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CANCER IMMUNOTHERAPY WITH ONCOLYTIC ADENOVIRUSES ENCODING INTERLEUKIN-2 PROTEINS

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ACADEMIC DISSERTATION

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To patients with cancer, and their families.

"The gift must be given back, sooner or later, willingly or unwillingly, and sadly it seems that I will be made to give it back before my time. I have learned much, experienced much, made many mistakes, enjoyed my triumphs, suffered my defeats, and, most vitally, experienced love. So many people live who never get that last one, and I have been lucky enough to."

Jake Seliger, 1983-2024, author and writer, documenting his experience with cancer and clinical trials in his blog "The Story's Story".

ABSTRACT

Cancer is a disease where uncontrolled cell growth can lead to morbidity. In the last two decades, immunotherapy has been established as a foundational form of cancer therapy. However, not all solid cancers respond to current immunotherapies, and not every patient benefits equally. Oncolytic adenoviruses are a form of immunotherapy, that can stimulate the immune system and produce transgenes inside tumors as a form of cancer gene therapy. Research in this thesis studied an oncolytic adenovirus encoding a modified interleukin-2 molecule (variant IL-2, Ad5/3-E2F-d24-vIL2) and an oncolytic adenovirus producing interleukin-2 (IL-2) in conjunction with tumor necrosis factor (TNF, Ad5/3-E2F-d24-hTNF-IRES-hIL2). Ad5/3-E2F-d24-vIL2 was evaluated in preclinical models in combination with chemotherapy and allogenic natural killer (NK) cell therapy, and Ad5/3-E2F-d24-hTNF-IRES-hIL2 was studied in a phase I clinical trial of patients with advanced solid tumors.

Combination of Ad5/3-E2F-d24-vIL2 to chemotherapy in the form of paclitaxel and gemcitabine showed synergistic findings in preclinical models of pancreatic cancer. When combined with microtubule stabilizing chemotherapy paclitaxel, the combination treatment led to aberrant cell cycle in the treated cells, and increased amounts of immunogenic cell death. In vivo studies confirmed the safety of the combination treatment with superior tumor control and survival benefit over solely chemotherapy treatment. This survival benefit was linked to enhanced immune cell activation and tumor microenvironment modulation. Furthermore, animals cured by the treatment had superior protection from subsequent tumor rechallenge.

The possibility of enhancing allogenic NK cell therapy with Ad5/3-E2F-d24-vIL2 was studied in vitro in clinical ovarian cancer specimens. Combination of Ad5/3-E2F-d24-vIL2 to NK cell therapy showed superior cell killing and NK cell activation over unarmed virus or NK cell monotherapy. Furthermore, an in vivo study confirmed the findings in a patient derived xenograft model of ovarian cancer.

Safety and preliminary efficacy of Ad5/3-E2F-d24-hTNF-IRES-hIL2 in humans was confirmed in a phase I trial of 20 patients with advanced solid cancers. The treatment was well tolerated, with three self-resolving grade 4 adverse events. Preliminary signs of efficacy were seen, with 20% or 60% of patients showing disease control, depending on the criteria used.

Mechanistic and biomarker assessment of samples collected during the trial showed systemic and tumor level proinflammatory changes after Ad5/3-E2F-d24-hTNF-IRES-hIL2 treatment. Decreasing lymphocyte count in the peripheral blood

was identified and validated as a promising prognostic marker of oncolytic adenovirus treatment.

ABSTRAKTI (FINNISH ABSTRACT)

Syöpä on sairaus, jossa solut lisääntyvät hallitsemattomasti, pahimmillaan johtaen kuolemaan. Viimeisen kahden vuosikymmenen aikana immunoterapia on muodostunut yhdeksi syövän hoidon kulmakivistä. Valitettavasti kaikki kiinteät kasvaimet eivät vastaa immunoterapiaan, ja kaikki potilaat eivät hyödy immunoterapiasta samalla tavalla. Onkolyyttiset adenovirukset ovat syövän immunoterapian muoto, jotka pystyvät stimuloimaan immuunijärjestelmää ja tuottamaan transgeenejä kasvainten sisällä, toimien siten syövän geeniterapiana. Tässä väitöskirjassa esitetyt osatyöt tutkivat kahta onkolyyttistä adenovirusta: adenovirusta joka tuottaa muokattua interleukiini-2 molekyyliä (variantti IL-2, Ad5/3-E2F-d24-vIL2), sekä virusta, joka tuottaa interleukiini-2:ta (IL-2) yhdessä tuumorinekroositekijä-alfan (TNF, Ad5/3-E2F-d24-hTNF-IRES-hIL2) kanssa. Ad5/3-E2F-d24-vIL2:ta tutkittiin prekliinisissä malleissa kemoterapiaan ja allogeeniseen luonnolliseen tappajasolu (NK)-terapiaan yhdistettynä. Ad5/3-E2F-d24-hTNF-IRES-hIL2:ta tutkittiin kliinisessä faasin I-kokeessa potilaissa, joilla oli todettu levinnyt kiinteä kasvain.

Ad5/3-E2F-d24-vIL2 viruksen yhdistäminen paklitakseli- ja gemsitabiini-kemoterapioihin johti synergistisiin vaikutuksiin haimasyövän prekliinisissä malleissa. Yhdistettynä mikrotubuleja stabiloivaan kemoterapiaan, paklitakseliin, tunnistettiin syöpäsoluissa häiriintynyt solusykli. Tämä puolestaan tehdosti solukuolemaa, joka aktivoi immuunipuolustusta. In vivo kokeet vahvistivat kyseisen yhdistelmähoidon turvallisuuden sekä hoidon yhteyden parempaan kasvaimen kontrolliin ja pidempää elossaoloaikaan. Hoidon hyödyllinen vaste assosioitui parempaan immuunisolujen aktivaatioon sekä kasvaimen mikroympäristön muokkaukseen. Tämän lisäksi hoidolla parantuneet eläimet olivat paremmin suojattuja myöhemmältä syövän uudelleenimplantaatiolta.

Ad5/3-E2F-d24-vIL2-viruksen kykyä tehostaa allogeenistä NK-terapiaa tutkittiin in vitro kliinisissä munasarjasyöpänäytteissä. Viruksen ja NK-soluterapian yhdistelmähoito näytti tuhoavan syöpäsoluja sekä aktivoivan NK-soluja tehokkaammin, kun yhdistelmähoitoa verrattiin pelkkään NK-terapiaan tai virushoitoon ilman transgeeniä. Myöhempi in vivo-tutkimus vahvisti löydökset potilaisnäytteistä kasvatetussa munasarjasyövän xenograftimallissa.

Ad5/3-E2F-d24-hTNF-IRES-hIL2-viruksen turvallisuus ja ensimmäiset hoidon tehon merkit kyettiin vahvistamaan faasin I kokeessa. Tutkimukseen osallistui 20 potilasta, jolla oli todettu levinnyt kiinteä kasvain. Hoito oli hyvin siedetty, ja kolme vakavuusluokan 4 sivuvaikutusta paranivat spontaanisti. Hoito oli tehokas:

riippuen käytetystä kuvantamiskriteeristöstä, syövän kasvu pysähtyi 20 tai 60 %:ssa tapauksista.

Ad5/3-E2F-d24-hTNF-IRES-hIL2-hoidon tarkempia ominaisuuksia sekä sen tehoa ennustavia biomarkkereita tutkittiin kliinisen kokeen aikana kerätyistä näytteistä. Tutkimuksessa havaittiin, että Ad5/3-E2F-d24-hTNF-IRES-hIL2-hoito johti sekä systeemiseen että kasvaimensisäiseen immuunijärjestelmän aktivaatioon. Lisäksi huomattiin, että veren lymfosyyttimäärän lasku ennustaa parempaa pidempää elossaoloaikaa.

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

I **S. Pakola**, D. C. A. Quixabeira, T. V. Kudling, J. H. A. Clubb, S. Grönberg-Vähä-Koskela, S. Basnet, E. Jirovec, V. Arias, L. Haybout, C. Heiniö, J. M. Santos, V. Cervera-Carrascon, R. Havunen, M. Anttila & A. Hemminki (2023). **An oncolytic adenovirus coding for a variant interleukin 2 cytokine improves response to chemotherapy through enhancement of effector lymphocyte cytotoxicity, fibroblast compartment modulation and mitotic slippage.** Frontiers in Immunology, 14:1171083

II D. C. A. Quixabeira, **S. Pakola**, E. Jirovec, R. Havunen, S. Basnet, J. M. Santos, T. V. Kudling, J. H. A. Clubb, L. Haybout, V. Arias, S. Grönberg-Vähä-Koskela, V. Cervera-Carrascon, E. Kerkelä, A. Pasanen, M. Anttila, J. Tapper, A. Kanerva & A. Hemminki (2023). **Boosting cytotoxicity of adoptive allogeneic NK cell therapy with an oncolytic adenovirus encoding a human vIL-2 cytokine for the treatment of human ovarian cancer.** Cancer Gene Therapy, 30(12):1679-1690.

III **S. A. Pakola***, K. J. Peltola*, J. H. A. Clubb, E. Jirovec, L. Haybout, T. V. Kudling, T. Alanko, R. Korpisaari, S. Juteau, M. Jaakkola, J. Sormunen, J. Kemppainen, A. Hemmes, T. Pellinen, M. van der Heijden, D. C. A. Quixabeira, C. Kistler, S. Sorsa, R. Havunen, J. M. Santos, V. Cervera-Carrascon & A. Hemminki (2024). **Safety, efficacy, and biological data of T cell-enabling oncolytic adenovirus TILT-123 in advanced solid cancers from the TUNIMO monotherapy phase I trial.** Clinical Cancer Research, 3;30(17):3715-3725.

IV **S. A. Pakola**, J. H. A. Clubb, T. V. Kudling, M. van der Heijden, E. Jirovec, V. Arias, L. Haybout, K. Peltola, T. Alanko, J. Sormunen, T. Pellinen, K. Taipale, D. C. A. Quixabeira, C. Kistler, R. Havunen, S. Sorsa, J. M. Santos, V. Cervera-Carrascon & Akseli Hemminki (2024). **Transient lymphocyte count decrease correlates with oncolytic adenovirus efficacy in humans: mechanistic and biomarker findings from TUNIMO phase I trial.** Submitted to Journal for ImmunoTherapy of Cancer.

The publications are referred to in the text by their roman numerals. Publication II is included in Dafne C. A. Quixabeira's thesis "Facilitating cancer immunotherapies with armed oncolytic adenoviruses". * Shared first authorship.

LIST OF ABBREVIATIONS

4-1BBL	Tumor necrosis factor ligand superfamily member 9
7AAD	7-aminoactinomycin d
ACK	Ammonium chloride potassium
ACT	Adoptive cell therapy
Ad2	Adenovirus serotype 2
Ad5	Adenovirus serotype 5
Ad5/3	Adenovirus serotype 5 with serotype 3 knob
ADCC	Antibody-dependent cellular cytotoxicity
ADT	Androgen deprivation therapy
AKT1	Rac(rho family)-alpha serine/threonine-protein kinase
ALK	Anaplastic lymphoma kinase
ALP	Alkaline phosphatase
ALT	Alanine transaminase
ANOVA	Analysis of variance
APC	Antigen presenting cell
aPTT	Activated partial thromboplastin time
AST	Aspartate transaminase
ATCC	American Type Culture Collection
ATM	Ataxia-telangiectasia mutated serine/theonine kinase
ATP	Adenosine triphosphate
BCG	Bacillus Calmette–Guérin
BCR	B cell receptor
bsAb	Bispecific antibody
CAF	Cancer-associated fibroblast
CAR	Coxsackievirus and adenovirus receptor
CAR T	Chimeric antigen receptor t cell
CBA	Cytokine-bead array
CCL	Chemokine
CD	Cluster of differentiation
COVID-19	Coronavirus disease 2019
CRC	Colorectal cancer
CsCl	Cesium chloride
CT	Computer tomography
CTCAE	Common terminology criteria for adverse events
CTLA-4	Cytotoxic t-lymphocyte associated protein 4
CXCL	C-X-C motif chemokine

DMEM	Dulbecco's modified Eagle's medium
dMMR	DNA mismatch repair deficiency
DNA	Deoxyribonucleic acid
E1A	Adenovirus early region 1A
E1B	Adenovirus early region 1B
E3	Adenovirus early region 3
E4	Adenovirus early region 4
ECG	Electrocardiogram
ECOG	Eastern Cooperative Oncology Group
eGFR	Estimated glomerular filtration rate
EGFR	Epidermal growth factor receptor
ELISpot	Enzyme-linked immunosorbent spot
EMA	European medicines agency
ER	Estrogen receptor
erbB2	Receptor tyrosine-protein kinase erbb-2
FBS	Fetal bovine serum
Fc	Fragment crystallizable region
FDA	Food and Drug Administration
FDG	Fluorodeoxyglucose
FELESA	Federation of European Laboratory Animal Science Associations
FLG	Filaggrin
FOLFOX	Folinic acid, 5-fluorouracil and oxaliplatin
FOXP3	Forkhead box p3
Gal-3	Galectin 3
GITRL	Tumor necrosis factor receptor superfamily member 18 ligand
GJ86	Gap junction beta-6 protein
GMCSF	Granulocyte-macrophage colony-stimulating factor
HD-IL-2	High-dose interleukin-2
HER2	Human epidermal growth factor receptor 2
HGSC	High-grade serous ovarian cancer
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HSV-1	Herpex simplex virus type 1
i.t.	Intratumoral
i.p.	Intraperitoneal
ICD	Immunogenic cell death
ICI	Immune checkpoint inhibitor
IFIT2	Interferon-induced protein with tetratricopeptide repeats 2
IFN	Interferon
IFNAR	Interferon-alpha/beta receptor
IHC	Immunohistochemistry

IHP	Isolated hepatic perfusion
IL	Interleukin
IL-2R	Interleukin 2 receptor
ILP	Isolated limb perfusion
ImmTAC	Immune mobilizing monoclonal T-cell receptors against cancer
INR	International normalized ratio
IRAE	Immune-related adverse event
IRES	Internal ribosome entry site
iRECIST	Modified RECIST 1.1 for immune based therapeutics
IRES	Internal ribosome entry site
ITR	Inverted terminal repeat
kDa	Kilodalton
KRT64	Keratin 64
KRTDAP	Keratinocyte differentiation-associated protein
LAG-3	Lymphocyte-activation gene 3
LDH	Lactate dehydrogenase
LG	L-glutamine
LPS	Lipopolysaccaride
LSECtin	Liver and lymph node sinusoidal endothelial cell c-type lectin
MEK	Mitogen-activated protein kinase kinase
MHC-II	Major histocombability complex II
MOI	Multiplicity of infection
mRNA	Messenger RNA
MSI-H	Microsatellite instability high
MTD	Maximum tolerable dose
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2h-tetrazolium
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK cell	Natural killer cell
NKT	Natural killer T cell
NSCLC	Non-small cell lung cancer
OS	Overall survival
OV	Oncolytic virus
OX40L	Tumor necrosis factor receptor superfamily member 4 ligand
P16	Cyclin-dependent kinase inhibitor 2a
P53	Transformation-related protein 53
PARP	Poly-ADP-ribose polymerase
PBK	Lymphokine-activated killer T-cell-originated protein kinase
PBMC	Peripheral blood mononuclear cell
PD-1	Programmed cell death protein 1
PDAC	Pancreatic ductal adenocarcinoma

PDCD1	Programmed cell death protein 1
PD-L1	Programmed death-ligand 1
PD-L2	Programmed death-ligand 2
PDX	Patient derived xenograft
PEG	Polyethylene glycol
PET	Positron emission tomography
PFS	Progression-free survival
PI	Propidium iodide
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha
PR	Progesterone receptor
PS	Penicillin streptomycin
PSA	Prostate specific antigen
PTEN	Phosphatase and tensin homolog
qPCR	Quantitative polymerase chain reaction
RANTES	Regulated on activation, normal T-cell expressed and secreted
Rb	Retinoblastoma
RECIST	Response evaluation criteria in solid tumors
RGD	Arginine-glycine-aspartate
RPMI	Roswell Park Memorial Insitute medium
RTCA	Real-time cell analysis
SBSN	Suprabasin
SOX	S-1 plus oxaliplatin
STAT1	Signal transducer and activator of transcription 1
STK11	Serine/threonine kinase 11
SUVmax	Maximum standardized uptake value
TAA	Tumor associated antigen
TCR	T cell receptor
TIL	Tumor infiltrating lymphocyte
TILT-123	Ad5/3-E2F-d24-hTNF-IRES-hIL2
TILT-452	Ad5/3-E2F-d24-vIL2
TLR	Toll-like receptor
TMB-H	Tumor mutational burden high
TME	Tumor microenvironment
TNF	Tumor necrosis factor
TNFR1	Tumor necrosis factor receptor 1
TNFR2	Tumor necrosis factor receptor 2
TTP	Time to progression
VEGFA	Vascular endothelial growth factor alpha
VEGFR	Vascular endothelial growth factor receptor
VP	Viral particle

WBC	White blood cell
WHO	World Health Organization

1 INTRODUCTION

Cancer is a disease of uncontrolled cell growth. The International Agency for Research on Cancer (IARC) estimated that in 2022, 20 million new cases were diagnosed and 9.7 million deaths occurred due to cancer (1). In Western countries, approximately one in two males and one in three females develop cancer in their lifetime, with one in five males and one in six females dying from cancer (2). Most humans succumbing to cancer die of solid cancers such as lung, colorectal and liver cancer, where curative treatment options in the metastatic setting are limited (1, 2).

Cancer is known to arise from mutations occurring in healthy cells, ultimately leading to malignant transformation of the cell. However, the human immune system actively clears malignant and pre-malignant cells, thus controlling cancer formation. Yet, at some point the balance between cancerous transformation and immunologic clearance is disturbed, and cancerous disease presents itself (3). In the past two decades, proceedings in laboratory research have enabled us to enhance function of immune cells to clear cancer cells even after cancerous disease has formed. Currently, in solid cancers, this is done using immune checkpoint inhibitors (ICIs) which block inhibitory signals present in the immune system, or using adoptive cell therapy, where immune cells better able to clear tumors are infused in to the patient (4). Cancer immunotherapy, compared to other traditional forms of cancer therapy such as surgery, radiotherapy or classical chemotherapy, has a unique attribute of sometimes delivering complete cures even in the metastatic setting (5). This ability to eradicate multiple metastatic tumors long term is thought to arise from re-establishment of the equilibrium between cancer and the immune system. In many cases immunotherapy has replaced traditional chemotherapy, and currently PD-1 and PD-L1 inhibitors have been approved for the treatment of most solid cancers. However, especially in the metastatic setting, immunotherapy does not benefit all patients, and not all responses are durable, and thus active efforts are ongoing to enable successful immunotherapy in more solid cancer types (6).

Oncolytic virotherapy is a form of immunotherapy, where viruses are engineered to replicate in cancer cells. Oncolytic viruses (OVs) have multiple features, such as cancer specificity, immunogenicity and customizability making them practical immunotherapeutics (7). OVs theoretically can combat many known resistance mechanisms to immunotherapy currently present in solid cancers, such as poor immune infiltration, immunosuppressive tumor microenvironment and limited antigen recognition (8). Due to these features, OVs are now considered a possible future treatment modality to enable immunotherapy in a larger groups of cancers,

but more research is needed to gain information on optimal combinations to OVs, toxicity of OVs and mechanism of action in humans (9).

2 REVIEW OF THE LITERATURE

2.1 CURRENT CANCER THERAPIES: PROGRESS FROM SYSTEMIC TREATMENTS TO HARNESSING THE IMMUNE SYSTEM

Treatment of cancer has been of interest for humans since at least ancient Egypt (10). Earliest forms of cancer therapy consisted of surgical excision of tumors and addition of different ointments on tumors. These treatments were not widely efficacious, but as societies developed, more cancer therapies became available with more sophisticated mechanisms of actions. The 21st century is marked by a great advance in cancer therapeutic developed, translating to better outcomes for cancer patients. At present day, four main pillars of cancer treatment consist of chemotherapy, radiation therapy, surgery and immunotherapy. Efficacious cancer therapy often consists of combination of these treatments and is dependent of disease stage and patient fitness.

2.1.1 Chemotherapy

Chemotherapy is method of treating cancer with chemicals, administered either orally, intravenously, intraperitoneally, or topically. Nowadays chemotherapeutics form a base of cancer therapy, and a medical specialty called medical oncology specializes in administration of these agents. Chemotherapeutics include chemicals which target common cell replication machinery (sometimes termed traditional chemotherapeutics) and are inherently toxic to cancer cells and healthy cells alike (11). Another class of chemotherapeutics are so called targeted therapeutics, which target specific molecules expressed more in cancer cells, or mutations specific in cancer cells. Targeted therapies allow for a more tailored treatment of cancer, with the potential of less side-effects.

