, K., Asakura, K., and Asamura, H. (2021). Debulking surgery for malignant tumors: the current status, evidence and future perspectives. Jpn J Clin Oncol.
Imyanitov, E. N., and Yanus, G. A. (2018). Neoadjuvant therapy: theoretical, biological and medical consideration. Chin Clin Oncol 7, 55.
Inge-Marie, S., Victor, C.-C., Joao, S., Riikka, H., Suvi, S., Marco, D., Amir, K., Brigitte, D., and Akseli, H. (2022). First-in-human clinical trial of an oncolytic adenovirus armed with TNFa and IL-2 in patients with advanced melanoma receiving adoptive cell transfer of tumor-infiltrating lymphocytes. In 2022 ASCO Annual Meeting.
Jazaeri, A. A., Zsiros, E., Amaria, R. N., Artz, A. S., Edwards, R. P., Wenham, R. M., Slomovitz, B. M., Walther, A., Thomas, S. S., Chesney, J. A., Morris, R., Matsuo, K., Gaillard, S., Rose, P. G., Donas, J. G., Tromp, J. M., Tavakkoli, F., Li, H., Fardis, M., and Monk, B. J. (2019). Safety
and efficacy of adoptive cell transfer using autologous tumor
infiltrating lymphocytes (LN-145) for treatment of recurrent,
metastatic, or persistent cervical carcinoma. Journal of Clinical Oncology 37.
Jiang, T., Zhou, C., and Ren, S. (2016). Role of IL-2 in cancer immunotherapy. Oncoimmunology 5, e1163462.