2.1.1.1 Traditional chemotherapeutics

Traditional chemotherapeutics include agents that target DNA replication, DNA synthesis, and cell division. These agents are used in cancer treatment, since cancer cells inherently are actively replicating cells. However, due to their nature of targeting replicating cells, traditional chemotherapy leads to toxicity in healthy replicating cells alike. Thus, cancer patients receiving traditional chemotherapy often experience such as hair loss, myelosuppression, and gastrointestinal

problems (12). Additionally, some chemotherapeutics have side effects inherent to the mechanism of action, such as neuropathic pain caused by paclitaxel, due to toxic effects of microtubule stabilization on peripheral nerve fibers (13). Currently chemotherapeutics have also been developed in more advanced formulations, such as formulations bound to albumin (nano albumin bound-paclitaxel), incorporated into liposomes (liposomal irinotecan) or conjugated to antibodies (trastuzumab deruxtecan) (14, 15). These formulations are designed to decrease toxic effects of chemotherapeutics and increase desired anti-cancer effects.

2.1.1.2 Hormone therapy and targeted therapies

Targeted therapies relate to therapies, which target a specific molecule or pathway present in cancer cells. The earliest form of targeted therapy, developed in the 1970s, was tamoxifen, which targeted estrogen receptors on cancer cells, blocking growth signals of estrogen to cancer cells (16). Another common form of hormone therapy used is androgen deprivation therapy (ADT) for prostate cancer, where androgen levels of the patient are lowered with therapeutics such as gonadotropin releasing hormone agonists, or more sophisticated newer androgen receptor antagonists, such as enzalutamide, leading to reduced cancer cell proliferation (17).

However, as cancer therapy developed, cell protein targeting was revolutionized by antibodies, which could be engineered to target other growth receptors on cancer and block their function. Example of these agents is trastuzumab, which targets HER2 (also known as erbB-2) on cancer cells. As therapeutics developed, agents targeting intracellular signaling cascades were also developed (12). This developed lead to an explosion in individualized cancer therapy, where targeted therapies could be tailored for the patients based on their tumor mutational landscape. Examples of targeted therapies targeting intracellular pathways include ALK inhibitor crizotinib and MEK inhibitor trametinib. These drugs can provide long term responses, lasting more than 5 years, although cancer is known to develop resistance to the agents at some point (18).

2.1.2 Radiotherapy

Radiotherapy is a form a cancer therapy, that utilizes the toxic effects of radiation to treat cancer. High doses of radiotherapy leads to DNA damage in cells, and due to often deficient DNA damage correction machinery in cancer cells, radiation of the tumor leads to profound cancer cell death. At present, multiple forms of different cancer radiotherapy is used, most common being external radiotherapy using a linear particle accelerator (19). Radiotherapy has also effects on normal cells, since the radiation beam often has to pass through layers of healthy tissue before reaching the targeted tumor. However, currently multiple ways of limiting

radiotoxicity is present, such as splitting the dose into multiple small amounts (fractionation), insertion of radiation sources inside or close to tumors (brachytherapy), targeting of radiotherapy to minimally harm normal tissue (stereotactic radiation) and utilization of natural tendency of tissues to take up specific periodic elements such as iodine (radioisotope therapy) (20).

2.1.3 Surgery

Surgery is the earliest form of cancer therapy, and arguably the most efficient if all cancer can be removed during surgery. Unfortunately, many cancers are diagnosed at advanced stages where surgical intervention is not able to remove all tumor. Nevertheless, surgery still has a vital role in current cancer therapy, and sometimes cancer which at presentation seems unresectable can be converted to a resectable form of cancer with chemotherapy or radiotherapy prior to surgery. Current surgical treatment of cancer often pursues a complete resection of malignant tissue, sometimes utilizing intraoperative tissue samples analyzed by a pathologist during surgery. In many cases, even with resectable cancer, surgery is combined with other cancer therapies prior to surgery (termed neoadjuvant therapy) and/or after surgery (termed adjuvant therapy). This has been shown to enhance the long-term success of surgery, since even if surgery reaches a complete resection, micrometatasis is possible prior to surgical intervention (21).

2.1.4 Immunotherapy

Immunotherapy is a form of cancer therapy, which takes advantage of the patient's own immune system to treat cancer. Immunotherapy is based on the fundamental role of the immune system in recognizing cancerous cells and clearing them. However, during cancer development, cancer finds ways to escape immune elimination, leading to unrestricted cancer growth (22). The aim of immunotherapy is to reinvigorate the immune system to eliminate cancer cells. Immunotherapy became a fundamental piece of cancer therapy during the 2010s, when checkpoint inhibitors were approved for treatment of metastatic melanoma (4). This part of the literature review focuses on approved forms of immunotherapy, with an illustration of discussed therapeutics shown in Figure 1.

2.1.4.1 Checkpoint inhibitors

Checkpoint inhibitors are a class of antibody drugs, which aim to block inhibitory signals experienced by immune cells, often arising from cancer cells or antigen presenting cells. The earliest form of immune checkpoint therapy was ipilimumab targeting CTLA-4 molecule on immune cells (23). CTLA-4 competes with CD28 for

binding to CD80 or CD86 in antigen presenting cells, where binding of CD28 with CD80 or CD86 leads to activation of the immune cell, whereas binding of CTLA-4 to CD80 or CD86 leads to anergy of the immune cell. Ipilimumab was approved in 2011 for the treatment of metastatic melanoma (23).

In addition to CTLA-4 inhibition, a class of antibodies targeting PD-1 and PD-L1 proteins have been developed, and have shown improved efficacy in many cancers, compared to CTLA-4 blockage. PD-1 is a receptor expressed on immune cells, with two known ligands, PD-L1 and PD-L2, expressed mostly on cancer cells, antigen presenting cells and other immune cells (23). Binding of PD-1 with its ligand leads to immune cell deactivation and anergy, and thus blocking this interaction protects the immune cell from these effects. Currently PD-1 and PD-L1 therapeutics have shown promising activity in melanoma, NSCLC, RCC, breast cancer, upper-GI cancers, bladder cancer, and subtypes of other solid cancers that harbor DNA mutation correction deficiencies (termed microsatellite instability high, MSI-H or mismatch repair deficient, dMMR) (24).

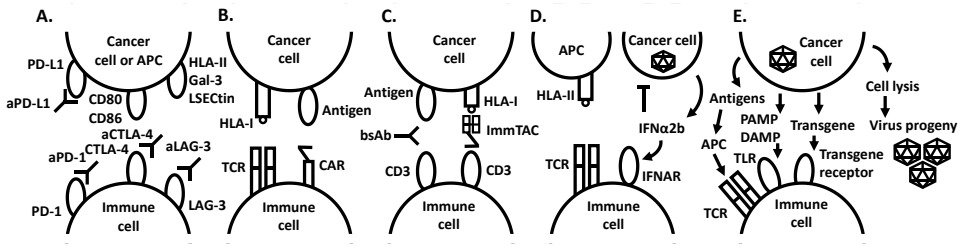

Figure 1. Currently approved form of cancer immunotherapy: checkpoint inhibitors (A), cell therapies (B), bsAbs and ImmTACs (C), cancer vaccines and immunostimulatory cancer gene therapy (D) and oncolytic viruses (E).

In addition to CTLA-4 and PD-1 axis inhibitors, in 2022 the FDA approved a LAG-3 inhibitor relatlimab for the treatment of metastatic melanoma, in combination with PD-1 inhibition (25). LAG-3 is a molecule expressed on immune cells, which binds molecules expressed on cancer cells, mainly MHC-II, Gal-3 and LSECtin. Binding of LAG-3 with its ligand leads to inhibition immune cell activation (25).

With their unique mechanism of action, checkpoint inhibitors brought with them unique side effects. These side effects, referred to as immune-related adverse events (IRAEs), stem from the normal functions of checkpoint molecules in human immunology. Although the goal of ICI therapy is to release immune attack against cancer cells, it is also able to induce immune activation against healthy tissues. Thus, IRAEs often present as autoimmune manifestations, such as hypo- or hyperthyroidism, colitis, uveitis, or rash (26). Interestingly, patients developing IRAEs often also develop an anti-cancer response as a result of immune checkpoint

therapy, highlighting the intricate balance between immunotherapy response and toxicity (27).

Although revolutionary in currently approved indications, checkpoint inhibitor therapy has not shown efficacy in all solid cancer types, and not all patients within approved indications respond (24). Reasons for checkpoint inhibitor therapy failure are multifaceted, but some recognized challenges include the paucity of anti-cancer immune cells, poor trafficking of immune cells to tumors, other immunosuppressive pathways presents in the tumor microenvironment (TME) and poor diffusion of antibody into tumors (8).

2.1.4.2 Cell therapies

Cell therapies are a form of cancer therapy, where anti-cancer cells are infused into the patient. Often cells are collected from the patient, followed by cell modification and/or activation in the laboratory to enhance the anti-cancer activity of the cells, and finally cells are administrated back into the patient. Alternatively, cells can be pre-prepared and administered to patients when needed in an "off-the-shelf" manner. Cell therapies are often called adoptive cell therapies (ACT), due to the methods used in modifying the cells in the laboratory (28). Modern ACT was pioneered in the 1980s by Steven A. Rosenberg, with experiments involving tumor infiltrating lymphocyte (TIL) therapy. In this approach, a tumor is removed from a patient, followed by a culture protocol in which lymphocytes are grown from the tumors tissue, expanded in number and reinfused back to the patient (29). In 2024, TIL therapy (lifileucel, Amtagvi) gained FDA approval for the treatment of metastatic melanoma that had progressed after immune checkpoint therapy (30).

Following TIL therapy, developments in synthetic biology of T cell receptors lead to the advent of chimeric antigen receptor T cells (CAR T). CAR T cells are T cells, which incorporate the binding part of an antibody (referred as Fv or variable fragment), along with an intracellular transduction domain from a normal T cell receptor. Development of CARs allowed scientists to produce adoptive cell therapies with designated specificity to a wanted antigen. CAR T cells were then subsequently designed to target cancer cell proteins and were tested in the clinic. Nowadays CAR T-cell therapy has revolutionized the treatment of B-cell leukemias and lymphomas, where cancers express a suitable antigen for the therapy (31). However, efficacy of CAR T-cell therapy in solid cancer has been limited, due to the absence or down-regulation of targets used in CAR T cells and other resistance mechanisms present in solid tumors (32). Furthermore, the long production timelines of CAR T cells limit their usability, as some cancer patients become unfit or succumb to the disease before CAR T-cell production is complete. Efforts to overcome these challenges include engineering of CAR T cells with multiple receptors, engineering of CAR T cells to produce cytokines promoting their survival,

and using off-the-shelf cell formulations (allogenic T cells or NK cells) (32). However, the obstacles of widespread use of CAR T cells in solid cancers are still present, and no CAR T-cell therapy has been approved for the treatment of solid cancers by the FDA or EMA.

A third important form of adoptive cell therapy is TCR T-cell therapy, where T cells are transduced with a synthetic T cell receptor targeting a set epitope present on cancer cells (33). TCR T-cell therapy has an inherent advantage over CAR T-cell therapy, in that a T-cell receptor can detect intracellular antigens presented on HLA I molecules, leading to larger antigen pool and lower activation threshold (33). In addition to TIL therapy approval, 2024 was marked by the approval of the first TCR T cell therapy, when MAGE-A4 targeted TCR T therapy afamitresgene autoleucel (Tecelra) was approved for synovial sarcoma (34).

NK-cell therapy has been proposed as a possible solution to overcome known limitations of CAR T-cell therapy and TCR T-cell therapy. Due to the inherent nature of NK cells' to recognize cells in an HLA-unrestricted manner, they can be administered allogenically, and doses can be manufactured ready for treatment in an off-the-shelf manner from allogenic sources (35). Early trials of CAR NK-cell therapy have shown promising efficacy in B-cell malignancies comparable to CAR T-cell therapy (36). Additionally, the safety profile of CAR NK-cell therapy seems advantageous over traditional CAR T-cell therapy. Patients receiving CAR NK-cell therapy rarely develop cytokine release syndrome or immune effector cell-associated neurotoxicity syndrome, which are common and sometimes fatal side effects of CAR T-cell therapy. The favorable safety profile of NK cells can make these therapies accessible to a wider population of cancer patients and allow multiple doses of NK cells to be administered to the same patient (35). Due to their early development stage, no NK-cell therapy is approved by the FDA or EMA, but multiple phase I and phase II trials are currently enrolling patients and results are eagerly awaited (37, 38).

2.1.4.3 Bispecific T cell engagers and immune mobilizing monoclonal TCRs against cancer

Bispecific T cell engagers (BsTEs) are a form of bispecific antibodies that utilize antibodies to bridge immune cells to target cells. Traditionally, BsTEs are constructed such that one variable region of the antibody targets a cancer antigen (such as EpCAM or CD19), and the other variable region targets an immune cell epitope (such as CD3). Thus, the binding of the antibody brings the target and immune cells in close contact with each other, facilitating recognition and immune clearance (39). Depending on the construct, BsTEs either lack or contain the natural constant region of the antibody. Multiple different BsTEs are already approved for cancer therapy, such as catumaxomab targeting EpCAM and CD3ε for malignant ovarian ascites, and blinatumomab targeting CD19 and CD3 for B-cell precursor

acute lymphocytic leukemia. However, the therapeutic effects have generally been limited in solid cancers (40).

A concept similar to BsTEs are immune mobilizing monoclonal TCRs against cancer (ImmTACs), which target peptides presented on HLA-I molecules (40). One end of an ImmTAC is a TCR designed to recognize a wanted antigen, and the other end of an ImmTAC is an antibody targeting the immune cells, often an antibody variable region targeting CD3. An ImmTAC targeting gp100 and CD3, tebentafusp, was approved in 2022 for the treatment of uveal melanoma, marking a notable milestone in the development of ImmTACs (41, 42).

2.1.4.4 Cancer vaccines and cancer gene therapy

The idea of vaccination against cancer stems from successful vaccination efforts against multiple different infectious agents. Although conceptually promising, vaccination against cancer has proven more difficult than against infectious agents. This may be due to multiple reasons including but not limited to lack of targetable antigens, evolution of antigen milieu in tumors due to selective pressure, immunosuppressive TME and damaged immune system of cancer patients due to previous lines of chemotherapy (43, 44). However, one cancer vaccine formulation, sipuleucel-T (also known as Provenge or APC8015), was approved in 2010 for the treatment metastatic castration-resistant prostate cancer. Sipuleucel-T is a unique formulation of autologous cell therapy combined with vaccine component in the form of prostatic acid phosphatase (PAP or PA2024). Sipuleucel-T is formulated by collecting peripheral blood mononuclear cells (PBMC) from the patient, followed by ex vivo cultivation of the cells with the PAP antigen, and intravenous reinfusion of the prepared product back to the patient (45, 46). The phase 3 trial of sipuleucel-T showed significantly improved in overall survival (4.1 months) and increased antibody and T-cell proliferation against PAP in the treated patients compared to placebo (46). However, after sipuleucel-T, no cancer vaccines have been approved by the FDA or EMA, although the recent advances in mRNA technology have shown promising results in early trials of neoadjuvant and adjuvant administration of cancer vaccines (47, 48).

Advancements in sequencing technology have enabled the generation of personalized cancer vaccines based on the expressed antigens present in the patient's individual tumor, termed individualized cancer immunotherapy. Furthest in development of these individualized cancer vaccines is Rocapuldencel-T (CMN-001), a therapy of dendritic cells loaded with antigens generated from whole-tumor mRNA. In a phase 3 trial of patients with metastatic renal cell carcinoma, Rocapuldencel-T was able to elicit changes in the patients' peripheral T cell populations, but no improvement in overall survival was observed, possibly due to the unexpectedly long survival of the control arm (49). Further advancements in

individualized cancer immunotherapy have focused on the development of tumor antigen mRNA encapsuled into lipid nanoparticles, similarly to COVID-19 vaccines, and multiple trials are currently ongoing with results awaited (50).

Although not classically considered immunotherapy, cancer gene therapy encoding immunostimulatory transgenes also has effects on the immune system. A recent success of the cancer gene therapy field was nadofaragene firadenovec (also known as Adstiladrin, Instilidrin or rAd-IFN/Syn3), a non-replicative adenovirus serotype 5 encoding IFNα2b, approved by the FDA in 2022 for the treatment of BCG-relapsed non-muscle invasive bladder cancer, administered via urethral catheter with Syn3 polyamide surfactant to enhance viral transduction (51-53). The approval leading trial of nadofaragene firadenovec showed an impressive 3-month complete response rate of 59.6%, with good tolerability of the intravesically administered drug (52). Although primarily designed to inhibit cancer cell growth by local IFNα2b production, IFNα2b has known activatory effects on immune cells, highlighting a possible immunologic mechanism behind the efficacy of nadofaragene firadenovec (54). Before the approval of nadofaragene firadenovec no non-replicative cancer gene therapy had been approved by the FDA or EMA, highlighting the promise of adenovirus-based therapeutics against cancer (55).

2.1.5 Oncolytic virotherapy

Oncolytic virotherapy uses viruses for the treatment of cancer and is considered a form of cancer immunotherapy. Some viruses, such as reovirus, are naturally tumor specific by replicating only in cancer cells, whereas some viruses, such as adenoviruses, can be engineered for specific replication (56). Oncolytic virotherapy has many advantageous qualities as a cancer therapeutic: engineered viruses cause minimal harm to normal cells, the virus replicates in tumors amplifying the input dose, and the transgenes of viruses can be easily switched and tailored. Moreover, the natural lytic cycle of the viruses releases tumor antigens (TAAs) and the inherent immunogenicity of the vectors provide an immunostimulatory effect resulting in an influx of lymphocytes to the tumors (7). Furthermore, some oncolytic viruses, such as adenovirus, have been used as vectors in vaccines, providing valuable safety information regarding possible side effects related to the therapy.

2.1.5.1 History of oncolytic virotherapy

Interest in the use of viruses for cancer treatment stemmed from observations in the late 1800s and early 1900s, when leukemia patients contracted influenza or tuberculosis and subsequently gained therapeutic effect for the leukemic disease (57). Additionally, until present day, case-reports have been published of cancer

patients contracting viral infections, with concurrent disappearance of cancerous disease (56, 58, 59). In the 1950s and 1960s, many scientists experimented with administration of different viruses for cancer patients, with variable clinical success (56). However, in the late 1980s and 1990s, developments in DNA technology enabled the genetic modification of viral genomes, and thus ushered forward a new age in oncolytic virotherapy. Viral genomes could be modified to introduce promoters specific for replication purely in cancer cells. These early or first-generation oncolytic viruses were naturally occurring or only slightly attenuated viruses without transgenes. Efficacy of first-generation OVs was mostly limited, although the Chinese National Medical Products Administration approved H101, an oncolytic adenovirus aimed to replicate only in p53-mutated cells, for the treatment of head and neck malignancies in 2005.

After the first generation of oncolytic viruses, the field moved forward with next generations of oncolytic viruses aimed to further enhance the safety and specificity of the vectors and added transgenes to the vectors. These developments culminated with T-VEC (talimogene laherparepvec), an oncolytic herpes virus encoding granulocyte macrophage colony stimulating factor (GMCSF). T-VEC was approved by the FDA and EMA for the treatment of melanoma in 2015. Although superior to injected GMCSF in the approval leading phase III trial, currently the use of T-VEC is limited due to more efficacious checkpoint inhibitors and limitation to intratumoral dosing. Additionally, recently in 2022, an engineered HSV-1 oncolytic virus Delytact (also known as teserpaturev or G47Δ) was approved for the treatment of malignant glioma in Japan (60).

After these developments, oncolytic virotherapy is now a recognized form of immunotherapy, but more research is needed to develop these agents further to facilitate greater efficacy in the clinic. Additionally, research on oncolytic viruses combined with other cancer therapeutics, namely chemotherapeutics and other immunotherapeutics other than checkpoint inhibitors is still lacking (9).

2.1.5.2 Oncolytic adenovirus

Adenovirus is the most used oncolytic virus in clinical trial development, with HSV-1 following closely behind. For oncolytic virotherapy, the most commonly used adenovirus serotype is the serotype 5, although other rarer subtypes such as 11 and 3 are used (61). Adenovirus is a nonenveloped virus, with a protein capsid constructed of hexon, penton and fiber proteins and a diameter of 70-100 nm. The adenovirus serotype 5 genome is a 36 kb double-stranded DNA genome. Normally, adenoviruses cause upper respiratory tract infections, but cases of conjunctivitis and gastrointestinal infections are also common for adenovirus (62).

Adenoviruses have multiple features making them suitable agents for oncolytic virus therapy. Firstly, the genome of adenoviruses is by nature very stable, easily

modifiable and easy to produce. Second, by nature adenoviruses are immunogenic, which can be utilized in cancer therapy to recruit immune cells to tumors. Thirdly, adenoviruses have been previously used also as vaccine backbones, which have proved the safety of administration of adenoviral particles to humans and possibility of large-scale production. Indeed, multiple oncolytic viruses are now in development in multiple different tumor indications. The list of current oncolytic adenovirus trials is shown in Table 1.

Table 1. Current recruiting or ongoing oncolytic adenovirus trials. Data from clinicaltrials.gov, accessed August 3rd 2024.

NCT Number	***Cancer type***	***Oncolytic adenovirus***	***Transgenes***	***Combination treatment***	***Phase***
NCT04673942	Sarcomas	AdAPT-001	TGFβR-Fc	PD-1 inhibitor	1/2
NCT06215846	Solid tumors	BioTTT001	nsIL12	-	1
NCT06283121	Gastric cancer	BioTTT001	nsIL12	SOX + PD-1 inhibitor	1
NCT06283134	Colorectal cancer	BioTTT001	nsIL12	PD-1 inhibitor + multikinase inhibitor	1
NCT03740256	Solid cancers	CAdVEC	IL-12, PD-L1 antibody	CAR T cell therapy	1
NCT06111235	Bladder cancer, urothelial cancer	CG0070	GMCSF	n-dodecyl-B-D-maltoside	3
NCT04452591	Bladder cancer	CG0070	GMCSF	n-dodecyl-B-D-maltoside	3
NCT06253845	Bladder cancer	CG0070	GMCSF	-	1
NCT03896568	Brain cancer	DNX-2401 loaded to mesenchymal cells	-	-	1
NCT06031636	Advanced malignant pleural mesothelioma	H101	-	PD-1 inhibitor	-
NCT05234905	Cervical cancer	H101	-	PD-1 inhibitor	2
NCT05124002	Bile duct cancer	H101	-	Hepatic arterial infusion of FOLFOX	4
NCT05564897	Bladder cancer	H101	-	PD-1 inhibitor	2
NCT05823987	Bile duct cancer	H101	-	PD-1 inhibitor + VEGFR inhibitor	2
NCT05675462	Liver cancer	H101	-	PD-1 inhibitor + VEGFR inhibitor	1

NCT03225989	Pancreatic cancer, ovarian cancer, bile duct cancer, colorectal cancer	LOAd703	TMZ-CD40L, 41BBL	-	1/2
NCT02705196	Pancreatic cancer	LOAd703	TMZ-CD40L, 41BBL	Gemcitabine + nab-paclitaxel + PD-L1 inhibitor	1/2
NCT05076760	Solid tumors	MEM-288	IFNβ, CD40L	PD-1 inhibitor	1
NCT04097002	Prostate cancer	ORCA-010	-	-	1/2
NCT04612504	Liver cancer	SynOV1.1	GMCSF	-	1
NCT04695327	Solid tumors	TILT-123	TNF, IL2	-	1
NCT04217473	Metastatic melanoma	TILT-123	TNF, IL2	TIL therapy	1
NCT06125197	NSCLC	TILT-123	TNF, IL2	PD-1 inhibitor	1
NCT05222932	Melanoma, head and neck cancers	TILT-123	TNF, IL2	PD-L1 inhibitor	1
NCT05271318	Ovarian cancer	TILT-123	TNF, IL2	PD-1 inhibitor + pegylated liposomal doxorubicin	1
NCT05914935	Brain cancer	YSCH-01	L-IFN	-	1

2.1.5.3 Other types of oncolytic viruses

In addition to adenovirus, other popular oncolytic viruses include herpes simplex viruses serotypes 1 and 2, reovirus, measles virus and vaccinia virus. Of these, HSV-1 therapeutics have been the most widely used due to their large genome, ease of modification comparable to adenovirus and natural neurovirulence. All oncolytic virus constructs have their own advantages and disadvantages, and active research is ongoing to identify which virus construct best suits which patients (9).

2.2 INTERLEUKIN-2: CYTOKINE CRUCIAL FOR T CELL GROWTH

2.2.1 History and biology of interleukin-2

In studies I-IV, all virus constructs contained a modified version of interleukin-2 or a normal interleukin-2 in combination with TNF. Interleukin-2, originally termed T-cell growth factor, was first identified in the mid-1970s, by stimulation of human

lymphocytes with phytohemagglutinin, a plant lectin protein (63, 64). Following stimulation, supernatant produced by stimulated lymphocytes was able to support growth of unstimulated T lymphocytes for up to 9 months, a feat unheard of at the time. As research progressed, in the 1980s it was shown that TCR interaction with its ligand, with co-stimulation from CD28 produces IL-2 and induces IL-2 receptor (IL-2R) expression on T cells, cementing the central role of IL-2 in T cell activation and proliferation (65). Currently, it is known that IL-2 also induces proliferative activity of other cells than T cells, such as NK and NKT cells.

In humans, interleukin-2 is a 15.5 kDa protein mainly produced by activated T cells. Human interleukin-2 receptors are made of three subunits, termed by Greek letters: IL-2Rα, IL-2Rβ and IL-2Rgamma. By combining these subunits, human cells can produce three different IL-2Rs with different affinities for IL-2: a low-affinity receptor (consisting of solely IL-2Rα), an intermediate affinity receptor (consisting of IL-2Rβ and IL-2Rgamma), or a high-affinity receptor (consisting of all three subunits). Of these different receptors, the high-affinity receptor is mostly expressed on activated CD4+ and CD8+ T cells and regulatory T cells (CD4+Foxp3+). Thus, along with supporting growth of activating T cells, IL-2 also induces the proliferation of suppressive regulatory T cells. This counterbalance to activation induced by IL-2 is thought to be needed to contain immune responses in peripheral tolerance (65, 66).

2.2.2 History of systemic interleukin-2 as cancer therapy

IL-2 has a long history as a cancer therapeutic, first trialed in the clinic in the mid-1980s (67). After developments in the recombinant production of IL-2 in E. coli, larger trials were possible with the administration of larger doses of IL-2. When given systemically, IL-2 has a short half-life of about 7 minutes. High-dose systemic IL-2 therapy is marked by significant toxicity due to effects of IL-2 on the vasculature and the immune compartment. Common side-effect syndrome of HD-IL-2 is vascular leak syndrome arising from IL-2 mediated endothelial cell activation, leading to fluid build-up in the lungs and other vascularized organs, along with weight gain. Additionally, the immune activation caused by IL-2 induces fever, chills, joint pain and general malaise, thus limiting the possible dose used and patient selection to fit patients able to tolerate the side effects (68).

Even with these severe side effects, the promising anti-cancer effects of IL-2 ushered forward more research. First results of HD-IL-2 showed complete or partial tumor regressions in half of the treated melanoma patients, and in all treated renal cell carcinoma patients when combined with autologous PBMCs stimulated with IL-2 (69). IL-2 therapy was also trialed in other solid cancers, but responses were sparse apart from a few lymphoma patients (70). Following these findings, HD-IL-2 was trialed in more patients focusing on renal cell carcinoma and

metastatic melanoma. After these studies, the FDA approved HD-IL-2 for the treatment of metastatic renal carcinoma in 1992, making IL-2 the first approved immunotherapy for cancer. Later in 1998, FDA similarly approved HD-IL-2 for the treatment of metastatic melanoma (71).

Due to the natural ability of IL-2 to induce proliferation of T cells, IL-2 therapy was also combined with the first form of T-cell therapy, TIL therapy, in the late 1980s (72). Metastatic lesions of patients with melanoma were excised, and lymphocytes were grown in the laboratory from these tumor samples. The multiplied and activated lymphocytes were then re-administered to the patients with concurrent IL-2 administration. 11 out of 20 patients responded to the therapy, which lead to larger trials with combinations with other synergistic cancer drugs, such as cyclophosphamide (73). These early trials of IL-2 therapy combined with TILs did not lead to regulatory approval, but in 2024 the FDA approved TIL therapy (Amtagvi, Iovance Biotherapeutics Inc) in combination with IL-2 post-conditioning, for the treatment of metastatic melanoma progressing after PD-1 therapy (30, 74).

2.2.3 History of vectored interleukin-2 as cancer therapy

Due to dose-limiting side-effects from systemic IL-2 therapy, vectored therapy of IL-2 has generated interest among scientists. Early trials of vectored IL-2 therapy consisted of transducing TIL samples with vectors, making these cells secrete IL-2 constitutively (75, 76). Later in the late 1990s and early 2000s, multiple reports were published on vectored IL-2 encoded by adenoviruses or other gene therapy constructs. These early trials mostly utilized non-replicative vectors, and clinical activity of these agents were varied (77-81).

Of note, promising efficacy was observed with two different adenovirus constructs encoding IL-2, AdCAIL-2 and TG-1042. Both of these constructs were non-replicative of nature, but regardless AdCAIL-2 showed promising decrease in PSA levels in patients with localized prostate cancer (81). Furthermore, tumors specimens showed accumulation of effector lymphocytes in tumors, confirming the mechanism of action of vectored IL-2 therapy from preclinical models. TG-1042 was studied in patients with metastatic melanoma, where complete remissions and multiple objective responses were seen (78).

Regardless of promising early results, the development of IL-2 gene therapy for cancer slowed, mirroring the general situation in the cancer gene therapy field. However, in the mid-2010s oncolytic viruses began to be armed with IL-2. The advantage of arming oncolytic viruses with transgenes, when compared to non-replicative vectors, is the long transgene expression available in the tumor. When new virions are produced by the natural oncolytic cycle of the virus, these new virions infect new cancer cells, producing more transgene. Research conducted in

the University of Helsinki Cancer Gene Therapy Group showed that arming oncolytic adenoviruses with IL-2 and TNF synergized with other cancer therapies harnessing T cell killing against cancer, such as checkpoint inhibitors, TIL therapy, and CAR T therapy (82-85). Following these preclinical developments, an oncolytic adenovirus encoding for TNF and IL-2, now known as TILT-123, entered clinical trials.

2.2.4 Modified interleukin-2 proteins as cancer therapy

Due to significant toxicity, and the Treg expanding nature of IL-2, research has also aimed to improve the pharmacokinetics and pharmacodynamics of IL-2, by modifying the IL-2 structure. Due to natural high expression of high-affinity IL-2 receptors on Treg cells, cancer therapy has aimed to bias binding from high-affinity receptors to intermediate affinity receptors, which are expressed on memory CD8+ T cells and NK cells. Popular methods of biasing the binding of the modified IL-2 molecule are to either block binding to the IL-2Rα subunit by antibodies, or by modifying the structure of IL-2 to ablate binding to IL-2Rα or bias the binding towards the other subunits (86). More recent research has also produced completely novel cytokine mimics, which activate same signaling cascades as IL-2 or modified IL-2 proteins but are structurally completely synthetic (87).

Regarding pharmacokinetics, due to the short half-life of IL-2 (less than 7 minutes), many efforts have been made to increase its half-life. Efforts in increasing IL-2 half-life have included binding the protein to albumin or immunoglobulins or coating the protein with PEG. Of considerable interest is the binding of IL-2 to tumor targeting immunoglobulins, thus circumventing both systemic toxicity of IL-2 and increasing the half-life (88).

All of the aforementioned ways of improving IL-2 therapy have shown improved efficacy over natural IL-2, but few constructs have progressed beyond phase I/II trials. Darleukin, IL-2 coupled to a L19-antibody targeting the ED-B domain of fibronectin expressed in tumor vasculature, is currently enrolling patients into a phase III trial studying the effect of neo-adjuvant Darleukin combined with Fibromun (same antibody with TNF coupling), followed by surgery and adjuvant immune checkpoint inhibition (89).

Regarding binding-biased IL-2 constructs, an IL-2Rgamma biased construct nemvaleukin alpha (ALKS 4230) has reached phase III trials in patients with platinum-resistant epithelial ovarian, fallopian or primary peritoneal cancer. In the ARTISTRY-7 trial, nemvaleukin alpha is combined with PD-1 inhibitor pembrolizumab, and the combination is compared to chemotherapy, and monotherapy of nemvaleukin alpha and pembrolizumab. The trial is currently recruiting with a target accrual of 376 patients (90).

Concerning PEGylated IL-2 constructs, the furthest in development is bempegaldesleukin (NKTR-214), currently in enrolling patients into 4 phase 3 trials. These trials enroll patients with metastatic melanoma, advanced renal cancer and muscle-invasive bladder cancer, and all trials combine bempegaldesleukin with the PD-1 inhibitor nivolumab. The combination of bempegaldesleukin with nivolumab in metastatic melanoma showed no improvement over nivolumab. Similarly, in advanced renal cancer, combination of bempegaldesleukin with nivolumab showed no improvement over VEGF-inhibition, leading to termination of clinical development. Results regarding bempegaldesleukin with nivolumab in muscle-invasive bladder cancer are awaited (88).

2.3 TUMOR NECROSIS FACTOR: A POTENT INFLAMMATORY CYTOKINE

2.3.1 History and biology of tumor necrosis factor

Tumor necrosis factor was first identified in the mid-1970s from the serum of mice infected with Bacillus Calmette-Guerin (BCG) and received its name from the ability of the serum to induce marked necrosis in tumors (91). TNF was in fact unknowingly used as a cancer therapeutic almost 100 years prior, when a US surgeon William Coley used bacterial extracts to treat tumors. Later it was shown that the mechanism behind Coley's treatment was not in fact the bacterial proteins such as LPS, but rather the TNF produced by the patients' macrophages following LPS stimulation (92). After recombinant TNF was produced, animal experiments showed marked tumor regression after local treatment of TNF, which expedited the development of TNF as a cancer therapy.

Normally in the body, TNF is mostly produced by activated macrophages, and functions as a key mediator of inflammatory processes. TNF has two known receptors, TNFR1 and TNFR2. TNFR1 is widely expressed on all cell types, whereas TNFR2 expression is mostly limited to immune cells (93). TNF binding to TNFR1 leads either to cell death or cell proliferation, dependent on the concentration of TNF binding TNFR1 and other cytokine signaling affecting the cell. TNF signaling through TNFR2 leads to cell survival through the NF-κB pathway (94). TNF has pleiotropic effects in cancer, affecting tumor fibroblast proliferation, blood vessel formation, cancer cell migration and immune responses against cancer (95).

2.3.2 History of systemic tumor necrosis factor as cancer therapy

After the first administrations of recombinant TNF to animals and humans, it became clear that high dose systemic TNF treatment is not possible due to serious

adverse events caused by TNF, namely endotoxic shock (96). This high toxicity limits the possible delivered systemic dose of TNF, and thus early phase I and II studies examining the anti-cancer effects of TNF were largely negative (97). However, later studies used TNF delivered in a locoregional way, where TNF is delivered close to the tumor with additional restriction of vasculature, limiting the systemic spread of TNF. Examples of the locoregional delivery systems include isolated limb perfusion (ILP) and isolated hepatic perfusion (IHP). Using these delivery mechanisms, almost tenfold higher doses of TNF could be used (98). However, TNF alone was not able to induce significant tumor reductions, but when combined with other cancer therapeutics (such as chemotherapeutics melphalan or doxorubicin), better responses were observed. In a phase II trial of patients with melanoma and sarcoma, high-dose TNF delivered via ILP and combined with IFN gamma (to induce upregulation of TNF receptors) and chemotherapy melphalan was able to induce 21 complete responses and two partial responses out of 23 patients treated (99). However, treatment with ILP and IHP are not curative for all patients, since distant metastases cannot be addressed with the therapy when delivered via these routes. Regardless, in 1999 the EMA approved recombinant TNF (tasonermin) for the treatment of soft tissue sarcoma, in combination with melphalan delivered with mild hyperthermic ILP. At present, recombinant TNF is a niche treatment modality of soft tissue sarcoma and melanoma, reserved for treatment of limb metastases with ILP, most commonly in combination with melphalan (100).

2.3.3 History of vectored tumor necrosis factor as cancer therapy

Due to the highly toxic effects of recombinant TNF, vectored delivery of TNF was of high interest during the early 2000s. These developments culminated with TNFerade, a non-replicative adenovirus vector where the expression of TNF is driven by an Egr-1 promoter, which is activated in cancer cells following radiation or cisplatin chemotherapy (101). Phase I trials of TNFerade showed impressive response rates in mixed solid tumors, sarcomas and head and neck cancer (102). Following a phase I/II study in locally advanced pancreatic cancer, TNFerade combined with fluorouracil chemotherapy and radiotherapy was compared to fluorouracil chemotherapy combined with radiotherapy in a phase III study of locally advanced pancreatic cancer (103). Sadly, the trial showed no improvement in overall survival time between the experimental arm and standard therapy, and further development of TNFerade was ceased (104).

The disappointment of the phase III trial of TNFerade after successful phase I trials was puzzling, but many lessons could be learned from the phase III trial of TNFerade. First, it was clear that administration of TNFerade to pancreatic tumors was challenging, due to the need of endoscopic equipment and specialized

personnel. Many of the phase I trials of TNFerade were conducted in tumor types where tumor injection was easier, such as head and neck cancers and melanoma. Second, since the TNF production by TNFerade was dependent on radiation of the tumor, or cisplatin chemotherapy, which was not included in the trial, it is likely that in many cases the radiation therapy was not able to induce TNF production in the tumors. Similarly, perhaps TNFerade therapy would have been better suited to cancers where tumor lesions are located more superficially, such as many head and neck cancers, or if TNFerade would have been combined to cisplatin therapy.

Regardless of the failed phase III trials, TNFerade motivated researchers to develop TNF-armed oncolytic viruses, where the problem of conditionally driven TNF expression would not exist. Arming oncolytic viruses with TNF has benefits beyond solely inducing cancer cells death, since TNF also promotes vascular permeability and attracts lymphocytes, thus enhancing the immunostimulatory effects of OV therapy (105, 106). Research in our laboratory demonstrated the potency of adding TNF to oncolytic adenovirus, with further synergy was observed when TNF arming was combined with IL-2 arming (107).

2.4 PREVIOUS TRIALS OF ONCOLYTIC ADENOVIRUS THERAPY IN HUMANS: LESSONS AND INNOVATIONS

Preclinical development of oncolytic adenoviruses is limited by available animal models. The most used preclinical cancer model, the laboratory mouse or Mus musculus, does not allow for adenovirus replication (108). This limitation forces scientists to choose between immune-competent models, where the virus does not replicate, or immune-compromised models with human tumors, where there is no functioning immune system, an important part of oncolytic adenovirus therapy. Solutions, such a humanized mouse models or cancer organoids circumvent some of these problems, but a relevant question of clinical translatability of findings in these settings still remain.

Due to these limitations in preclinical oncolytic adenovirus research, high importance is based on learning from early human trials of oncolytic adenoviruses. This part of the literature review aims to highlight some important oncolytic adenoviruses that have been used in humans.

2.4.1 H101 and ONYX-015

H101, also known as Oncorine, and ONYX-015, also known as dl1520, are two highly similar oncolytic adenoviruses. ONYX-015 represents the earlier form of the virus generated from an Ad2 and Ad5 chimera, and H101 represents the genetically engineered and manufactured Ad5 version used in clinic currently. Both are viruses

harbor deletions in the E1B gene leading to loss of production of 55 kDa protein, that inactivates p53 (109). In addition to the E1B deletion, H101 also harbors a partial deletion in the E3 region (110). Although initially thought to be specific to p53 mutated cells, later studies have suggested that ONYX-015 is able to replicate also in p53 wild-type cells, and the exact tumor specificity of ONYX-015 is most likely due to other effects of E1B-55kDa, such as late viral mRNA transport (111). ONYX-015 was the first oncolytic adenovirus to be widely tested in clinical trials, and thus ONYX-015 has provided valuable information on the use of oncolytic adenoviruses in humans.

Early trials of ONYX-015 focused on intratumoral dosing. These trials showed that administration of oncolytic adenovirus is safe in humans, even at relatively high intratumoral doses of $4x10^{12}$ viral particles. In terms of adverse events, intratumoral ONYX-015 treatment led to side effects resembling a transient flu infection including: fever, chills, asthenia and nausea (112). These effects were most likely due to immune activation caused by the agent. Additionally, as is common with intratumoral therapeutics, a common side effect of intratumoral ONYX-015 was injection site pain (113).