Jounaidi, Y., Doloff, J. C., and Waxman, D. J. (2007). Conditionally replicating adenoviruses for cancer treatment. Curr Cancer Drug Targets 7, 285.
Kabbinavar, F., Irl, C., Zurlo, A., and Hurwitz, H. (2008). Bevacizumab improves the overall and progression-free survival of patients with metastatic colorectal cancer treated with 5-fluorouracil-based regimens irrespective of baseline risk. Oncology 75, 215.
Kanerva, A., Nokisalmi, P., Diaconu, I., Koski, A., Cerullo, V., Liikanen, I., Tähtinen, S., Oksanen, M., Heiskanen, R., Pesonen, S., Joensuu, T., Alanko, T., Partanen, K., Laasonen, L., Kairemo, K., Kangasniemi, L., and Hemminki, A. (2013). Antiviral and antitumor T-cell immunity in patients treated with GM-CSF-coding oncolytic adenovirus. Clin Cancer Res 19, 2734.
Karapanagiotou, E. M., Roulstone, V., Twigger, K., Ball, M., Tanay, M., Nutting, C., Newbold, K., Gore, M. E., Larkin, J., Syrigos, K. N., Coffey, M., Thompson, B., Mettinger, K., Vile, R. G., Pandha, H. S., Hall, G. D., Melcher, A. A., Chester, J., and Harrington, K. J. (2012). Phase I/II trial of carboplatin and paclitaxel chemotherapy in combination with intravenous oncolytic reovirus in patients with advanced malignancies. Clin Cancer Res 18, 2080.
Kennedy, M. A., and Parks, R. J. (2009). Adenovirus virion stability and the viral genome: size matters. Mol Ther 17, 1664.
Knezevic, C. E., and Clarke, W. (2020). Cancer Chemotherapy: The Case for Therapeutic Drug Monitoring. Ther Drug Monit 42, 6.
Kucerova, P., and Cervinkova, M. (2016). Spontaneous regression of tumour and the role of microbial infection--possibilities for cancer treatment. Anticancer Drugs 27, 269.
Lang, F. F., Conrad, C., Gomez-Manzano, C., Yung, W. K. A., Sawaya, R., Weinberg, J. S., Prabhu, S. S., Rao, G., Fuller, G. N., Aldape, K. D., Gumin, J., Vence, L. M., Wistuba, I., Rodriguez-Canales, J., Villalobos, P. A., Dirven, C. M. F., Tejada, S., Valle, R. D., Alonso, M. M., Ewald, B., Peterkin, J. J., Tufaro, F., and Fueyo, J. (2018). Phase I Study of DNX-2401 (Delta-24-RGD) Oncolytic Adenovirus: Replication and Immunotherapeutic Effects in Recurrent Malignant Glioma. J Clin Oncol 36, 1419.
Lemos de Matos, A., Franco, L. S., and McFadden, G. (2020). Oncolytic Viruses and the Immune System: The Dynamic Duo. Mol Ther Methods Clin Dev 17, 349.
Liu, Y., and Zeng, G. (2012). Cancer and innate immune system interactions: translational potentials for cancer immunotherapy. J Immunother 35, 299.
Locke, F. L., Ghobadi, A., Jacobson, C. A., Miklos, D. B., Lekakis, L. J., Oluwole, O. O., Lin, Y., Braunschweig, I., Hill, B. T., Timmerman, J. M., Deol, A., Reagan, P. M., Stiff, P., Flinn, I. W., Farooq, U., Goy, A., McSweeney, P. A., Munoz, J., Siddiqi, T., Chavez, J. C., Herrera, A. F., Bartlett, N. L., Wiezorek, J. S., Navale, L., Xue, A., Jiang, Y., Bot, A., Rossi, J. M., Kim, J. J., Go, W. Y., and Neelapu, S. S. (2019). Long-term safety and activity of axicabtagene ciloleucel in refractory large B-cell lymphoma (ZUMA-1): a single-arm, multicentre, phase 1-2 trial. Lancet Oncol 20, 31.
Lynch, J. P., and Kajon, A. E. (2016). Adenovirus: Epidemiology, Global Spread of Novel Serotypes, and Advances in Treatment and Prevention. Semin Respir Crit Care Med 37, 586.
Macedo, N., Miller, D. M., Haq, R., and Kaufman, H. L. (2020). Clinical landscape of oncolytic virus research in 2020. J Immunother Cancer 8.
Mach, N., Gao, J., Schaffarczyk, L., Janz, S., Ehrke-Schulz, E., Dittmar, T., Ehrhardt, A., and Zhang, W. (2020). Spectrum-Wide Exploration of Human Adenoviruses for Breast Cancer Therapy. Cancers (Basel) 12.
Malhotra, V., and Perry, M. C. (2003). Classical chemotherapy: mechanisms, toxicities and the therapeutic window. Cancer Biol Ther 2, S2.
Man, S. M., Karki, R., and Kanneganti, T. D. (2016). AIM2 inflammasome in infection, cancer, and autoimmunity: Role in DNA sensing, inflammation, and innate immunity. Eur J Immunol 46, 269.
Manfredi, F., Cianciotti, B. C., Potenza, A., Tassi, E., Noviello, M., Biondi, A., Ciceri, F., Bonini, C., and Ruggiero, E. (2020). TCR Redirected T Cells for Cancer Treatment: Achievements, Hurdles, and Goals. Front Immunol 11, 1689.

Mantwill, K., Klein, F. G., Wang, D., Hindupur, S. V., Ehrenfeld, M., Holm, P. S., and Nawroth, R. (2021). Concepts in Oncolytic Adenovirus Therapy. Int J Mol Sci 22.

McKenna, M. K., Rosewell-Shaw, A., and Suzuki, M. (2020). Modeling the Efficacy of Oncolytic Adenoviruses In Vitro and In Vivo: Current and Future Perspectives. Cancers (Basel) 12.

Mita, Y., Kimura, M. Y., Hayashizaki, K., Koyama-Nasu, R., Ito, T., Motohashi, S., Okamoto, Y., and Nakayama, T. (2018). Crucial role of CD69 in anti-tumor immunity through regulating the exhaustion of tumor-infiltrating T cells. Int Immunol 30, 559.

Moehler, M., Heo, J., Lee, H. C., Tak, W. Y., Chao, Y., Paik, S. W., Yim, H. J., Byun, K. S., Baron, A., Ungerechts, G., Jonker, D., Ruo, L., Cho, M., Kaubisch, A., Wege, H., Merle, P., Ebert, O., Habersetzer, F., Blanc, J. F., Rosmorduc, O., Lencioni, R., Patt, R., Leen, A. M., Foerster, F., Homerin, M., Stojkowitz, N., Lusky, M., Limacher, J. M., Hennequi, M., Gaspar, N., McFadden, B., De Silva, N., Shen, D., Pelusio, A., Kirn, D. H., Breitbach, C. J., and Burke, J. M. (2019). Vaccinia-based oncolytic immunotherapy Pexastimogene Devacirepvec in patients with advanced hepatocellular carcinoma after sorafenib failure: a randomized multicenter Phase IIb trial (TRAVERSE). Oncoimmunology 8, 1615817.