Additionally, ONYX-015 was trialed also in intravenous, intra-arterial, and intraperitoneal administration routes. These trials included even higher doses of up to 10^{13} viral particles, and fever and chills were experienced in almost all patients. Regarding intravenous dosing, liver tropism of adenoviruses was also present, with transient transaminitis occurring in two thirds of patients (114-116).

Early ONYX-015 trials also incorporated qPCR analysis of tumors and plasma, where virus could be detected 1-3 days post treatment, but by days 14-17 all samples were negative. These findings gave insights into the biology of oncolytic adenoviruses in humans: the human immune system was able to clear the virus quickly, a feat not present in immune-deficient animal models (112).

Regarding other correlative findings, the early ONYX-015 trials showed an expected increase in neutralizing antibodies against the virus in all patients, along with high amounts of pro-inflammatory cytokines being detected in serums after ONYX-015 therapy (112).

Although providing valuable information about biology of oncolytic adenoviruses in humans, the early ONYX-015 trials showed only a few responses to virus monotherapy. However, later studies of ONYX-015 combined with chemotherapy and checkpoint inhibitors showed more responses to the therapy (117, 118). H101 was approved in 2005 in China for the treatment of refractory nasopharyngeal cancer, combined with cisplatin and/or fluorouracil. Development of ONYX-015 continues to this day, in the form of Oncorine or H101, and multiple trials of Oncorine are underway (110).

2.4.2 DNX-2401

DNX-2401, also known as Delta-24-RGD, is an oncolytic adenovirus developed for the treatment of brain cancers. DNX-2401 is an oncolytic adenovirus based on the serotype 5, with 24 bp E1A deletion, and RGD-fiber (119). Development of DNX-2401 mirrored the challenges and concerns of oncolytic adenoviruses developed before, such as ONYX-015, and the construct included developments in specificity and targeting of the virus. DNX-2401 incorporates a 24 bp deletion in the adenoviral E1A gene, making the virus able to replicate only in cells deficient in the p16-Rb-E2F-pathway, a common pathway mutated in cancer. Normally, the adenoviral E1A protein binds Rb protein in cells, leading to E2F transcription and progress to cell cycle phase S, enhancing viral replication. 24 bp deletion in the E1A region abrogates the ability of E1A to bind Rb, and thus viruses with this deletion are able to replicate only in cells deficient in the p16-Rb-E2F pathway.

Additionally, DNX-2401 has a modified fiber knob to facilitate better cancer cell transduction (119). Normally, serotype 5 adenoviruses utilize the coxsackie and adenovirus receptor (CAR) to enter cells, but many cancers such as glioblastoma express relatively low levels of CAR. Thus, the fiber knob of DNX-2401 incorporates an arginine-glycine-aspartate (RGD) peptide, which allows the virus to utilize integrins for cell entry, thus enhancing infectivity against most cancer types, including glioma.

DNX-2401 has been trialed in multiple trials of brain cancer, mostly composing of recurrent glioblastoma and diffuse intrinsic pontine glioma. Results in these challenging-to-treat patients have been encouraging, with some patients receiving long-term responses of more than 3 years, in comparison to the normal expectation of overall survival of 12 months. DNX-2401 trials also incorporated post-treatment tumor sampling, where DNX-2401 spread could be seen, confirming preclinical studies on oncolytic adenovirus replication in brain tumors. Tumor specimens also showed marked infiltration of cytotoxic CD8+ T cells, confirming that treatment with oncolytic adenovirus can elicit lymphocyte activation also in more immune-protected organs such as the brain (120, 121).

DNX-2401 trials have also incorporated inventive ways of systemically administering DNX-2401 to the brain. One of these examples include loading mesenchymal stem cells with DNX-2401, following systematic administration of these cells intra-arterially via transfemoral endovascular intracranial injection. In addition, DNX-2401 has been studied in combination with temozolomide chemotherapy and checkpoint inhibitors, providing valuable information on the importance of order of combination treatments. DNX-2401 combined with temozolomide showed better efficacy over DNX-2401 only when temozolomide was administered after DNX-2401. Comparative tumor analysis of temozolomide before versus after DNX-2401 showed that pre-treating with temozolomide lead to decreased amounts of dendritic cells and CD8+ T cells in the tumors, most likely

due to dampened antiviral response when temozolomide was given before DNX-2401 (119, 122).

DNX-2401 combined with PD-1 inhibitor pembrolizumab completed a phase I/II trial in 2023. The trial showed that administration of DNX-2401 combined with pembrolizumab in glioblastoma was safe, however the trial failed to show statistical significance in response rate, although the response rate of 10.4% was more than double of prespecified control rate of 5%. In addition, the trial showed promising overall survival time, with 52.7% patients alive at 12 months, compared to prespecified rate of 20%, along with two patients with durable complete response (123).

Future development of DNX-2401 is currently ongoing, with future generations of viruses encoding immunostimulatory OX40L (Delta-24-RGDOX) and GITRL (Delta-24-GREAT) in preclinical evaluation (119).

2.4.3 ICOVIR-5

ICOVIR-5 is a third-generation version of the original 24 bp deleted adenovirus serotype 5 vector, and can be considered the next generation after DNX-2401. ICOVIR-5 was produced with the goal of generating a vector for systemic delivery, and in order to circumvent possible toxicity of high E1A expression, an E2F1 promoter was incorporated upstream of E1A, making the vector double specific for cells with mutations in the p16-Rb-E2F pathway (124).

After first preclinical studies published in 2007, ICOVIR-5 was first trialed in humans in an exploratory study of four children with metastatic and refractory neuroblastoma (125). In this trial, mesenchymal stem cells were collected from the patients' bone marrow, infected with ICOVIR-5, and re-infused back to the patients. Side-effects to the therapy were mild, and one of the four patients who received a durable complete response was alive at the time of the original report (125). A further developed version of ICOVIR-5 was also developed, named ICOVIR-7, with additional E2F-response palindromes in the E2F promoter, making the viral replication more specific for E2F overexpressing cells. ICOVIR-7 was tested as an intratumoral injection in 20 patients with mixed solid tumors in 2010, and the treatment showed good tolerability with some responses (126).

After a few years of silence, ICOVIR-5 continued into trials in solid tumors. In 2019, a trial of intravenous ICOVIR-5 in patients with metastatic melanoma was reported (127). Doses up to $1x10^{13}$ VPs were infused as a single dose, with $1x10^{13}$ VPs identified as the MTD due to grade 3 transaminitis present in two patients. No grade 4 adverse events were encountered. The trial showed ICOVIR-5 presence in biopsies by PCR in 4/11 patients evaluated, with virus detectable in the blood up to 26 days after infusion in some patients. Disease control was seen in 7/11 assessed patients on day 26, although no objective responses were observed. No further

publications of ICOVIR-5 have been published since 2021, and development has mostly focused of DNX-2401 and other developments of the ICOVIR-5 backbone.

2.4.4 VCN-01

VCN-01 is an oncolytic adenovirus, with a E2F promoter driving 24 bp deleted E1A expression, thus targeting cells mutant in the p16-Rb-E2F pathway. VCN-01 also incorporates the RGD-motif in the fiber knob, similarly to DNX-2401 and ICOVIR-5. Although sharing a similar backbone to ICOVIR-5, VCN-01 encodes a unique transgene in hyaluronidase (PH20), an enzyme targeted to break down stroma in tumors (128). This unique feature of VCN-01 is well rationalized, since many human tumors are rich in stroma, and this avid stroma inhibits the diffusion of cancer therapeutics inside tumors, leading to therapy resistance (129). VCN-01 has been developed for the treatment of stroma rich tumors, such as pancreatic and ovarian tumors, and completed phase I trials in advanced solid tumors and PDAC as monotherapy and in combination with nab-paclitaxel and gemcitabine (130, 131). In addition to a unique transgene, VCN-01 administration also used a high intravenous dose: up to $1x10^{13}$ viral particles were given intravenously, with good tolerability. In the PDAC phase I trials, nab-paclitaxel with gemcitabine was administrated per usual strategy after VCN-01 administration. Viral replication, as measured by PH20 in serum, was detected up to 28 days after single dose (130).

VCN-01 is currently in a randomized phase II trial for PDAC, comparing VCN-01 combined with nab-paclitaxel and gemcitabine to chemotherapy alone (132). In addition, VCN-01 is being trialed in combination with CAR-T therapy against mesothelin expressing cancers, such as ovarian and pancreatic cancer, showing promising efficacy in settings where no lymphodepleting chemotherapy or post-conditioning IL-2 is given (133).

2.4.5 ColoAd1

ColoAd1, also known as enadenotucirev, is an oncolytic adenovirus with a completely unique development history. ColoAd1 arises from a directed evolution experiment, where different cancer cell lines were infected simultaneously with a pool of different adenoviruses with different serotypes (134). Following 20 serial passages in vitro, ColoAd1 was selected from a colorectal adenocarcinoma cell line, showing over 1000-fold greater infectivity of the cells, when compared to wild-type adenovirus serotype 5. After sequencing, it was discovered that ColoAd1 was a chimeric virus between serotypes 11 and 3, with near-complete E3 deletion and a small E4 region deletion. After preclinical development ColoAd1 entered clinical trials (135-137).

Clinical trials of ColoAd1 also utilized an inventive administration scheme (138, 139). ColoAd1 trials focused on intravenous dosing to facilitate transduction of multiple metastases and to ease the clinical translation. The intravenous doses of ColoAd1 were administered on three consecutive visits, on days 1, 3 and 5. Following treatment with ColoAd1, patients underwent primary surgery (138). Tumor samples showed presence of the virus in select patients, up to 40 days after last intravenous dose. More recent studies combined ColoAd1 with PD-1 inhibitor nivolumab or chemoradiotherapy, but unfortunately responses in these trials were limited, although treatment was well tolerated (140, 141).

Development of ColoAd1 continues as an armed form of the virus. Further generations developed on ColoAd1 backbone include viruses encoding for CD40 agonistic antibody (NG-350A) and CD80 plus CD3 antibody (NG-348) (142, 143).

2.4.6 CG0070

CG0070, also known as cretostimogene grenaderepvec, is a serotype 5 oncolytic adenovirus, where the expression of early adenoviral genes is driven by a E2F promoter, making the virus specific to E2F overexpressing cells. Additionally, CG0070 encodes an immunomodulatory cytokine, GMCSF, aimed to enhance the immune effects of the therapy (144).

CG0070 began development in the mid 2000's, with a target tumor type of bladder cancer (144). Bladder cancer is an interesting and well-suited tumor indication for OVs, since although being a solid tumor and challenging to treat with current therapies, the bladder is easily accessible via urinary catheter, and thus high doses of virus can be delivered close to the tumor location without the need for intratumoral dosing (145). Additionally, bladder cancer is one of the few solid cancers where immunotherapy already has an established role: intravesicular BCG has been a standard treatment for localized bladder cancer for more than 50 years.

CG0070 completed the first phase I trials in BCG-failed non-muscle invasive bladder cancer in 2012, showing promising complete response rate of close to 50% (144). In addition to the unique administration route, CG0070 therapy also incorporates a unique pre-treatment scheme to optimize viral infection: bladders of patients are rinsed with 0.1% dodecyl maltoside, a mild detergent, to enhance virus penetration trough the mucosal layer of the bladder. After this rinsing, the bladder is washed with saline and CG0070 is administered (144).

CG0070 as a monotherapy has now entered phase III trials in BCG unresponsive non-muscle invasive bladder cancer, and phase II trials with combination of CG0070 with pembrolizumab have shown impressive complete response rates of over 57% at 12-months with good tolerability (146, 147).

2.5 BIOMARKERS OF ONCOLYTIC VIRUS THERAPY: A DEVELOPING FIELD

Following early trials of oncolytic viruses, it was clear that not every patient responded to therapy, but long-term responses could be seen in select patients. However, since early trials aimed to study the safety and preliminary efficacy, far less information was gained on the characteristics of the responses. After it was clear that not every patient benefitted from therapy, clinical trials also started to include investigations into patients who demonstrated treatment benefit. Earliest reports of these investigations were related to the mechanism of action of the agent: in a trial of ONYX-015 in patients with metastatic colorectal cancer, wild-type p53 status predicted better overall survival, a finding contradictory to the mechanism of action of ONYX-015 but possibly linked to the more aggressive nature of p53 mutated cancers (148). Similarly, but with non-contradictive results, early trials of CG0070 found that phosphorylated Rb was evident in almost all patients with complete responses (144). Numerous clinical trials have included in depth descriptions of patients with exceptional responses, but these reports were more anecdotal in nature rather than aiming to provide conclusions with wide applicability in the clinical use of OVs.

However, clinical trials from mid 2010s started to include progressively more investigations into different biomarkers and characteristics of responses. As information amassed, numerous fundamental questions of oncolytic virus therapy started to clarify. Reports showed that generally higher doses were beneficial, most likely due to the ability of higher doses to transduce more tumors, combating hepatic elimination and neutralizing antibodies present in the patients prior to administration (127, 149-151). Interestingly, based on preclinical evidence, the presence of neutralizing antibodies was thought to limit oncolytic virus efficacy. However, trial results have shown that presence or induction of neutralizing antibodies was not a hindrance to therapy, and in fact presence of neutralizing antibodies or production of neutralizing antibodies were often reported to correlate with beneficial clinical results (120, 122, 130, 152-156). Additionally, production of both anti-tumoral and anti-viral responses, measured with either flow cytometry or ELISpot based methods, correlated with better clinical results (157-160). These results provided compelling evidence to the multifaceted mechanism of action of oncolytic viruses: agents first thought to solely derive their effect from oncolytic elimination of cancer cells, turned out to be potent immune activators when administrated to humans.

Regarding patient demographics, oncolytic virus clinical trials have enrolled patients from most different solid and liquid cancers. Responses have been seen in most types, and further development from phase I trials to later stage trials has mostly been driven by a clinical need in the chosen cancer type. However, these

studies have been able to show that patients with less advanced disease and with fewer prior treatment lines (ICI or chemotherapy) respond better to oncolytic virus therapy (154, 155, 161-168).

In the 2010s and 2020s, as sequencing technologies and other assays developed, these were also utilized to study the molecular underpinnings behind responses in oncolytic virus trials more in depth. Due to marked expansion in possibilities to study virtually every single gene, RNA or protein in immune cells, cancer cells or in soluble body mediums, a collection of different biomarkers for response were reported. Regarding tumor mutations, *AKT1, ATM, P53, PIK3CA, PTEN* and *STK11* mutations seemed to correlate with favorable clinical outcomes, however the difference in underlying disease pathology and treatment sensitivity could not be dissected in these early phase trials (169, 170).

Regarding intratumoral transcriptomics, higher expression of *CD3, FLG, GJB6, IFIT2, KRT64, KRTDAP, LAG3, PBK, PDCD-1, PDL1, SBSN* and *STAT1* pre-treatment have been reported to correlate with better responses to oncolytic virus therapy (123, 160, 167, 171). On a more holistic level, comprehensive IHC profiling combined with transcriptomic evaluation has shown that responders to oncolytic virus therapy show more pro-inflammatory TME, with increased infiltration of CD8+ T cells and expression of pro-inflammatory gene pathways (122, 123, 141, 150, 171-176). These intratumoral findings support the notion that the mechanism of action of oncolytic viruses is as much, if not mostly, tied to the pro-inflammatory and lymphocyte attracting properties of the agents, compared to pure oncolysis.

Analysis of intratumoral samples provide valuable information regarding the oncolytic virus therapy, but collecting intratumoral biopsy samples in the clinic can be challenging and time consuming. Additionally, a single biopsy from a single tumor most likely doesn't accurately describe the whole tumor, not to mention the other metastases present in the patient. For these reasons, peripheral blood is of major interest for biomarker development. Venous blood samples are readily available from patients, and immune cell subsets and soluble protein levels are relative stable across the circulating blood system. Of the circulating blood subsets, the peripheral blood mononuclear cell (PBMC) compartment is the most studied, as it contains most lymphocyte populations in addition with innate immune cells such as monocytes. Analysis of PBMCs has shown favorable response in patients with more activated and memory-like CD8+ and CD4+ T cells after therapy (157, 171, 177, 178). More in depth analysis of these PBMCs has shown that higher clonality (less unique TCRs/BCRs) also correlates with favorable clinical outcomes, possibly arising from generation of anti-viral and/or anti-tumor lymphocyte clones (179, 180).

Regarding serum proteins, higher post-treatment amounts of serum IL6, IL8, VEGFA, RANTES and fractalkine have been associated with poor clinical outcomes in OV therapy (177). Conversely, higher post-treatment amounts of pro-

inflammatory mediators IFNβ, IL23, CCL22 and CXCL10 have been associated with response to OV therapy (153, 172, 177, 181). Serum proteomic findings are well suitable for clinical use, since kits available for detection of different proteins are readily available, but in-depth mechanistic conclusions are difficult to form from single protein analytes.

In conclusion, the field of OV therapy has progressed far from preclinical models focusing on pure oncolytic potential. Biomarkers of response discussed previously arise from multiple different trials with different cancer indications, viruses, dosing schemes and combination therapies. Thus, much work is still needed to accurately decipher the mechanism of different oncolytic treatments and to find optimal combinatorial treatments with different forms of OV therapy.

3 AIMS OF THE STUDY

The aims of this study are:

1. To evaluate the combination of oncolytic adenovirus producing a modified IL-2 molecule with standard chemotherapy of pancreatic cancer in a pre-clinical setting (I).

2. To study the combination of oncolytic adenovirus producing a modified IL-2 molecule, with allogenic NK cell therapy for ovarian cancer in a pre-clinical setting (II).

3. To assess the safety, pharmacokinetics, pharmacodynamics, and efficacy of oncolytic adenovirus producing IL-2 and TNF in a human phase I trial (III).

4. To assay the mechanism of action and correlates of response of oncolytic adenovirus producing IL-2 and TNF in humans (IV).

4 MATERIALS AND METHODS

4.1 Virus constructs

4.1.1 Ad5/3-E2F-d24-vIL2

In studies I and II, an oncolytic adenovirus Ad5/3-E2F-d24-vIL2, also known as TILT-452, was used. Ad5/3-E2F-d24-vIL2 is an oncolytic adenovirus based on the adenovirus serotype 5 backbone, with the fiber knob changed to serotype 3 knob, to facilitate better cancer cell entry, intravenous delivery and protection from neutralizing antibodies (182, 183). Ad5/3-E2F-d24-vIL2 harbors a E2F promoter upstream of early adenoviral genes, in order to facilitate replication only in cells with E2F overexpression, which is common in cancer cells (184). Additionally, the virus harbors a 24 bp deletion in the adenoviral E1A gene, making the virus able to replicate only in cells with defects in the Rb-p16 pathway, a pathway commonly mutated in cancer cells (185, 186). Furthermore, the E1B/19k gene is deleted, to induce enhanced apoptosis of cancer cells by limiting the anti-apoptotic effects of normal E1B/19k (187).

Ad5/3-E2F-d24-vIL2 encodes a modified interleukin-2 transgene. In the virus, the interleukin-2 molecule harbors five amino acid modifications, specifically L80 to F, L81 to D, L85 to V, I86 to V and I92 to F. These mutations lead to preferential binding to the gamma-subunit of the human interleukin-2 receptor.

Ad5/3-E2F-d24-vIL2 construct was produced in SW102 cells utilizing GalK and AmpR mediated recombineering. Plasmids containing the virus were expanded in DH5α cells (Thermo Fisher Scientific), and viral genomes were released with PacI digestion. Viral genomes were transfected to A549 cells using lipofectamine, and concentrated viral preps were produced with CsCl ultracentrifugation and dialysis.

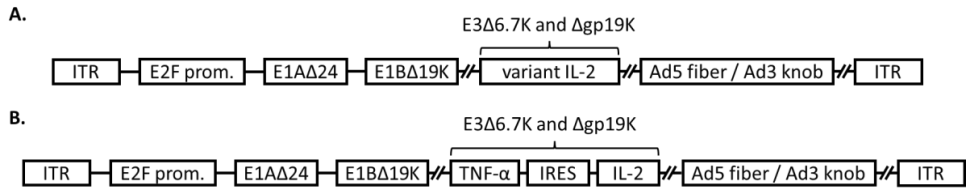

Figure 2. Genomic constructs of viral vectors used in the study. A. Construct of Ad5/3-E2F-d24-vIL2 (TILT-452). B. Construct of Ad5/3-E2F-d24-TNFα-IRES-IL-2 (also known as TILT-123). Both vectors are built upon adenovirus serotype 5 backbone.

4.1.2 Ad5/3-E2F-d24-hTNF-IRES-hIL2

In studies III and IV, Ad5/3-E2F-d24-hTNF-IRES-hIL2, also known as TILT-123, was used. TILT-123 shares the same backbone as TILT-452, but the vIL2 transgene is replaced with an expression cassette producing TNF and IL-2. The genetic elements of TNF and IL-2 are separated with an IRES-linker, ensuring balanced expression of both transgenes. TILT-123 was generated in the same method as TILT-452. For clinical trial usage, TILT-123 was produced in an approved GMP-facility. Graphical representation of TILT-452 and TILT-123 are shown in Figure 2.

4.2 Preclinical models of cancer

4.2.1 In vitro models of human cancer and other models

4.2.1.1 Cell lines

In study I, a panel of human PDAC cell lines were utilized to assess replication of Ad5/3-E2F-d24-vIL2 in human PDAC. Additionally, in study I, mouse PDAC cell line Panc02 and mouse CRC cell line MC-38 were used for in vivo studies. In study II, cell line derived from human high-grade serous ovarian cancer was similarly cultured in vitro prior to in vivo inoculation. Virus was produced in A549 lung adenocarcinoma cells in all studies. In study I, mouse CTLL-2 cell line was used to test potency of virally produced transgene. Cell lines used, their origin, growth media and study are denoted in Table 2.

Table 2. Cell lines used in this thesis work.

Cell line	*Species*	*Cancer type*	*Growth media*	*Origin*	*Study*
Panc-1	Homo sapiens	PDAC	DMEM, 10% FBS, 1% PS, 1% LG	ATCC	I
Capan-2	Homo sapiens	PDAC	DMEM, 10% FBS, 1% PS, 1% LG	ATCC	I
BxPC-3	Homo sapiens	PDAC	RPMI, 10% FBS, 1% PS, 1% LG	Gift from Hanna Seppänen	I
MIA PaCa-2	Homo sapiens	PDAC	DMEM, 10% FBS, 1% PS, 1% LG	Gift from Hanna Seppänen	I
HPAF-II	Homo sapiens	PDAC	DMEM, 10% FBS, 1% PS, 1% LG	Gift from Hanna Seppänen	I
PDX-OvCa	Homo sapiens	HGSOC	RPMI, 10% FBS, 1% PS, 1% LG	Helsinki University Hospital	II
A549	Homo sapiens	NSCLC	DMEM, 10% FBS, 1% PS, 1% LG	ATCC	I-IV
Panc02	Mus muculus (C57BL/6)	PDAC	RPMI, 10% FBS, 1% PS, 1% LG	Gift from Kayoko Hosaka	I
MC-38	Mus muculus (C57BL/6)	CRC	RPMI, 10% FBS, 1% PS, 1% LG	ATCC	I
CTLL-2	Mus muculus (C57BL/6)	TLL	RPMI, 10% FBS, 10% T-STIM, 1% PS, 1% LG	ATCC	I

4.2.1.2 Patient-derived histocultures

In study II, surgical ovarian cancer samples were used to study clinical human ovarian cancers. Resected samples were collected in 10% FBS RPMI, transported to the laboratory, dissected to 1-2 mm pieces using scalped and forceps, and incubated in an enzyme mix consisting of 170 mg/l collagenase type I, 170 mg/l collagenase type IV, 25 mg/ml DNaseI and 25 mg/ml elastase. Samples were incubated in the mix until sufficient breakdown of tissue was observed, or overnight. Samples were filtered through 70 µm filter, washed and centrifuged, and stored in -140'C until analysis. For analysis effects of virus therapy and NK cell therapy on human ovarian samples, histocultures were established from frozen samples by seeding 50 000 cells per well in RPMI, 10% FBS, 1% PS, 1% LG, followed by 24-hour rest, after which samples were treated with virus and/or NK cells.

4.2.2 Animal models of human cancer

4.2.2.1 C57BL6 (Panc02, pancreatic ductal adenocarcinoma)

In study I, to study the effects of Ad5/3-E2F-d24-vIL2 combined with nano albumin bound-paclitaxel and gemcitabine in an immunocompetent setting, 1 x 10^6 Panc02 cell were subcutaneously injected to 5-week-old C57BL/6J female mice. After initial tumor growth of diameter to 3 to 4 mm, mice were randomized to different treatment groups, consisting of mock treatment, Ad5/3-E2F-d24-vIL2 treatment (3 x 10^9 viral particles i.t. every two days), chemotherapy (nano albumin bound-paclitaxel 50 mg/kg every 7 days and gemcitabine i.p. 100 mg/kg every 7 days), or combination of virotherapy and chemotherapy. Mice were treated for 50 days, after which treatment was ceased and tumor growth followed.

4.2.2.2 NOD.Cg-Prkdc^{scid}Il2rg^{tm1Sug}/JicTac (PDX-OvCa, ovarian high-grade serous carcinoma)

In study II, to study the effects Ad5/3-E2F-d24-vIL2 combined with allogenic NK cell therapy, 3.5 x 10^6 PDX-OvCa cells were subcutaneously injected to 5-10 week old NOD.Cg-PrkdcscidIl2rgtm1Sug/JicTac female mice. After initial tumor growth to diameter of 5 to 6 mm, animals' immune systems were humanized by intraperitoneal injection of 5x10^6 autologous PBMCs from the PDX-OvCa patient. After humanization, animals were randomized to different treatment groups, consisting of mock treatment, NK cell treatment (1 x 10^7 allogenic NK cells i.p., on day 1), Ad5/3-E2F-d24 or Ad5/3-E2F-d24-vIL2 (both 1 x 10^9 viral particles i.t., on days 0, 3, 6 and 9). Tumor growth was followed every 1 to 2 days. Animal were euthanized on day 12 due to signs of graft-versus-host disease, and samples were collected for analysis.

4.3 Clinical trial and patients

4.3.1 Trial aim, timeline and collected samples

Studies III and IV are based on research conducted within TUNIMO (NCT04695327) clinical trial. TUNIMO was a single-arm, dose-escalation, open-label, phase I trial conducted in two cancer centers in Finland, Docrates Cancer Center and HUS Comprehensive Cancer Center. The primary aim of TUNIMO was to study the safety of TILT-123 treatment in humans. Secondary aims included maximum tolerable dose of TILT-123, immune effects of TILT-123, virus persistence, virus shedding and efficacy.

In TUNIMO, patients received one intravenous dose of TILT-123 followed with five intratumoral doses. Both intravenous and intratumoral doses were escalated across cohorts, as shown in Table 3.

Table 3. Dose escalation scheme of TUNIMO trial.

Cohort (patients enrolled)	*Intravenous dose, VP*	*Intratumoral dose, VP*
1 (2)	$3x10^9$	$3x10^9$
2 (4)	$3x10^{10}$	$3x10^{10}$
3 (3)	$3x10^{11}$	$1x10^{11}$
4 (3)	$1x10^{12}$	$3x10^{11}$
5 (3)	$2x10^{12}$	$3x10^{11}$
6 (5)	$4x10^{12}$	$5x10^{11}$

During TUNIMO, samples from patients were collected in order to study the mechanism of action of TILT-123. These samples included biopsies, whole blood, serum and PBMCs. TUNIMO trial timeline and collected samples used in studies III and IV are shown in Figure 3.

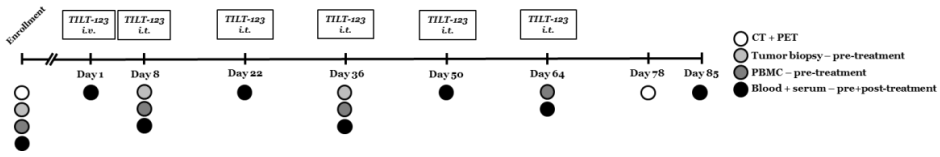

Figure 3. TUNIMO (NCT04695327) trial treatment scheme, samples collected and imaging.

4.3.2 Enrollment criteria

TUNIMO trial included multiple inclusion and exclusion criteria. Inclusion criteria for TUNIMO included age over 18 years, solid tumor that had failed standard therapy or no standard therapy existed or was unlikely to result in meaningful clinical benefit. At least one tumor had to more than 14 mm in diameter to facilitate biopsy analysis and intratumoral treatment. Patients had to have adequate hepatic and renal function (platelet count more than 75 000 /mm^3, hemoglobin more than 100 g/l, AST and ALT less than 3 times the upper limit of normal, bilirubin less than 1,5 time the upper limit of normal, eGFR more than 60 ml/min, leukocyte count more than $3x10^9$). Patients had to adhere to adequate form of contraception. The performance score as assessed by WHO/ECOG criteria had to be 0 or 1 at screening, with a life expectancy longer than 3 months.

Exclusion criteria for TUNIMO included immunosuppressive drugs, such as corticosteroids more than 20 mg daily prednisone or biologics. Patients had to have 30 days without any cancer therapy prior to first injection. Palliative radiation therapy was not allowed 14 days before enrollment. Other exclusion criteria included uncontrolled cardiac/vascular disease, myocardial infarction or cerebral stroke with 12 months, history of severe hepatic dysfunction, hepatitis or HIV, history of coagulation disorder, current pregnancy or breastfeeding, untreated brain metastases or treated brain metastases that had progressed 3 months prior to screening, or allergy to ingredients present in TILT-123 formulation.

4.3.3 Patients

Research presented in studies III and IV is based on patients enrolled to the TUNIMO trial. In total, 30 patients were evaluated for enrollment, and 20 were enrolled after fulfilling enrollment criteria. The 20 patients are shown in Table 4.

Table 4. Patient characteristics of TUNIMO trial.

Patient	*Cohort*	*Age*	*Sex*	*ECOG*	*Tumor type*	*Stage*
20202	1	65	F	1	NSCLC	IV
20203	1	60	F	1	Melanoma of unknown primary	IV
20204	2	50	M	0	Myxoid liposarcoma	IV
20205	2	64	F	1	Ovarian serous cystadenocarcinoma	IV
20101	2	52	F	1	Breast carcinoma, HER2+, ER-, PR-	IV
20206	2	71	F	1	High grade serous ovarian carcinoma	IIIC
20103	3	47	M	0	Anaplastic thyroid carcinoma	IV
20102	3	38	M	1	Rhabdomyosarcoma	IV
20211	3	66	F	1	Cutaneous melanoma	IV
20212	4	63	F	1	Leiomyosarcoma	IV
20104	4	56	M	1	Leiomyosarcoma	IV
20107	4	51	M	0	Chondrosarcoma	IV
20213	5	66	M	1	Neuroendocrine carcinoma of the bladder	IV
20108	5	52	F	1	Adenoid cystic carcinoma	IV
20214	5	72	F	1	Mucinous carcinoma of the appendix	IV
20216	6	33	F	1	Cutaneous melanoma	IV
20109	6	61	F	0	Leiomyosarcoma	IV
20217	6	51	F	1	Myxoid liposarcoma	IV
20219	6	42	M	1	High grade mucoepidermoid carcinoma of the parotid gland	IV
20111	6	63	F	0	High grade serous carcinoma of the peritoneum	IV

4.3.4 Safety evaluation

In study III, safety of TILT-123 therapy in humans was evaluated with adverse events. Patients were monitored on each treatment visit, vital signs were documented and safety laboratory tests were performed on each visit before and after TILT-123 administration. All AEs were recorded according to CTCAE 5.0 adverse event documentation guidelines. Additionally, ECGs were performed to assess any cardiac effects of TILT-123.

4.3.5 Response evaluation

4.3.5.1 CT and FDG-PET

In studies III and IV, patient response to TILT-123 therapy was assessed with contrast enhanced CT and FDG PET on day 78 of the trial. Regarding CT, RECIST 1.1 and iRECIST criteria were used to classify patient response (188, 189). Regarding PET, a criterion shown in Table 5 was used to assess response.

Table 5. PET evaluation criteria used in TUNIMO trial.

Response to therapy	*Radiographical finding*
Complete metabolic response (CMR)	Complete resolution of FDG activity within measurable lesions and all reliably assessable lesions to background levels. No new PET-avid lesions in pattern typical of cancer*. Lymph nodes may remain metabolically active due to immune response.
Partial metabolic response (PMR)	> 30 percent decrease in FDG activity measured as the summed SUVmax of measurable lesions (up to 5 lesions, max 2/organ). No new PET-avid lesions in pattern typical of cancer*.
Minor metabolic response (MMR)	10-29 percent decrease in FDG summed SUVmax. No new PET-avid lesions in pattern typical of cancer*.
Stable metabolic disease (SMD)	0-9 percent decrease or up to < 30 percent increase in FDG summed SUVmax. No new PET-avid lesions in pattern typical of cancer*.
Progressive metabolic disease (PMD)	≥ 30 percent increase in FDG summed SUVmax in pattern typical of tumor, or new clearly PET-avid clinically significant lesions in pattern typical of cancer*. Increase in metabolic activity in lymph nodes should not result in PMD if no progression is detected elsewhere, since it might reflect immunological activation and not progression.

** "clinically significant lesions in pattern typical of cancer" defined as lesions associated with a CT abnormality most consistent with cancer (≥ 2 cm in diameter), and clearly not because of inflammation of infection or related to treatment response.*

4.3.5.2 Overall survival, progression-free survival, and time to progression

In studies III and IV, response to TILT-123 therapy was also studied by assessing patient survival. Additionally, in study III, progression-free survival and time to progression were calculated, to assess the duration of response to TILT-123. Regarding overall survival, patient survival was calculated from enrollment to death or censoring, if patient was alive at data cutoff. Progression-free survival was calculated from enrollment to disease progression or death, depending on which occurred earlier, or the patient was censored if the patient was alive without progression at data cutoff. Time to progression was calculated from enrollment to disease progression, or patient was censored if progression did not happen before data cutoff time.

4.4 Combinational therapeutics in animal models of human cancer

4.4.1 Chemotherapeutics

In in vivo model of study I, Ad5/3-E2F-d24-vIL2 was combined with standard chemotherapy of PDAC used in humans, nano albumin-bound paclitaxel and gemcitabine. Nano albumin-bound paclitaxel (Bristol Myers Squibb, NY, USA) was diluted in 0,9% sterile saline, and administered to mice intraperitoneally with a dose of 50 mg/kg, once a week. Animal weight was measured prior to administration, and dose was calculated according to weight.

Additionally, in vivo model of study I, gemcitabine (Accord Healthcare, UK) was diluted in 0,9% sterile saline, and administrated to mice intraperitoneally with a dose of 100 mg/kg, once a week. Similarly to nano albumin bound-paclitaxel, animal weight was measured prior to administration and dose calculated accordingly.

4.4.2 Adoptive NK cell therapy

In study II, allogeneic NK cell therapy was used to study the combination of Ad5/3-E2F-d24-vIL2 to NK cell therapy. To produce NK cells, blood samples from healthy donors (Finnish Red Cross, Finland) were used to extract PBMCs using Ficoli-density based centrifugation followed by ACK lysis and storage in -140'C prior to usage. Later, NK cells were expanded from frozen samples using NK cell activation and expansion kit (Miltenyi Biotec, Germany) for 18 days. Following expansion, NK cells were isolated using NK cell isolation kit (Miltenyi Biotec, Germany). NK cells were not frozen after isolation, and used fresh for in vitro and in vivo studies, and the same donor NK cells were used for all in vitro and in vivo studies.

4.5 Biological assays

4.5.1 Cell death analysis

4.5.1.1 MTS analysis

In study I, for assessment of human PDAC cell line sensitivity to infection by different virus constructs, human pancreatic cancer cell lines were seeded in flat-bottom 96-well plates in concentration of 10 000 cells per well. After 24 hours of incubation, medium was aspirated, and different viruses were added in 1 to 1000 MOI, and paclitaxel and gemcitabine were added in concentration of 1 to 1000 nM.

Cells were incubated for 3 to 7 days, after which medium was aspirated, and CellTiter 96 Aqueous MTS reagent (Promega) was added in 20% diluted to original medium. Cells were incubated for 2 hours, after which absorbance was assessed with 96-well plate reader (Hidex) at absorbance of 490 nm. Controls without cells, virus or chemotherapeutics were used.

4.5.1.2 Real time cytotoxicity analysis

For real time cytotoxicity analysis used in study II, frozen ovarian cancer samples were thawed, centrifuged and resuspended, and added to xCELLigence Real-Time Cell Analysis (RTCA) 16-well plates (Agilent), pre-coated with 5 µg/ml of fibronectin. 50 000 cells were added per well, allowed to grow for 24 hours, after which expanded allogeneic NK cells were added on top in in ratio of 8 effector cells per one cancer cell, with or without addition of viruses at 100 MOI. Cell impedance was recorded for 200 hours post original ovarian sample seeding. Controls without NK cells and/or virus were used.

4.5.2 Immunogenic cell death analysis

In study I, the effect of virus and chemotherapeutic combination to immunogenic cell death was assessed with release of ATP to cell culture supernatant and expression of calreticulin in treated cells. 100 000 Panc-1 cells were seeded to 12-well plates, allowed to rest for 24 hours, after which Ad5/3-E2F-d24-vIL2 was added with or without paclitaxel or gemcitabine. After 48 hours post-treatment, supernatant was collected, and extracellular ATP amounts were determined with ATP Determination Kit (Thermo Fisher). Similarly, after 48 hours cells were detached from plates using trypsin and stained with anti-calreticulin antibody (PA3-900, Thermo Fisher) and secondary donkey anti-rabbit antibody conjugated to AF750 (A-21039, Thermo Fisher). Amounts of cells expressing calreticulin were assessed with Novocyte Quanteon flow cytometer (Agilent).

4.5.3 Safety laboratory analysis

In studies III-IV, safety of TILT-123 therapy was assessed with safety laboratory testing. Peripheral venous blood samples were collected on each visit before therapy, and 16 hours post-therapy. From the samples, a complete blood count was done, along with assays analyzing liver status and function (INR, aPTT, bilirubin, ALT, AST, ALP), kidney function and electrolytes (creatinine, sodium, potassium) with addition of LDH.

4.5.4 Virus distribution analysis

In study III, TILT-123 distribution was assessed with qPCR targeting the viral IRES-IL2 region, not present normally in human genome. Peripheral whole blood samples, tumor samples, saliva, urine and feces samples were collected, from which DNA was extracted with MagMAX DNA isolation kit (Thermo Fisher). qPCR was run with QuantStudio 7 Flex (Thermo Fisher). Acquired CT values were transformed to VP per unit volume or weight using standard curves.

4.5.5 Immunohistochemistry analysis

4.5.5.1 Tissue preparation

In studies I-IV, mouse or human tissues were prepared for immunohistochemistry by submerging collected samples to 10% formalin for 24 hours, followed by dehydration and storage in 70% ethanol before preparation of tissue blocks. To prepared paraffin embedded tissue blocks, samples were subjected to increasing concentrations of ethanol, followed by xylene substitution followed by addition of paraffin wax. Paraffin blocks were sectioned for staining using a microtome.

4.5.5.2 Hematoxylin and eosin staining

In studies I-IV, human or mouse tissue were stained with hematoxylin and eosin staining to study tissue morphology and cancer type. Following preparation of paraffin sections, sections were mounted on glass slides and deparaffinized, followed with hematoxylin and eosin staining. Stained samples were digitized with a slide scanner. Scanned slides or original slides were analyzed by qualified pathologist or veterinary pathologist.

4.5.5.3 Multiplex IHC

In studies III-IV, multiplexed IHC was used to assess immune cell infiltration and status in collected biopsies. Paraffin sections were stained with a two-step staining protocol, using antibodies shown in Table 6 and described more in depth previously (190). Stained samples were scanned with Zeiss Axioscan Z1 (Carl Zeiss) and cell amounts were quantified with CellProfiler version 4.2.5.

Table 6. Antibodies and conjugations used in immunohistochemistry.