Morgan, R. A., Dudley, M. E., Wunderlich, J. R., Hughes, M. S., Yang, J. C., Sherry, R. M., Royal, R. E., Topalian, S. L., Kammula, U. S., Restifo, N. P., Zheng, Z., Nahvi, A., de Vries, C. R., Rogers-Freezer, L. J., Mavroukakis, S. A., and Rosenberg, S. A. (2006). Cancer regression in patients after transfer of genetically engineered lymphocytes. Science 314, 126.

Nish, S. A., Schenten, D., Wunderlich, F. T., Pope, S. D., Gao, Y., Hoshi, N., Yu, S., Yan, X., Lee, H. K., Pasman, L., Brodsky, I., Yordy, B., Zhao, H., Brüning, J., and Medzhitov, R. (2014). T cell-intrinsic role of IL-6 signaling in primary and memory responses. Elife 3, e01949.

Papaioannou, N. E., Beniata, O. V., Vitsos, P., Tsitsilonis, O., and Samara, P. (2016). Harnessing the immune system to improve cancer therapy. Ann Transl Med 4, 261.

Ponterio, E., and Gnessi, L. (2015). Adenovirus 36 and Obesity: An Overview. Viruses 7, 3719.

Quixabeira, D. C. A., Cervera-Carrascon, V., Santos, J. M., Clubb, J. H. A., Kudling, T. V., Basnet, S., Heiniö, C., Grönberg-Vähä-Koskela, S., Anttila, M., Havunen, R., Kanerva, A., and Hemminki, A. (2022). Local therapy with an engineered oncolytic adenovirus enables antitumor response in non-injected melanoma tumors in mice treated with aPD-1. Oncoimmunology 11, 2028960.

Quixabeira, D. C. A., Zafar, S., Santos, J. M., Cervera-Carrascon, V., Havunen, R., Kudling, T. V., Basnet, S., Anttila, M., Kanerva, A., and Hemminki, A. (2021). Oncolytic Adenovirus Coding for a Variant Interleukin 2 (vIL-2) Cytokine Re-Programs the Tumor Microenvironment and Confers Enhanced Tumor Control. Front Immunol 12, 674400.

Ranki, T., Pesonen, S., Hemminki, A., Partanen, K., Kairemo, K., Alanko, T., Lundin, J., Linder, N., Turkki, R., Ristimäki, A., Jäger, E., Karbach, J., Wahle, C., Kankainen, M., Backman, C., von Euler, M., Haavisto, E., Hakonen, T., Heiskanen, R., Jaderberg, M., Juhila, J., Priha, P., Suoranta, L., Vassilev, L., Vuolanto, A., and Joensuu, T. (2016). Phase I study with ONCOS-102 for the treatment of solid tumors - an evaluation of clinical response and exploratory analyses of immune markers. J Immunother Cancer 4, 17.

Rehman, H., Silk, A. W., Kane, M. P., and Kaufman, H. L. (2016). Into the clinic: Talimogene laherparepvec (T-VEC), a first-in-class intratumoral oncolytic viral therapy. J Immunother Cancer 4, 53.

Riley, R. S., June, C. H., Langer, R., and Mitchell, M. J. (2019). Delivery technologies for cancer immunotherapy. Nat Rev Drug Discov 18, 175.

Rincón, E., Cejalvo, T., Kanojia, D., Alfranca, A., Rodríguez-Milla, M., Gil Hoyos, R. A., Han, Y., Zhang, L., Alemany, R., Lesniak, M. S., and García-Castro, J. (2017). Mesenchymal stem cell carriers enhance antitumor efficacy of oncolytic adenoviruses in an immunocompetent mouse model. Oncotarget 8, 45415.

Robbins, P. F., Kassim, S. H., Tran, T. L., Crystal, J. S., Morgan, R. A., Feldman, S. A., Yang, J. C., Dudley, M. E., Wunderlich, J. R., Sherry, R. M., Kammula, U. S., Hughes, M. S., Restifo, N. P., Raffeld, M., Lee, C. C., Li, Y. F., El-Gamil, M., and Rosenberg, S. A. (2015). A pilot trial using lymphocytes genetically engineered with an NY-ESO-1-reactive T-cell receptor: long-term follow-up and correlates with response. Clin Cancer Res 21, 1019.

Rosen, L., Evans, H. E., and Spickard, A. (1963). Reovirus infections in human volunteers. Am J Hyg 77, 29.

Rosenberg, S. A. (2014). IL-2: the first effective immunotherapy for human cancer. J Immunol 192, 5451.

Rosenberg, S. A., and Restifo, N. P. (2015). Adoptive cell transfer as personalized immunotherapy for human cancer. Science 348, 62.

Roy, D. G., and Bell, J. C. (2013). Cell carriers for oncolytic viruses: current challenges and future directions. Oncolytic Virother 2, 47.

Santos, J., Heiniö, C., Quixabeira, D., Zafar, S., Clubb, J., Pakola, S., Cervera-Carrascon, V., Havunen, R., Kanerva, A., and Hemminki, A. (2021). Systemic Delivery of Oncolytic Adenovirus to Tumors Using Tumor-Infiltrating Lymphocytes as Carriers. Cells 10.

Santos, J. M., Cervera-Carrascon, V., Havunen, R., Zafar, S., Siurala, M., Sorsa, S., Anttila, M., Kanerva, A., and Hemminki, A. (2018). Adenovirus Coding for Interleukin-2 and Tumor Necrosis Factor Alpha Replaces Lymphodepleting Chemotherapy in Adoptive T Cell Therapy. Mol Ther 26, 2243.

Santos, J. M., Heiniö, C., Cervera-Carrascon, V., Quixabeira, D. C. A., Siurala, M., Havunen, R., Butzow, R., Zafar, S., de Gruijl, T., Lassus, H., Kanerva, A., and Hemminki, A. (2020). Oncolytic adenovirus shapes the ovarian tumor microenvironment for potent tumor-infiltrating lymphocyte tumor reactivity. J Immunother Cancer 8.

Sauthoff, H., Pipiya, T., Heitner, S., Chen, S., Bleck, B., Reibman, J., Chang, W., Norman, R. G., Rom, W. N., and Hay, J. G. (2004). Impact of E1a modifications on tumor-selective adenoviral replication and toxicity. Mol Ther 10, 749.

Schaft, N. (2020). The Landscape of CAR-T Cell Clinical Trials against Solid Tumors-A Comprehensive Overview. Cancers (Basel) 12.

Sengupta, S., Ulasov, I. V., Thaci, B., Ahmed, A. U., and Lesniak, M. S. (2011). Enhanced transduction and replication of RGD-fiber modified adenovirus in primary T cells. PLoS One 6, e18091.

Sia, J., Neeson, P. J., and Haynes, N. M. (2022). Basic cancer immunology for radiation oncologists. J Med Imaging Radiat Oncol.

Siurala, M., Havunen, R., Saha, D., Lumen, D., Airaksinen, A. J., Tähtinen, S., Cervera-Carrascon, V., Bramante, S., Parviainen, S., Vähä-Koskela, M., Kanerva, A., and Hemminki, A. (2016a). Adenoviral Delivery of Tumor Necrosis Factor-α and Interleukin-2 Enables Successful Adoptive Cell Therapy of Immunosuppressive Melanoma. Mol Ther 24, 1435.

Siurala, M., Vähä-Koskela, M., Havunen, R., Tähtinen, S., Bramante, S., Parviainen, S., Mathis, J. M., Kanerva, A., and Hemminki, A. (2016b). Syngeneic syrian hamster tumors feature tumor-infiltrating lymphocytes allowing adoptive cell therapy enhanced by oncolytic adenovirus in a replication permissive setting. Oncoimmunology 5, e1136046.

Son, J., George, G. C., Nardo, M., Krause, K. J., Jazaeri, A. A., Biter, A. B., and Hong, D. S. (2022). Adoptive cell therapy in gynecologic cancers: A systematic review and meta-analysis. Gynecol Oncol 165, 664.