IHC PANEL A (Studies III-IV)			
Target	**Clone/Catalog number**	**Secondary antibody**	**Staining round**
CD56	156R-94	TSA-488	1
CD8	M7103	TSA-555	1
PD1	LSB12784	AlexaFluor 647	1
Granzyme B	Ab4059	AlexaFluor 750	1
CD45	M0701	AlexaFluor 647	2
Epi	CST 3195	AlexaFluor 750	2
Cytokeratin	ab9377	AlexaFluor 750	2
IHC PANEL B (Studies III-IV)			
Target	**Clone/Catalog number**	**Secondary antibody**	**Staining round**
CD4	ab133616	TSA-488	1
CD20	MS-340	TSA-555	1
FoxP3	ab20034	AlexaFluor 647	1
Granzyme B	ab4059	AlexaFluor 750	1
CD45	M0701	AlexaFluor 647	2
Epi	CST 3195	AlexaFluor 750	2
Cytokeratin	ab9377	AlexaFluor 750	2
IHC PANEL C (Studies III-IV)			
Target	**Clone/Catalog number**	**Secondary antibody**	**Staining round**
CD68	168M-94	TSA-488	1
CD11c	ab52632	TSA-555	1
PDL1	CST 13684	AlexaFluor 647	1
CD45	M0701	AlexaFluor 750	1
CD16	CM 116R-14	AlexaFluor 647	2
Cytokeratin	ab7753	AlexaFluor 750	2
Cytokeratin	MA5-13156	AlexaFluor 750	2
E-Cadherin	BD 610182	AlexaFluor 750	2

4.5.5.4 Monochrome IHC analysis

Monochrome IHC was used in study II to assess amounts of immune cells and PDL1 in collected ovarian cancer samples. After staining with antibodies targeting CD4 (104R-16, Cell Marque), CD8 (NCL-CD8-4B11, N-C), CD56 (156R-96, Cell Marque) and PDL1 (741-4860, Roche), samples were analyzed by a trained pathologist and cell counting was performed. Monochrome IHC targeting the E1A was used in study III to assess TILT-123 positivity in collected samples.

4.5.6 Neutralizing antibody analysis

In study III, neutralizing antibody responses against TILT-123 were studied by a neutralizing antibody assay. Serum from patients was collected and stored in -20'C prior to analysis. To analyze the amount of neutralizing antibodies in serum, 10 000 A549 cells were seeded to white-walled flat bottom 96-well plates in medium without serum. 24 hours later, patient serum in dilutions from 1:64 to 1:16384 was mixed with Ad5/3-Luc1 and incubated at room temperature for 1 hour, followed by addition of the mix to A549 cells. Cells were incubated with serum-virus mix for 1 hour, followed by addition of 10% DMEM medium, after which cells were incubated for 24 hours. After this, Reporter lysis buffer (Promega) was added to cells, followed by a freeze-thaw cycle and addition of Luciferase Assay Reagent (Promega) and record of luminescence with a luminometer (Hidex). The lowest serum sample dilution able to neutralize 80% of the signal compared to non-serum well was considered as the neutralizing titer.

4.5.7 Cytokine analyses

4.5.7.1 Bead-based assays

In study I, two different bead-based cytokine kits were: CBA (BD Biosciences) and LegendPlex (Biolegend). CBA was used to assess and compare cytokine production of Ad5/3-E2F-d24-vIL2 and Ad5/3-E2F-d24-hIL2, whereas LegendPlex was used to assess effects of virus treatment to intratumoral cytokine amounts. Both assays were conducted according to manufacturer's recommended protocol, and bead samples were acquired with BD Accuri C6 (BD Biosciences) or Novocyte Quanteon (Agilent) flow cytometers.

4.5.7.2 Proximity extension assay

For studies III-IV, a proximity extension based assay (Olink Target 96 Immuno-Oncology, Thermo Fisher) was used to study cytokine amounts in patients serum. Frozen serum samples were thawed, and randomized to 96-well PCR-plates, and Olink Target 96 Panel was performed with Fluidigm Biomark HD (Standard BioTools). Different plate batches were normalized to with bridging samples utilizing R package Olink Analyze v3.8.2.

4.5.8 Flow cytometry analysis

Flow cytometry was used in studies I and II to study effects of virotherapy to immune cells and other intratumoral cell types. Additionally, in study I flow

cytometry was used to assess different states of cell cycle after cancer cell infection with virus and treated with paclitaxel. In study IV, flow cytometry was performed to study the effects of TILT-123 therapy on memory subsets in PBMC samples collected from patients.

Flow cytometry was performed with extracellular or intracellular staining protocol, depending on the location of target antigen. Prior to staining with antibodies, Fc-receptors on cells were blocked with human or mouse Fc-block (BD Biosciences). Following blocking, cells were stained with antibodies listed in Table 7. For intracellular staining, cells were permeabilized and fixed with Transcription Factor Buffer Kit (BD Biosciences, NJ, USA). For dead cell discrimination, 7AAD or PI was utilized.

Table 7. Antibodies and conjugations used for flow cytometric analysis.

Study I				
Target	**Fluorochrome**	**Clone**	**Catalog**	**Producer**
DNA	7AAD	-	420404	Biolegend
Ki-67	AF488	B56	561165	BD Biosciences
H3Ser10	AF488	11D8	650804	Biolegend
Calreticulin	Non-conjugated	Polyclonal	PA3900	Thermo Fisher Scientific
Anti-Rabbit IgG (H+L)	AF750	-	A-21039	Thermo Fisher Scientific
CD45	AF700	30-F11	560510	BD Biosciences
CD3	BV786	17A2	564010	BD Biosciences
CD4	FITC	GK1.5	100406	Biolegend
CD8	BV510	53-6.7	100752	Biolegend
CD25	BV605	PC61	563061	BD Biosciences
FOXP3	BV421	MF23	562996	BD Biosciences
NK1.1	PE-CF594	PK136	562864	Biolegend
PD1	PE	RMP1-30	566831	BD Biosciences
LAG3	APC	C9B7W	562346	BD Biosciences
Perforin	PE	S16009A	154306	Biolegend
EpCAM	AF488	G8.8	118210	Biolegend
PDPN	APC-Cy7	8.1.1.	127418	Biolegend
PDGFR (CD140a)	PE	APA5	135905	Biolegend
CD45	V500	30-F11	561487	BD Biosciences
Ly6C	APC	HK1.4	128016	Biolegend
MHC-II (I-A/I-E)	BV785	M5/114.15.2	107645	Biolegend
CD62L	Pacific Blue	MEL-14	104424	Biolegend
CD44	APC-Cy7	IM7	560568	BD Biosciences
CD69	BV605	H1.2F3	104530	Biolegend
Study II				
Target	**Fluorochrome**	**Clone**	**Catalog**	**Producer**
EpCAM	FITC	9C4	324204	Biolegend

MICA/B	APC	6D4	320908	Biolegend
HLA-ABC	PE-Cy7	W6/32	25-9983-42	Thermo Fisher Scientific
HLA-E	PE	3D12HLA-E	12-9953-42	Thermo Fisher Scientific
CD155	AF700	SKII.4	337630	Biolegend
CD112	PerCP-Cy5.5	TX31	337416	Biolegend
DNA	PI	-	421301	Biolegend
CD3	AF700	SK7	344822	Biolegend
CD4	BV570	RPA-T4	300534	Biolegend
CD8	BV510	RPA-T8	563256	BD Biosciences
PD1	APC-Cy7	EH12.2H7	329922	Biolegend
CD25	BV711	M-A25	356138	Biolegend
FoxP3	PE-Dazzle594	206D1	320126	Biolegend
Granzyme B	PE	GB11	561142	BD Biosciences
EpCAM	PE-Dazzle594	9C4	324232	Biolegend
CD56	BV510	HCD56	318340	Biolegend
CD158b	FITC	DX27	312604	Biolegend
Study IV				
Target	**Fluorochrome**	**Clone**	**Catalog**	**Producer**
DNA	7AAD	-	420404	Biolegend
CD3	BV711	UCHT1	563725	BD Biosciences
CD4	BV605	RPA-T4	562658	BD Biosciences
CD8	BV510	SK1	563919	BD Biosciences
CD45RA	AF700	HI100	560673	BD Biosciences
CD197	PE-Cy7	G043H7	353226	Biolegend

4.5.9 Transcriptomic analysis

In study IV, transcriptomic analysis of immune-related mRNA transcripts was performed on biopsies collected before and after TILT-123 therapy. mRNA was extracted from biopsies using RNA purification kit (Macherey-Nagel, Germany). mRNA transcript amounts were quantified with nCounter PanCancer Immune Profiling Panel (Nanostring, WA, USA), with additional probes targeting the adenovirus serotype hexon sequence, 24 bp deleted E1A sequence, and adenovirus serotype 3 fiber sequence. mRNA counts were normalized with bridge samples across different batches. Transcript count analysis and pathway analysis was conducted in Rstudio utilizing package clusterProfiler.

4.6 Statistics

4.6.1 Survival and other time-to-event analyses

In studies I, III and IV, time-to-event analyses were performed by utilizing Mantel-Cox tests, Gehan-Breslow-Wilcoxon tests, or MaxCombo tests. In studies III and IV, overall survival was calculated from enrollment to death of any cause. Progression-free survival was calculated from enrollment to cancer progression or death of any cause. Time to progression was calculated from enrollment to cancer progression.

4.6.2 Grouped analysis

In all studies, comparison of groups was done with t-tests, ANOVA with post-hoc analyses for normally distributed samples or Mann-Whitney tests for non-normally distributed samples. P-value of less than 0.05 was considered statistically significant.

4.7 Ethics

4.7.1 Preclinical experiments

Animal experiments of studies I and II were conducted under approval Animal Experimentation Board of the Provincial Government of Southern Finland (license number ESAVI/12559/2021), following FELESA guidelines for humane treatment of research animals.

Collection and research of human ovarian cancer samples was approved by the Ethics Board of Helsinki University Hospital (permit 120/13/03/02/16). All patients gave written informed consent prior to sample collection.

4.7.2 Clinical trial

TUNIMO trial ethics were evaluated by Ethics Board of Helsinki University Hospital (statement HUS/1804/2020). TUNIMO trial protocol was evaluated and approved by the Finnish Medical Agency (approval 49/2020). All patients enrolled to the study gave informed consent.

4.7.3 Validation patient dataset

Advanced Therapy Access Program ethics were reviewed and approved by Helsinki and Uusimaa Hospital District Ethical Board (statement HUS/62/13/03/02/2013). All patients gave informed consent.

5 RESULTS AND DISCUSSION

Despite major advances in multiple different cancer types, many patients with solid cancers face a poor prognosis. This is especially evident in the metastatic setting, where most solid cancers remain an incurable disease. Thus, new treatment modalities are needed. The study results discussed herein represent results from two preclinical studies and two studies from phase I trial of advanced solid tumors. The preclinical research included in this thesis focused on oncolytic adenovirus encoding a modified interleukin-2 molecule ("variant IL-2"), representing the next generation of oncolytic adenoviruses aiming to harness the immunostimulatory benefits of IL-2. The variant IL-2 encoding virus was tested in pancreatic cancer and ovarian cancer. The two clinical/translational studies included in this thesis were based on a phase I trial of oncolytic adenovirus encoding tumor necrosis and normal human interleukin-2. These studies aimed to study the safety, efficacy, pharmacokinetics, pharmacodynamics, and markers of response to oncolytic adenovirus therapy in this trial.

5.1 ONCOLYTIC ADENOVIRUS PRODUCING A VARIANT IL-2 MOLECULE SYNERGIZES WITH STANDARD CHEMOTHERAPY OF PANCREATIC CANCER (STUDY I)

Metastatic pancreatic cancer remains a disease with extremely dismal prognosis, and no major advances in the treatment of pancreatic cancer have been approved in decades, albeit approvals of more sophisticated chemotherapy regimens (191). Pancreatic cancer currently ranks seventh in cancer related deaths worldwide, and is expected to soon surpass breast cancer as the third leading cause of cancer mortality in the European Union (191). Pancreatic cancer is markedly treatment resistant, thought to arise from the thick stroma in pancreatic tumors. Additionally, regardless of major efforts, immunotherapies have not shown major efficacy in pancreatic cancer (192). Study I aimed to study the efficacy of oncolytic adenovirus Ad5/3-E2F-d24-vIL2 in combination with standard chemotherapy of nab-paclitaxel and gemcitabine for the treatment of pancreatic cancer. Ad5/3-E2F-d24-vIL2 represents a more developed version of oncolytic adenovirus encoding IL-2, and builds on previous pre-clinical research with Ad5/3-E2F-d24-hTNF-IRES-hIL2.

5.1.1 Ad5/3-E2F-d24-vIL2 replicates in human PDAC cell lines producing a functional cytokine and synergizes with paclitaxel and gemcitabine (Study I)

Firstly, we aimed to study if Ad5/3-E2F-d24-vIL2 was able to replicate, lyse and produce the transgene in pancreatic cancer cell lines. The virus produced dose dependent cell killing in five different cell lines assayed (Figure 1B, study I). Transgene expression was assayed from the supernatant following 48 hours of infection, and the virus was able to produce cytokines in similar levels to a virus producing an unmodified interleukin-2 molecule (Figure 1C, study I). Similarly, the supernatant was able to produce similar expansion of mouse CTLL-2 lymphocytes compared to recombinant human IL-2 and supernatant from virus producing unmodified interleukin-2 (Figure 1D, study I).

Next, the combination of Ad5/3-E2F-d24-vIL2 with standard chemotherapeutics of pancreatic cancer, paclitaxel and gemcitabine, was assessed in co-cultures and cell killing assays. Both paclitaxel and gemcitabine synergized with Ad5/3-E2F-d24-vIL2, and no hinderance of cell killing was seen when combining these chemotherapeutics to oncolytic virus therapy (Figure 1E, study I).

Due to cell cycle modifying effects of both paclitaxel and oncolytic adenoviruses, next we assayed the different cell cycle states after paclitaxel, virus monotherapy or combination treatment. In agreement with previously published reports, the treatment with paclitaxel led to increased apoptosis and M-phase arrest of pancreatic cancer cells (Figure 2B, study I). Additionally, when paclitaxel was combined with Ad5/3-E2F-d24-vIL2, markedly more cells with high DNA amount (7-AAD) with concurrent low expression of mitotic markers Ki-67 and H3-histone were observed. These findings agree with previous reports of a cell state called mitotic slippage, where a cell after replicating its DNA fails to complete mitosis and instead produces a dysfunctional cell with doubled DNA amount (Figure 2E, study I). Mitotic slippage is a known effect of microtubule stabilizing chemotherapeutics such as paclitaxel, and it seems that concurrent infection with Ad5/3-E2F-d24-vIL2 also increases the amount of mitotic slippage in pancreatic cancer cells (Figure 2D, study I). Additionally, this translated into enhanced cell killing of pancreatic cancer cells after therapy (Figure 2F, study I).

Following these observations, we aimed to study if combination of paclitaxel and gemcitabine with Ad5/3-E2F-d24-vIL2 therapy led to enhanced amounts of immunogenic cell death (ICD). ICD is a known effect of both oncolytic adenoviruses and some therapeutics, and thus we aimed to assess if combining these treatments also led to synergistic ICD effects (193). Indeed, when assaying markers of ICD by extracellular ATP release and translocation of calreticulin, both combination of paclitaxel and of gemcitabine to Ad5/3-E2F-d24-vIL2 led to increased amount of ICD in pancreatic cancer cells (Figure 3A-D, study I).

5.1.2 Combination of Ad5/3-E2F-d24-vIL2 to standard chemotherapy shows enhanced tumor growth control and survival in a mouse model through immune cell influx and TME adaptation (Study I)

Following these in vitro investigations, we aimed to study the feasibility of the combination of chemotherapy with oncolytic virus therapy in an animal model. We utilized a subcutaneous model of mouse pancreatic cancer. This animal experiment utilized an immunocompetent mouse model, C57BL6, which allows for the study of the immune effects of the treatment. However, mouse cells are generally not suitable for productive replication of human adenoviruses. Similarly, the mouse cell line Panc02 used in the animal experiment was not lysed by Ad5/3-E2F-d24-vIL2, but did produce the transgene in a dose-dependent fashion (Supplementary Figure 3A-B, study I). Thus, although the animal model did consider the important immunomodulatory effects of the treatment, it did not facilitate studying the oncolytic effects of the treatment, likely understating the potential of the treatment.

Mice were engrafted with the cell line, and after tumor growth to 3-5 mm, animals were randomized to four treatment groups: control with no treatment, virus monotherapy, chemotherapy monotherapy or combination of virotherapy and chemotherapy. Animals received nab-paclitaxel and gemcitabine once a week, and virus treatments every two days to control for the lack of replication in the model (Figure 4A, study I). Treatment was given until day 50, after which animals were monitored for tumor growth. Mechanistic samples were collected on day 10 by sacrificing randomly 5 animals per group. The combination treatment showed superior tumor control already at an early timepoint (Figure 4B, study I), without marks of toxicity when assayed by animal weight gain (Figure 4c, study I). The combination treatment also facilitated long-term tumor control with improved survival compared to all other groups (Figure 4E-F, study I).

To study the mechanism behind the enhanced tumor control, we assayed the mechanism of action samples collected on day 10 by utilizing flow cytometry and soluble protein analysis. Flow cytometric analysis of immune cells showed increased amounts of CD4+ T cells and CD8+ T cells infiltrating into tumors, when comparing the combination treatment to chemotherapy treatment (Figure 5A, study I). Furthermore, the combination treatment showed increased amount of perforin expression in CD4+ T cells and CD8+ T cells, indicating more activated lymphocytes in the combination treatment group (Figure 5C, study I). Parallelly, the combination group had markedly decreased expression of inhibitory markers PD1 and LAG3 on CD4+ T cells, CD8+ T cells and NK cells (Figure 5B, study I).

In order to assay the TME more closely, we also performed flow cytometry looking at a major component of pancreatic cancer stroma, cancer associated fibroblasts (CAF). Cancer associated fibroblasts are known to be a key player of treatment resistance in pancreatic cancer, through stroma production and

inflammatory signaling (194). Combination treatment led to no changes in myofibroblastic CAFs, a decrease of inflammatory CAFs and an increase in antigen-presenting CAFs (Figure 6B, study I). These findings support the multifaceted effects of oncolytic viruses, beyond pure oncolysis. Additionally, we assayed the proteomic milieu of the tumors by assessing the amounts of different immune-related cytokines. Comparing the combination treatment to chemotherapy monotherapy, the combination treatment showed more immune-related proteins CCL2, CCL3, CCL4, IFN-gamma and TNF (Figure 6C, study I).

In conclusion, these results show that combination of chemotherapy with oncolytic virotherapy leads to enhanced tumor control and survival, and on the other hand combining chemotherapy to virotherapy is able to negate the negative immunosuppressive effects of chemotherapy.

5.1.3 Combination of Ad5/3-E2F-d24-vIL2 with standard chemotherapy provides enhanced tumor rechallenge protection

Finally, we aimed to assay the surviving animals for generation of immune memory. This was done by engrafting animals with complete response with two cell lines: the original pancreatic cancer cell line Panc02, in addition to a new cell line, a colorectal cancer cell line MC-38 (Figure 7A, study I). The animals received no additional treatment. Animals treated with the combination therapy showed enhanced growth control of the original cell line, in addition to improved control of the new cell line (Figure 7B-C, study I). These results support generation of immune memory in animals receiving the combination treatment, possibly through epitope spread arising from the more efficient immune effect and circumvention of immune suppression. By analysis of spleens of these rechallenged animals, spleens from the combination treatment showed less CD4+ T cells and more CD8+ T cells, with increased amounts of CD62L+ CD4+ T cells, supportive of enhanced immune activation (Figure 7D, study I).

5.2 ONCOLYTIC ADENOVIRUS PRODUCING A VARIANT IL-2 MOLECULE ENABLES ALLOGENIC NK CELL THERAPY OF OVARIAN CANCER (STUDY II)

Similarly to pancreatic cancer, ovarian cancer is a malignancy with limited treatment options and a high degree of development of treatment resistance (195). Recently, PARP-inhibitors have become a new valuable tool for treating ovarian cancer (196). However, not all patients are eligible for PARP inhibition, and similarly to pancreatic cancer, ovarian cancer is currently markedly resistant to immunotherapy (196, 197). Immunotherapies, in the form of checkpoint inhibition

or cellular therapies, have not demonstrated efficacy in ovarian cancer thus far (197). In study II, we aimed to assess if oncolytic virotherapy in the form of Ad5/3-E2F-d24-vIL2 could enable NK cell therapy in ovarian cancer.

NK cell therapy is a promising form of cellular therapy, which tackles many problems that T cell-based therapeutics face (198). Firstly, NK cell therapy can be dosed allogenically, allowing for quicker ("off-the-shelf") administration of cell compared to T cells. Traditionally T-cell therapeutics, such as CAR T cells and modified TCR T cells, are produced in an autologous manner where immune cells are collected from the patient, followed by transduction and expansion of the collected cells. This production usually takes at least 2 weeks to complete, time during which the disease of the patient can progress such that treatment with the manufactured product is not anymore feasible. Additionally, CAR T cells and TCR T cells are prone to downregulation of the target molecule, either the protein targeted by the CAR or the MHC-I complex needed for TCR activation. NK cells on the other hand regulate their activation and inhibition by multiple different cell surface proteins, where inhibition of NK cell activation is more complex to manipulate by the cancer cell (198). Finally, NK cell therapeutics have shown superior safety profile to CAR T cells, where cytokine release syndrome is observed in more than 75% patients receiving CAR T cells for hematologic malignancies (199). This leads to limited availability of the cell therapeutics to more frail patients to whom a cytokine release syndrome could be fatal.

5.2.1 Clinical ovarian cancer specimens show variable PD-L1 expression and immune cell infiltration

With the aim to study clinically relevant ovarian cancer, we collected tumor samples from patients undergoing ovarian cancer surgery. Histologically, the patients exhibited a range of various ovarian cancer subtypes, predominantly high-grade serous carcinoma (HGSC) of fallopian tube or ovary (Figure 2A, study II). As is common for ovarian cancer, the disease had already metastasized outside the ovary, and majority of the samples were collected from the greater omentum (Figure 2B, study II). Regarding PDL1 and immune cell infiltration, the tissue samples exhibited varying levels of expression and infiltration. The PD-L1 expression, a general marker of immune activity and suitability for checkpoint inhibition, was generally less 1% (Figure 1D, study II). Immune cell infiltrates in the samples were generally CD4+ T cells, with low amounts of CD8+ T cells and NK cells, supporting the general lack of anti-tumor immune activity in ovarian cancer (Figure 2E-H, study II).

5.2.2 Clinical ovarian cancer specimens are efficiently killed by Ad5/3-E2F-d24-vIL2 combined with allogenic NK cell therapy and demonstrate enhanced immune activation

To study the feasibility of allogenic NK cell therapy combined with Ad5/3-E2F-d24-vIL2, we first isolated and expanded NK cells from a healthy donors from the Finnish Red Cross. To assess the ability of these cells to kill ovarian cancer samples, we seeded ovarian cancer cells to impedance measuring plates, after which NK cells were added with unarmed oncolytic adenovirus or oncolytic adenovirus armed with variant IL-2. Generally, Ad5/3-E2F-d24-vIL2 combined with NK cell therapy showed superior cell killing in most samples compared to NK cell monotherapy or NK cell therapy combined with the unarmed virus (Figure 2A-L, study II). These results underline the variability of responses in different patients with the same diagnosis and same therapy, although results presented in this study can also be affected by the in vitro system utilized.

To further study the responses observed, we utilized flow cytometry to assess immune populations in concurrent histocultures of four patients with suitable amount of tissue material. These assessments showed higher expression of inhibitory CD158b molecule on NK cells, with generally highest granzyme B expression on both NK cells and CD8+ T cells (Figure 3B, D and F, study II). These findings highlight the complexity of assessing immune responses, since often immune activation is followed by a negative regulation and a decrease in the immune activation. Additionally, two samples were analyzed for the amounts of T regulatory cells, due to the mechanism of action of encoded variant IL-2 molecule aiming to expand preferentially CD8+ T cells and NK cells. Accordingly, we observed no significant increase of T regulatory cells in the assayed samples (Figure 3I, study II).

Finally, we analyzed the expression of multiple different activating and inhibiting NK cell ligands on the ovarian cancer cells. Adenoviruses are known to modulate cell surface expression of different immune ligands, in order to evade immune clearance of infected cells (200). Additionally, one of the key immunomodulatory genes of the adenovirus, the early gene 3 (E3) is partially deleted in the variant 2 encoding virus to make room for the transgene. However, the unarmed virus still harbors the intact E3 region. We assessed the different NK cell ligands in the clinical ovarian samples 48h post-infection. Interestingly, the different samples showed widely variable changes in the expression profile of the NK cells ligands, with no clear coherent changes in any of the assayed ligands (Figure 4A-E, study II). These results emphasize the variability of response to oncolytic virus therapy, and further research is needed to assess expression changes in different timepoints of viral infection.

5.2.3 Combination of Ad5/3-E2F-d24-vIL2 with allogenic NK cell therapy shows enhanced tumor control in vivo through increased amounts of activated intratumoral effector lymphocytes

After these in vitro studies, we continued into an in vivo feasibility experiment combining oncolytic virotherapy with allogenic NK cell therapy. To facilitate the animal experiment, we utilized a previously established patient-derived xenograft model of ovarian cancer. This PDX cell line was engrafted subcutaneously into immunodeficient NOD/JicTac mice, and following establishment of treatable tumors, autologous T cells were engrafted intraperitoneally to reconstitute a human immune system in the mice. Following reconstitution, animals received virus treatment every three days and one dose of allogenic expanded NK cells intraperitoneally (Figure 5A, study II).

Treatment with NK cell therapy combined with oncolytic virus encoding variant IL-2 showed significantly better tumor control compared to mock treatment and NK cell therapy (Figure 5F, study II). Superior tumor control compared to unarmed virus combined to NK cell therapy did not reach significance, but the combination treatment of unarmed virus failed to show superior control over NK cell therapy without virus, supporting the superior synergism of variant IL-2 encoding virus over unarmed virus.

Next, we assessed the immune populations present in the animals. Surprisingly, influx of CD56 cells was the highest in the group treated with the NK cell monotherapy, however, treatment with the variant IL-2 virus showed greatest amounts of CD4+ T cells and CD8+ T cells (Figure 6A, C and E, study II). When assessing the cytotoxicity of these cell subsets, we could see the greatest amount of activated NK cells, CD4+ T cells and CD8+ T cells in the group receiving variant IL-2 virus (Figure 2B, D, F, study II). These results indicated that treatment combination of variant IL-2 producing virus with allogenic NK cells was able to support the immune function of the cells, and most likely the quality of the infiltrated cells matters more than the pure number of cells. Additionally, we assessed the amount of T regulatory cells in this in vivo setting, and we could see the highest amounts of T regulatory cells in the group receiving variant IL-2 virus with NK cells (Figure 6G, study II). Although seemingly counterintuitive at first glance, due to the inability of the variant IL-2 molecule to expand T regulatory cells, we hypothesize that this effect is due to production of normal IL-2 by CD4+ T cells, arising from the immune activation ignited by the variant IL-2 virus.

5.3 ONCOLYTIC ADENOVIRUS PRODUCING TNF-A AND IL-2 IS SAFE AND PRODUCES ANTI-TUMOR EFFECTS IN HUMANS (STUDY III)

Study III focused on a dose-escalation phase I clinical trial, TUNIMO (NCT04695327), where oncolytic adenovirus encoding tumor necrosis and interleukin-2 (Ad5/3-E2F-d24-hTNF-IRES-hIL2, TILT-123, igrelimogene litadenorepvec) was administered to patients with advanced solid tumors. The goal of the trial was to study the safety and efficacy of TILT-123, along with pharmacokinetic and pharmacodynamic analysis. 20 patients were enrolled in two Finnish cancer centers, and 10 were available for response evaluation. Median age of the patients was 58 years, 13 patients were female, and the most common cancer type were different sarcomas. The patients had received median 4.5 lines of previous systemic cancer treatment, and 5 patients had received checkpoint inhibitors before the trial (Table 1, study III).

5.3.1 Ad5/3-E2F-d24-hTNF-IRES-hIL2 administration is safe producing mostly infection-like adverse events

20 patients received TILT-123 administered intravenously on day 1, followed by five intratumoral doses on days 8, 22, 36, 50 and 64. The intravenous dose escalated from 3×10^9 to 4×10^{12} viral particles across six cohorts. Similarly, the intratumoral dose escalated from 3×10^9 to 5×10^{11} viral particles in the same cohorts. 7 patients discontinued the trial treatment due to progressive disease, and 3 patients discontinued due to worsening of the general condition (Figure 1A, study III). TILT-123 could be detected in blood 1 and 16 hours after treatment, with highest amount of virus detected after the intravenous dose (Figure 1E, study III). Amount of circulating TILT-123 increased with increasing dose (Figure 1F-G, study III).

Regarding adverse events, the most common events related to TILT-123 were fever (18 occurrences), chills (14 occurrences) and fatigue (9 occurrences). Severity of these events were mostly grade 1 to 2, with one grade 3 fatigue (Table 2, study III). Most adverse events per patient were seen in dose level 4 with 8.7 events per patient, with generally more adverse events in the three highest dose levels compared to the three lowest dose levels. Generally, the treatment was well tolerated, with three grade 4 adverse events occuring. The first was neutropenia in a patient 20104 with leiomyosarcoma, where after the day 8 intratumoral dose the neutrophil decreased below 0.5×10^9 cells/L on day 10 (Supplementary Figure 1A, study III). The patient received no granulocyte stimulating growth factor, and the neutrophil count normalized by the next treatment cycle on day 22. The patient experienced similar decreases in the neutrophil count decreases on days 22 and 36,

with spontaneous recovery and finally a neutrophil count above the normal limit at the end of the trial.

The second case of grade 4 adverse event was pseudothrombocytopenia occurring in patient 20108 with adenoid cystic carcinoma of the face. The patient's platelet count decreased from 360 x 10^9 cells/L to 137 x 10^9 cells/L between the day 1 post-treatment and day 8 pre-treatment. Additionally, 5 days after the day 8 intratumoral dose, the platelet count decreased further down to 19 x 10^9 cells/L, with significant platelet aggregation in the tubes (Supplementary Figure 1B, study III). A clinical suspicion of pseudothrombocytopenia was raised, prompting a repeat blood draw in citrate tubes. The platelet count subsequently increased to 391 x 10^9 cells/L, a value similar to pre-treatment levels. All further blood draws were done in citrate tubes, and no thrombocytopenia occurred in the patient during the remainder of the study.

The third grade 4 adverse was lymphopenia occurring in patient 20219 with mucoepidermoid carcinoma of the parotid gland. After the intravenous dose, the post-treatment blood draw on day showed a lymphocyte count of 0.14 x 10^9 cells/L. Due to known effect of oncolytic viruses and viral infections inducing transient lymphocyte count decreases, the patient was monitored, and normalization of lymphocyte count was seen by day 8 (Supplementary Figure 1C, study III). The patient experienced similar decreases in the lymphocyte count following subsequent TILT-123 administrations. Lymphocyte count decrease was also observed generally in all patients treated, with a decrease 1-2 days post-treatment and normalization 6-15 days after treatment (Figure 1B, study III). Similar, but less pronounced decreases were observed for total leukocytes and neutrophils (Figure 1C-D, study III).

5.3.2 Ad5/3-E2F-d24-hTNF-IRES-hIL2 induces disease control in both injected and non-injected lesions

Treatment efficacy was assessed on day 78 with contrast-enhanced computer tomography (CT) and radiolabeled glucose positron emission tomography (FDG-PET). Utilizing RECIST 1.1, iRECIST and PET-criteria, disease control rate was 20%, 20% and 60% of evaluated patients, respectively. One patient, 20103 with anaplastic thyroid carcinoma, achieved a partial response by RECIST 1.1 and iRECIST evaluation, and two patients achieved a partial metabolic response (Supplementary Table 3, study III).

When evaluating individual lesions, lesion control could be seen in 9/19 (47.4%) injected lesions by CT evaluation and in 11/17 (64.7%) injected lesions by PET evaluation (Figure 2A-b, study III). Regarding non-injected lesions, lesion control could be seen in 9/13 (69.2%) lesions by CT evaluation and in 11/14 (78.6%) lesions by PET evaluation (Figure 2C-D, study III). These results suggested the ability of

the combined intravenous and intratumoral administration to facilitate control of both injected and non-injected tumors. Interestingly, and linear increase in PET SUVmax reading could be seen with an increase in both i.v. and i.t. dose given, possibly arising from immune cell trafficking to tumors leading to increased glucose consumption in the tumors, inferring that PET might have an inherent bias when assessing oncolytic adenovirus efficacy (Figure 2E-F).

The median overall survival of enrolled patients was 124.5 days. By evaluating disease control utilizing PET-criteria, patients with PET disease control showed longer overall survival of 213.5 days compared to 109 days in patients without disease control in PET (p=0.165, Figure 2G, study III). The median PFS and TTP of the patients enrolled were 87.5 days and 97 days, respectively (Figure 2H-I, study III).

4 patients survived for more than one year after enrollment (Figure 2J, study III). These patients included a patient 20204 with myxoid liposarcoma, patient 20103 with anaplastic thyroid carcinoma, patient 20212 with leiomyosarcoma and patient 20211 with nodular melanoma. Three of the four patients with long survival (20204, 20212 and 20211) were heavily pretreated, having received a minimum of three prior lines of systemic oncological therapy (Supplementary Table 3, study III). Patient 20204 had received a total of 10 previous forms of cancer treatment (surgery, radiation and chemotherapy) prior to enrollment to TUNIMO, and completed the trial with progressive disease when evaluated with RECIST 1.1 and stable metabolic disease when evaluated with PET-criteria. The patient received 4 additional doses of intratumoral TILT-123 after the trial, after which the patient was followed up without systemic treatment other than palliative radiotherapy to a groin metastasis. The patient finally progressed 518 days after enrollment and succumbed to the disease 821 days after enrollment.

Patient 20103 with anaplastic thyroid carcinoma was the patient achieving the best radiographical RECIST 1.1 response in the trial, with a partial response. Examples of this patient's abdominal and lung metastases responses are shown in Figure 3A of study III. The patient was relatively treatment naïve, receiving surgery and adjuvant radiotherapy in the first line, and paclitaxel combined to radiotherapy in the metastatic setting, before enrolling in the trial. Regarding tumor mutational status, the patient was evaluated as microsatellite stable with high tumor mutational burden. Interestingly, this patient population had previously benefitted from checkpoint inhibitor immunotherapy, even though checkpoint inhibitors are generally more efficacious in solid tumors with microsatellite instability (201). The patient completed the trial, and received 4 additional intratumoral doses and 3 additional intravenous doses of TILT-123, followed by radiotherapy and pembrolizumab. The patient was alive at the time of writing of study III, with 739 days of overall survival, and is still alive at the of writing of this thesis, with close to 1000 days of overall survival and stable disease.

Beneficial treatment effects of TILT-123 treatment were also observed in checkpoint inhibitor refractory setting. Patient 20202 with metastatic non-small cell lung cancer with wild-type EGFR and ALK status enrolled in the trial after receiving chemotherapy in the forms of cisplatin, pemetrexed and nano albumin-bound paclitaxel, radiation therapy and immune checkpoint inhibition in the forms of pembrolizumab and nivolumab. The original PDL1 expression in the tumor tissue was 60%. Neck lesions and axillar lesions of the patient responded well to TILT-123 therapy, with 62% and 54% SUVmax decrease, respectively (Figure 3B, study III). Comparing patients with or without previous checkpoint inhibitor therapy, responses could be seen in both patient groups, without clear trends, however the injected lesions of patients without previous checkpoint inhibitor therapy did seem to respond better when evaluating response with CT, however this evaluation is prone to bias due to pseudoprogression (Supplementary Figure 4, study III). Additionally, we observed limitations to imaging measurements, where tumor diameter change was not able to capture marked necrosis present in the tumors (Figure 3C, study III).

Results in the efficacy part of study III highlights the difficulty in assessing treatment response in immunotherapy trials. Current imaging criteria such as RECIST 1.1 were developed and refined in the era where chemotherapy and targeted therapies were advanced and are thus not optimal in capturing treatment benefit in immunotherapy settings (202, 203).

5.3.3 Interaction of Ad5/3-E2F-d24-hTNF-IRES-hIL2 with the immune system leads to immune activation

Next in study III, we assessed signs of tumor transduction of TILT-123 and its immune effects. Patient 20202 with checkpoint inhibitor refractory non-small cell lung cancer showed positive immunohistochemistry staining for the adenoviral E1A protein after intravenous delivery, and in injected and non-injected lesions from day 36 (Figure 4A, study III). Positive staining for E1A supports the ability of TILT-123 to transduce tumors via the intratumoral and intravenous routes. Concomitantly, we observed increased infiltration of both CD8+ T cells and NK cells in the tumor samples after intravenous or intratumoral administration (Figure 4B, study III).

Regarding neutralizing antibodies, 9 out of 20 patients had measurable neutralizing antibodies at baseline, defined as a titer of 1:64 or higher (Figure 4C, study III). Neutralizing antibody titer did not associate with disease control by PET-criteria, although patients with positive baseline neutralizing antibodies did show a tail of long-term survivors (Figure 4D-E, study III). Generally, all patients developed increasing neutralizing antibody titers (Figure 4F, study III).

Serum proteomics showed increase in the amounts of IFN-gamma in the serum after intravenous delivery, with accompanying high expression of chemokines CCL3, CCl4, CCL19, CXCL9, CXCL10 and CXCL11 (Figure 4G, study III). Similarly, after intravenous dosing, an increase in the amounts of interleukins IL-10 and IL-15 could be observed. When assessing serum proteomics after intratumoral dosing, relatively small serum proteomic changes were observed after the first intratumoral dose (Figure 4H, study III), possibly due to the already upregulated serum proteome (Supplementary Figure 6, study III) prior to administration, thus limiting the potential for further pro-inflammatory effects. After the second intratumoral dose, high upregulation of IFN gamma, chemokines and interleukins were observed, similarly to the intravenous setting (Figure 4I, study III).

5.4 MECHANISM OF ACTION AND BIOMARKERS OF RESPONSE OF ONCOLYTIC ADENOVIRUS PRODUCING TNF-A AND IL-2 IN HUMANS (STUDY IV)

Due to limitations in preclinical animal models, the study of human specimens is of key interest for oncolytic adenovirus research. Additionally, the field of OVs still currently lacks established biomarkers of response, and thus more research is needed. In study IV, we aimed to assess the mechanism of action of TILT-123, and to generate predictive biomarkers of response, by analysis of specimens collected during the TUNIMO trial.

5.4.1 Ad5/3-E2F-d24-hTNF-IRES-hIL2 produces cyclical changes in major blood components with lymphocyte decrease correlating with CT tumor decrease

In TUNIMO, samples were collected in the form of tumor biopsies, whole blood, serum and PBMC samples (Figure 1A, study IV). Samples were utilized in downstream analysis by multiplex immunohistochemistry, Nanostring transcriptomic analysis, blood safety laboratory testing, serum proteomic profiling, and flow cytometry staining of PBMC samples. Findings from these specimens were then correlated to tumor growth by imaging, and overall survival time.

Blood safety laboratory testing in TUNIMO showed cyclical changes in the major blood components (Figure 1B-C, study IV). Most noticeably, decreases in the white blood cell (WBC) count and lymphocyte count were observed after each TILT-123 administration (Figure 1B, study IV). At baseline, a lower amount of WBCs correlated with slower tumor growth, and lower amount of lymphocytes similarly trended to better response, suggesting that lower amounts of these cell correlated with slower tumor growth or better tumor control following therapy (Figure 1D, study IV). Other major blood cell components did not significantly correlate with

smaller tumor growth (Figure 1E, study IV). Due to cyclical changes in both the total WBCs and lymphocytes, we next aimed to assess if changes in WBC count or lymphocyte count, from pre-treatment to 1 to 2 days after therapy, correlated with smaller tumor growth. We observed that lymphocyte count decrease associated with smaller tumor growth in all treatment point assayed (Figure 1F, study IV). Changes in the WBC count showed no correlation to imaging change in any of the treatment timepoints (Supplementary Figure 1A, study IV). Accordingly, the average lymphocyte count decrease predicted imaging change, and while average WBC count change did not (Figure 1G and Supplementary Figure 1B, study IV). No predictive value of other blood cell component change could be seen to radiographical change (Supplementary Figure 1C, study IV). Interesting, regarding PET imaging, a decrease in peripheral blood lymphocyte count on day 64 correlated with an increase in PET signal change from baseline to day 85, highlighting that the possible pseudoprogression was also present in PET imaging, even 20 days after treatment administration (Supplementary Figure 1D, study IV).

These findings showcase the immune interaction of TILT-123 therapy in humans. Decreases in lymphocytes, leukocytes and platelets have been reported in previous oncolytic virus trials and its now a known side-effect of oncolytic virotherapy (204-211). However, in depth analysis in relation to treatment efficacy has not been conducted, other than one study of oncolytic reovirus combined with FOLFOX6 and bevacizumab in metastatic CRC patients, reporting PFS detriment in patients not experiencing lymphopenia by CTCAE classification (212). Our findings support that decrease in lymphocytes accurately predicts tumor growth after TILT-123 therapy.