Spiess, P. J., Yang, J. C., and Rosenberg, S. A. (1987). In vivo antitumor activity of tumor-infiltrating lymphocytes expanded in recombinant interleukin-2. J Natl Cancer Inst 79, 1067.

Sun, D., Gao, W., Hu, H., and Zhou, S. (2022). Why 90% of clinical drug development fails and how to improve it? Acta Pharm Sin B 12, 3049.

THL, T. j. H. L. (2022). Syövän hoito.

Tähtinen, S., Kaikkonen, S., Merisalo-Soikkeli, M., Grönberg-Vähä-Koskela, S., Kanerva, A., Parviainen, S., Vähä-Koskela, M., and Hemminki, A. (2015). Favorable alteration of tumor microenvironment by immunomodulatory cytokines for efficient T-cell therapy in solid tumors. PLoS One 10, e0131242.

UK, C. R. (Accessed 3/2022). Cancer Research UK. https://www.cancerresearchuk.org/health-professional/cancer-statistics/risk/lifetime-risk.

van den Berg, J. H., Heemskerk, B., van Rooij, N., Gomez-Eerland, R., Michels, S., van Zon, M., de Boer, R., Bakker, N. A. M., Jorritsma-Smit, A., van Buuren, M. M., Kvistborg, P., Spits, H., Schotte, R., Mallo, H., Karger, M., van der Hage, J. A., Wouters, M. W. J. M., Pronk, L. M., Geukes Foppen, M. H., Blank, C. U., Beijnen, J. H., Nuijen, B., Schumacher, T. N., and Haanen, J. B. A. G. (2020). Tumor infiltrating lymphocytes (TIL) therapy in metastatic melanoma: boosting of neoantigen-specific T cell reactivity and long-term follow-up. J Immunother Cancer 8.

Waldman, A. D., Fritz, J. M., and Lenardo, M. J. (2020). A guide to cancer immunotherapy: from T cell basic science to clinical practice. Nat Rev Immunol 20, 651.

Wang, B., and Yin, Q. (2017). AIM2 inflammasome activation and regulation: A structural perspective. J Struct Biol 200, 279.

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

WHO, W. H. O. (2022). Cancer incidents.

Wyld, L., Audisio, R. A., and Poston, G. J. (2015). The evolution of cancer surgery and future perspectives. Nat Rev Clin Oncol 12, 115.

Yamamoto, Y., Nagasato, M., Yoshida, T., and Aoki, K. (2017). Recent advances in genetic modification of adenovirus vectors for cancer treatment. Cancer Sci 108, 831.

Zacharakis, N., Huq, L. M., Seitter, S. J., Kim, S. P., Gartner, J. J., Sindiri, S., Hill, V. K., Li, Y. F., Paria, B. C., Ray, S., Gasmi, B., Lee, C. C., Prickett, T. D., Parkhurst, M. R., Robbins, P. F., Langhan, M. M., Shelton, T. E., Parikh, A. Y., Levi, S. T., Hernandez, J. M., Hoang, C. D., Sherry, R. M., Yang, J. C., Feldman, S. A., Goff, S. L., and Rosenberg, S. A. (2022). Breast Cancers Are Immunogenic: Immunologic Analyses and a Phase II Pilot Clinical Trial Using Mutation-Reactive Autologous Lymphocytes. J Clin Oncol, JCO2102170.

Zafar, S., Quixabeira, D. C. A., Kudling, T. V., Cervera-Carrascon, V., Santos, J. M., Grönberg-Vähä-Koskela, S., Zhao, F., Aronen, P., Heiniö, C., Havunen, R., Sorsa, S., Kanerva, A., and Hemminki, A. (2020). Ad5/3 is able to avoid neutralization by binding to erythrocytes and lymphocytes. Cancer Gene Ther.

Zhang, S., and Rabkin, S. D. (2021). The discovery and development of oncolytic viruses: are they the future of cancer immunotherapy? Expert Opin Drug Discov 16, 391.

Zhao, L., and Cao, Y. J. (2019). Engineered T Cell Therapy for Cancer in the Clinic. Front Immunol 10, 2250.

Zivcec, M., Safronetz, D., Haddock, E., Feldmann, H., and Ebihara, H. (2011). Validation of assays to monitor immune responses in the Syrian golden hamster (Mesocricetus auratus). J Immunol Methods 368, 24.