5.4.2 Ad5/3-E2F-d24-hTNF-IRES-hIL2 promotes lymphocyte accumulation in tumors, with baseline PD-L1, CD16 and CD11c expression correlating with longer overall survival

Next in study IV, we aimed to assess the intratumoral immune cell subset changes after TILT-123, to study if lymphocyte count decrease in the peripheral blood mirrored immune changes at the tumoral level. After intravenous TILT-123 therapy, increases in the amounts of immune cells and more specifically CD8+ T cells, CD4+ T cells, NK cells and B cells was observed, of which the increase in CD8+ T cells was significant (Figure 2A, study IV). Additionally, we assessed granzyme B and PD-1 expression on these cell populations to identify changes in activation status. We could observe increases in granzyme B and PD-1 expression on both CD8+ T cells and -NK cells, supporting the notion that TILT-123 can attract cells to tumors and support their activation status (Figure 2B, study IV). However, in day 36 samples, which represent the effects of intratumoral therapy, no significant chances to baseline could be seen. This observation could be rationalized by at least

two explanations. All of the tumor samples in the trial were collected prior to treatment administration, thus day 8 samples reflect the effect of the intravenous dose given on day 1, and similarly the day 36 samples reflect the effects of the intratumoral dose given on day 22. Thus, the time between the last administration in the day 36 biopsies is twice as long, and it is possible that the immune activation is by nature stronger 7 days after the therapy rather than 14 days. Another explanation for the observed effect could be more efficient tumor transduction by the intravenous dose. Theoretically, intravenous doses of the virus would have better ability to transduce multiple metastases, where as the intratumoral dose requires dissemination from the injected tumor to other metastases. Additionally, it is possible that the effects of oncolytic virotherapy are transient, and that less dramatic changes are seen in repeated dosing. More research on the timing of the immune effect is needed to confirm the findings.

Regarding lymphocyte count decrease and immune infiltrate in the tumors, we could observe that larger lymphocyte count decrease on day 22 correlated with increased amounts of activated NK cells in the tumors on day 36, supporting the notion that peripheral lymphocyte changes in fact reflect tumor level changes (Figure 2C, study IV). Further, to study the prognostic factors of baseline tumor characteristics in relation to overall survival time, we found that baseline tumor infiltration of CD8+ T cells or NK cells did not correlate with overall survival time (Figure 2D, study IV). However, when assessing PD-L1, CD16 and CD11c expression in stromal areas at baseline, a positive correlation of all three markers to overall survival time could be observed, but more studies are needed to assess if these results are purely prognostic or actually predictive of response (Figure 2F, study IV).

PD-L1 expression level is a known biomarker of response in checkpoint inhibitors targeting the PD-1/PD-L1 axis (213). However, PD-L1 expression in the tumor might be a predictive factor also for other immunotherapies, since immune activation with subsequent interferon expression is known to upregulate PD-L1 expression on a variety of cell types (214-216). Thus, assessment of only PD-L1 expression is not able to describe the complexity of immune activation or suppression status of the TME. CD16 is a receptor of Fc-portions of antibodies, and is expressed widely on different immune cells, such as macrophages, NK cells, neutrophils and even on activated T cells (217, 218). Binding of CD16 to the antibody Fc-region leads to activation and antibody-dependent cell-mediated cytotoxicity (ADCC) of NK cells and monocytes (217). Oncolytic adenoviruses are known to induce high amounts of neutralizing antibodies, but generally production of neutralizing antibodies is not detrimental to OV therapy, and often better tumor control to therapy are seen in patients who develop high neutralizing titers (120, 122, 130, 152, 153, 219). CD16 mediated ADCC is possible mechanism behind this observation, due to enhanced clearance of tumor cells infected with oncolytic virus,

leading to enhanced tumor clearance by ADCC, assuming enough new virions can still be generated in non-cleared cells.

Similarly to CD16, CD11c is expressed widely in multiple immune cell subsets, such as dendritic cells, macrophages, neutrophils and B cells. CD11c is an integrin molecule, and binding of CD11c to CD18 leads to formation of complement receptors and promotion of innate immune response (220, 221). CD11c has also been shown to participate in leukocyte chemotaxis (220). Correlation of CD11c to overall survival could be reasoned by at least two mechanisms: increased amounts of dendritic cells and beneficial immune subsets in the tumors, or stronger complement mediated cell cytotoxicity in tumors with high CD11c expression. Interaction of cancer and complement is a multifaceted process, but theoretically complement activation can induce anti-tumor effects through antibody-mediated complement activation via the classical pathway (222). Additionally, adenovirus is known to induce complement activation, however this has been mostly studied in terms of vector neutralization and not as an anti-tumor effect (223-225).

5.4.3 Ad5/3-E2F-d24-hTNF-IRES-hIL2 produces pro-inflammatory intratumoral transcriptional changes with responders showing stronger inflammatory changes

In addition to immunohistochemistry analysis, we also aimed to study the transcriptional changes in the collected tumor biopsies. The effect of intravenous TILT-123 was studied by analyzing tumor samples collected pre-treatment on day 8, thus showing effects of intravenous TILT-123 dosing 7 days post treatment. Transcriptomic gene-set analysis showed upregulation of gene sets related to acute immunological activation, such as complement activation, humoral immune response and antigen processing (Figure 3B, study IV). Conversely, downregulation of gene sets related to type-I interferon signaling and viral replication was seen, reminiscent of active anti-viral response and viral inhibition of anti-viral responses (Figure 3B, study IV). Adenoviruses include multiple genetic components to facilitate immune evasion in infected hosts, and thus infectious adenoviral particles are often found long after primary infection (200, 226-228). Most studied of these are the proteins encoded by the E1 and E3 regions, in addition to virus associated (VA) RNAs encoded by the adenoviral genome. E1A encoded proteins are known to protect infected cells from interferon response by interfering with the formation interferon stimulated gene factors (229). Two other E1 encoded proteins. E1B-19K and E1B-55K. are involved in inhibition of cell apoptosis after infections, thus limiting cell death and subsequent inflammation. E1B-19K, which is deleted in TILT-123, is able to interrupt death receptor signaling downstream of TNF-receptors, and thus inhibiting apoptosis through the caspase 3 pathway. This is thought to arise from E1B-19K binding to Bax and Bak proteins, which normally

activate caspase 3 through cytochrome c release from the mitochondria (230). E1B-55K on the other hand inhibits apoptosis through inhibition of p53 transcription. In infected cells, E1B-55K is known to inhibit p53 function by multiple ways: 1) nuclear export of p53 2) transcriptional suppression and 3) proteasome mediated degradation of p53 (231).

Of E3 region encoded proteins, E3-gp19K, E3-14.7K, E3-10.4K, E3-14.5K and E3-6.7K are known to have roles in immune evasion of adenovirus. E3-gp19K is known to block HLA-I transport to the cell membrane, in addition to suppressing the expression of NK cell activating ligands (232). The other aforementioned E3 encoded proteins are associated with interference of apoptotic pathways by downregulating death receptors and blocking NF-κB activation (229).

Interestingly, in study IV, when assessing the transcriptomics of injected tumors on day 36, very little pro-inflammatory changes were seen (Figure 3C, study IV). Upregulated gene sets of these tumors included sets not related to immune activity, but rather normal physiologic signaling (Figure 3D, study IV). Conversely, downregulated gene sets included sets related to interferon-I and viral replication, possibly linked to already cleared viral infection (Figure 3D, study IV). However, when assessing the non-injected tumors of from day 36, upregulation of gene sets related to immune cell chemotaxis and migration was noted. This was found in addition to downregulation of gene sets related to viral infection, possibly reminiscent of early viral infection of tumors (Figure 3F, study IV). This contrast between non-injected and injected tumors on day 36 could be reasoned by viral spread between metastases: it is possible that in injected tumors potent immune activity had already been completed by the time of biopsy collection, and conversely the non-injected tumors were now being transduced by the virus which had been able to escape the immune response in the injected tumors. Interestingly, when assessing transcriptomic differences between patients with long and short survival (more or less than 120 days), we observed that patients benefitting from the treatment showed downregulation of immune activated gene sets and viral replication on all available timepoints (Figure 3G, study IV). Although counterintuitive at first, we reasoned that this could be arising from a more fit immune system of the patients who benefitted from the therapy: these patients mounted stronger responses against TILT-123 and conversely cleared the virus from the tumors quicker, leading to downregulation of immune activity at the biopsy sampling time.

5.4.4 Intratumoral Ad5/3-E2F-d24-hTNF-IRES-hIL2 mRNA transcripts correlate with longer overall survival and lymphocyte count decrease

To further study the transcriptomics of the tumors, we assayed mRNA transcripts targeting mRNA molecules in TILT-123, more specifically the 24 bp deleted E1A region of TILT-123, in addition to hexon and fiber mRNA transcripts. No significant differences in upregulation of any transcripts could be seen between timepoints, although day 36 injected tumors showed greatest amounts of transcripts (Figure 4A, study IV). However, correlation of these transcripts to overall survival showed interesting findings. The amount of transcripts in non-injected tumors on day 36 correlated significantly to overall survival time, possibly mirroring successful systematic spread of the vector (Figure 4B, study IV). Additionally, when assessing the relation of lymphocyte count decrease to the amount of TILT-123 mRNA transcripts, pre-treatment levels of transcripts in day 8 and day 36 non-injected tumors samples showed a correlation of higher TILT-123 mRNA transcripts to lymphocyte count decrease post-therapy (Figure 4C-D, study IV), This finding linked our finding of lymphocyte count decrease to the presence of TILT-123 in the tumor, shedding light on the mechanism behind lymphocyte count decrease and TILT-123 treatment.

5.4.5 Ad5/3-E2F-d24-hTNF-IRES-hIL2 clinical benefit is linked to stronger systemic pro-inflammatory changes and absolute lymphocyte count decrease associates with longer overall survival

In study IV, we further aimed to characterize the systemic effects present after TILT-123 therapy, and to see if these observations correlated with clinical benefit. Memory subset analysis of PBMCs collected during the trial showed increased amounts of effector memory CD8+ T cells, without significant changes in other memory populations (Figure 5A, study IV). Interestingly, the amount of effector memory cells present also correlated to longer overall survival time (Figure 5B, study IV). No significant changes in the amounts of CD4+ memory subsets could be observed (Supplementary Figure 4B, study IV).

We then aimed to assess if the patients displaying large lymphocyte decreases after therapy showed differential peripheral immune status prior to therapy. When looking at blood proteomics, patients with large lymphocyte count decrease showed markedly low amounts of immune activity prior to treatment (Figure 5C, study IV). Accordingly, enrichment for gene sets showed downregulation of pathways related to immune cell chemotaxis and response (Figure 5D, study IV). However, after administration of the 1st dose of TILT-123 we could see markedly more interferon gamma and IL-2 in the serum, suggestive of a stronger immune activity (Figure 5E).

Finally in study IV we aimed to study if lymphocyte count decrease could predict overall survival. Both, larger average relative and absolute lymphocyte count decrease predicted longer overall survival when patients were split into two groups by the median change (Figure 6A-B, study IV). Additionally, by just utilizing day 1 absolute lymphocyte count change, a larger decrease similarly predicted longer survival (Figure 6C, study IV). Finally, we aimed to validate our findings in a separate cohort of patients treated in an individualized therapy program of oncolytic adenoviruses. These patients were of similar advanced disease and refractory to current treatment, as patients in TUNIMO (Supplementary Table 5). A total of 96 patients were available with lymphocyte counts before therapy and one day after therapy. Patients in this validation set showed a median decrease of 42.1% in peripheral lymphocyte count after the first therapy (Figure 6D, study IV). When stratifying patients into groups by the level of lymphocyte count decrease, we could observe that patients with the largest lymphocyte count decrease (-50% or less), had significantly longer survival when compared to other groups (Figure 6E, study IV).

These results highlight the interaction of TILT-123 therapy with the patients' immune system. Furthermore, these studies provide information regarding rational design of biomarkers of oncolytic virus therapy. Our results presented in study IV show that immune fitness could be a key contributor to the success of oncolytic adenovirus therapy. Similarly, other studies have noted that stronger proinflammatory changes after OV therapy correlate with better tumor control, but thus far no conclusive biomarker have been proposed to measure this fitness (123, 158, 171, 172, 174, 175, 177, 181). Our results in study IV propose, that lymphocyte count measurements can be a usable biomarker of OV therapy. Lymphocyte count measurements are available at all hospitals and are easily included in laboratory testing done during standard oncological treatment. However, further studies are needed to identify a possible threshold level for lymphocyte decrease needed for beneficial treatment outcome, if such level exists. Additionally, it should be noted that this biomarker is by definition only able to be assessed in the current form on-treatment, and more research should be done to translate this into a biomarker used before any treatment is given.

LIMITATIONS OF THE STUDIES

All research is conducted within the limits of the model systems utilized and outcomes measured. Thus, in addition to acknowledging the results of the research, also the limitations of the research should be addressed.

Studies I and II present pre-clinical research of oncolytic adenovirus in vitro, ex vivo and in vivo. An important limitation of cancer models utilized in laboratory cancer research is the difference of these models to actual human cancer. This is especially prevalent in cancer cell lines, which represent an aggressively dividing and homogenous cell population, whereas most human tumors are a heterogenous group of cells with only a certain population of cells replicating (233). Similarly, tumors established from cell lines often lack this heterogeneity present in human tumors, and important hurdles of therapeutics such as stromal barriers can be left underappreciated (234).

Study II utilized samples collected from human patients undergoing cancer surgery, and utilization of such ex vivo models help to reduce some difference between laboratory research and human subjects. However, as in study II, ex vivo experiments are usually conducted after samples are dissociated mechanically and enzymatically in order to utilize them for different laboratory research tools, but this dissociation also leads to loss of tissue architecture, an important consideration for therapeutic development. Additionally, in the laboratory, samples are often cultivated in medium rich in nutrients and oxygen, which are often limited in actual human tumors (235).

Studies III and IV present clinical and translational research of oncolytic adenovirus TILT-123 in humans in a phase I trial. Although the first step in uncovering the effects of TILT-123 in humans, important limitations are included in clinical trials. Clinical trials are by nature designed to answer certain questions, such as safety, pharmacodynamics, pharmacokinetics, and efficacy. Thus, clinical trials enroll patients by specific criteria, in order to answer these questions most efficiently. These enrollment criteria often leave out patients with attributes that increase the risk for unexpected outcomes, such as extremely advanced cancer and unmanaged co-morbidities (236). Thus, results of clinical trials can only be applied to the patient population included in the trial. The clinical trial in studies III and IV enrolled patients with different types of advanced solid cancer refractory to conventional treatments. This creates bias to many measured outcomes, especially overall survival since different cancer types often have widely different aggressiveness. Additionally, imaging techniques can lead to bias, since both CT and PET imaging have known faults in judging treatment response, most

importantly due to pseudoprogression arising from infiltrating lymphocytes and tumor swelling (237). This was also evident in studies III and IV, since dose given and lymphocyte decrease seemed to affect PET imaging results, along with patients with progressive disease by CT being alive for extended periods after therapy cessation. Furthermore, since the trial was a phase I clinical trial, naturally no control arm was present, and no blinding was done.

Study IV aimed to develop biomarkers of oncolytic adenovirus therapy and profile the immunological changes in the patients by utilizing samples collected along the trial. The most important limitation of the study was the heterogenous and limited number of patients in the study, along with the fact that all sample types were not available from all patients due to known challenges of clinical research and sample collection. Thus, results of the study should be interpreted with caution, and ideally repeated in other clinical trials of oncolytic adenoviruses. The promising biomarker of lymphocyte decrease identified in study IV also has a built-in limitation that the assessment is by definition made after the patient receives TILT-123, and thus is not useful in predicting patients' response to TILT-123 before enrollment. Furthermore, since studies III and IV focused on the virus further in development (TILT-123), the results of these studies are not necessarily applicable for future generations of IL-2 encoding oncolytic viruses, such as Ad5/3-E2F-d24-vIL2 utilized in studies I and II.

SUMMARY AND CONCLUSIONS

This thesis presents translational research on oncolytic adenoviruses encoding for modified or unmodified versions of IL-2. Oncolytic viruses have been used in humans for 25 years, and although great progress has been made, more research is needed to establish optimal combinational treatments to OVs, in addition to effects of OV therapy in humans. The findings presented in this thesis show that oncolytic adenoviruses therapy synergizes with paclitaxel and gemcitabine therapy. Mechanism behind the synergy is likely due to synergistic cell cycle modulatory effects of OVs and paclitaxel, and potentiation of immunogenic cell death induced by these agents. Furthermore, the in vivo evaluation highlighted the beneficial role of the immunostimulatory effect of oncolytic virus treatment when combining the treatment to chemotherapy. The second study presented in the thesis highlights the possibility of combining oncolytic adenovirus to allogenic NK cell therapy. The study showed enhanced cytotoxicity of NK cells when NK cell therapy was combined with OV therapy in vitro. These findings were confirmed in vivo, where combination of allogenic NK cell therapy to OV therapy showed superior tumor control and immune cell activation.

The studies arising from the TUNIMO clinical trial provided information on the effects of oncolytic adenovirus therapy in humans. The third study of this thesis showed that the administration of TILT-123 oncolytic adenovirus was safe in humans, and that favorable tumor control as well as long overall survival was seen in selected patients. The final study of this thesis focused on the immunostimulatory effects of TILT-123 therapy in humans. The results showed systemic and intratumoral proinflammatory changes in patients treated with TILT-123. Furthermore, the study was able to show that lymphocyte count decrease was a prognostic marker of TILT-123 therapy, producing valuable information for future studies of oncolytic viruses.

In conclusion, oncolytic adenoviruses encoding different forms of IL-2 can be combined with different oncological treatments safely and with synergistic anti-tumor effects. The interaction of the human immune system with oncolytic virus treatment is a key determinant of therapy success. Further studies will show how combination of other oncological treatments affects oncolytic virus therapy and the interaction with the human immune system.

6 FUTURE PROSPECTS

Great strides have been made in the treatment of cancer during the past decades. The era of immunotherapy has opened completely new avenues for treatment of cancer, and the field of oncology is likely just starting to tap the potential of harnessing the immune system for the treatment of cancer. Although checkpoint inhibitors revolutionized treatment of metastatic melanoma and select other cancers, similarly drastic responses have not been seen in most cancer types. It is thus likely that either 1) new immunotherapeutics with different of mechanism of action or 2) immunotherapeutic combinations will usher forward the next wave of immunotherapy innovation in oncology.

Oncolytic viruses have now been used in humans for roughly 25 years. Cures and responses have been observed in various solid cancers, but responses rates have generally been lower than the expected complete cures often observed in laboratory animals. However, the rationale behind oncolytic viruses remains solid, and thus future clinical research with oncolytic viruses will show how the field progresses in the future. As is evident from previous clinical trials of oncolytic viruses, likely to achieve high response rates, oncolytic viruses should be combined with other oncological treatments, such as chemotherapy, checkpoint inhibitors or cell therapy. Additionally, clinical translation has provided new challenges, especially relating to the treatment administration route: most oncolytic viruses are developed pre-clinically as intratumoral treatments due to ease of administrability in animal models compared to intravenous administration. However, in humans, the situation is reversed: intratumoral administration is challenging to perform except for superficial lesions due to the need for specialized personnel and equipment needed to perform injections to deep tumor lesions. Thus, additional development is needed to generate agents for intravenous administration, in order to facilitate therapy for most cancer patients.

The oncolytic adenovirus investigated in studies III and IV, TILT-123, is currently being evaluated in multiple different combinatorial studies: NCT04217473 is combining TILT-123 with TIL therapy for the treatment of checkpoint inhibitor refractory melanoma, and has now concluded recruitment and published reports are awaited, with a case-report of a complete response published (238). Other ongoing combinatory trials of TILT-123 include combination with anti-PD-L1 ICI in checkpoint inhibitor refractory melanoma or head and neck cancer (NCT05222932), combination with anti-PD-1 ICI in NSCLC (NCT06125197) and combination with anti-PD-1 ICI and pegylated liposomal doxorubicin in ovarian cancer (NCT05271318). Simultaneously, some oncolytic viruses such as

CG0070 are at advanced stages of clinical trials, with regulatory approval possibly on the horizon.

However, interest should also be placed on the human side of the advancement: as new immunotherapeutics and combinations are evaluated, knowledge and treatment of toxicities related to immunotherapy should be advanced concurrently to treatment innovation. Additionally, many immunotherapies are becoming increasingly expensive due to increasing development costs, and thus even if great new therapeutics are approved, those might only be available for the most affluent nations and citizens. Careful evaluation should be placed on the development of the clinical trial landscape to safely reduce regulatory barriers. The COVID-19 pandemic proved that even advanced therapeutics can be safely and swiftly developed when there is joint interest and investment. Learnings from the pandemic hopefully lead to innovations in the clinical trial landscape, leading to decreases in regulatory time approval of cancer therapeutics, ultimately translating to the meaningful end product of increased healthy years for current and future patients with cancer.

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