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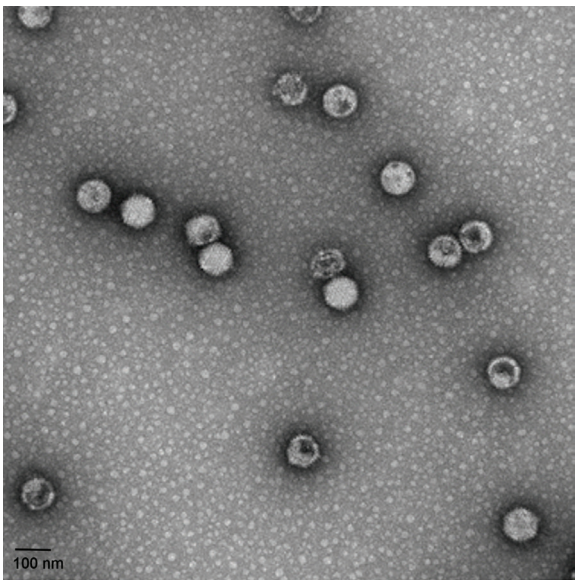
Enhancing Adoptive Cell Therapy of Solid Tumours with Armed Oncolytic Adenoviruses



CANCER GENE THERAPY GROUP
DEPARTMENT OF ONCOLOGY
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DOCTORAL PROGRAMME IN CLINICAL RESEARCH
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ENHANCING ADOPTIVE CELL THERAPY OF SOLID TUMOURS WITH ARMED ONCOLYTIC ADENOVIRUSES

Riikka Havunen

ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Medicine of the University of Helsinki, for public examination in lecture room 1, Haartman Institute, on 9.11.2018, at 12 noon.

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Image on cover: Scanning electron microscopy image on Ad5/3-E2F-d24-hTNFa-IRES-hTNFa (data provided by Cristina Peixoto, Erin M. Tranfield, and Ana Laura Sousa from Instituto de Biologia Experimental e Tecnológica (iBET) and the Electron Microscopy facility (IGC), Oeiras, Portugal).

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ABSTRACT

Adoptive T-cell therapies (ACT) are emerging as essential treatments for cancer patients with immunologically active tumours, such as melanoma. Immunologically silent tumours, however, require further stimulation. Oncolytic viruses provide an intriguing option for immune system activation, as they induce danger signalling, immunogenic cell death, and tumour epitope spreading. This study investigated oncolytic adenovirus coding for human Tumour Necrosis Factor alpha (TNFα) and Interleukin 2 (IL2) as an enhancer for ACT. Syrian hamsters permissive for human adenovirus replication provided a model for tumour-infiltrating lymphocyte (TIL) therapy with oncolytic Ad5/3-E2F-d24-hTNFα-IRES-hIL2. Complementary studies were conducted in mice with replication-incompetent viruses, providing data on adenovirally-delivered transgenes with receptor-modified T cells. Both replication-incompetent viruses and oncolytic viruses were able to enhance the antitumour efficacy of ACT. Combined with TIL therapy, Ad5/3-E2F-d24-hTNFα-IRES-hIL2 was able to cure 100% of the animals from tumours. The cured animals resisted tumour rechallenge, indicating formation of immunologic memory. TNFα enhanced chemokine expression in tumours, which attracted the infused cell graft into tumours. The transgenes also induced the presence of T cells, B cells, natural killer cells, and antigen-presenting cells in tumours, yet they lowered the levels of immunosuppressive M2 macrophages. Moreover, local treatment induced systemic antitumour efficacy in non-injected distant tumours. Both tumours showed similar profiles in intratumoural immune cells, indicating systemic changes in the immune system. The animals treated with cytokine-armed viruses showed no signs of systemic toxicity. Furthermore, the local delivery of IL2 was safer and more efficient than systemic IL2 in regard to ACT, suggesting that adenovirally delivered IL2 could replace the toxic, systemic IL2 in ACT protocols. To conclude, oncolytic adenovirus coding for immunostimulatory cytokines is a potential enabler for T-cell therapies.

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Riikka Havunen

Helsinki, October 2018

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

I. Siurala M., **Havunen R.**, Saha D., Lumen D., Airaksinen A. J., Tähtinen S., Cervera-Carrascon V., Bramante S., Parviainen S., Vähä-Koskela M., Kanerva A., and Hemminki A. **Adenoviral Delivery of Tumor Necrosis Factor-α and Interleukin-2 Enables Successful Adoptive Cell Therapy of Immunosuppressive Melanoma.** Molecular Therapy (2016); 24(8): 1435–1443.

II. **Havunen R.**, Siurala M., Sorsa S., Grönberg-Vaha-Koskela S., Behr M., Tähtinen S., Santos J. M., Karell P., Rusanen J., Nettelbeck D. M., Ehrhardt A., Kanerva A., and Hemminki A. **Oncolytic Adenoviruses Armed with Tumor Necrosis Factor Alpha and Interleukin-2 Enable Successful Adoptive Cell Therapy.** Mol Ther Oncolytics (2016); 31(4): 77-86.

III. **Havunen R.**, Santos J. M., Sorsa S., Rantapero T., Lumen D., Siurala M., Airaksinen A. J., Cervera-Carrascon V., Tähtinen S., Kanerva A., and Hemminki A. **Abscopal Effect in Non-injected Tumors Achieved with Cytokine-Armed Oncolytic Adenovirus.** Submitted.

IV. Santos J. M., **Havunen R.**, Siurala M., Cervera-Carrascon V., Tähtinen S., Sorsa S., Anttila M., Karell P., Kanerva A., and Hemminki A. **Adenoviral production of interleukin-2 at the tumor site removes the need for systemic postconditioning in adoptive cell therapy.** Int J Cancer (2017); 141(7): 1458-1468

The publications are referenced in the text by their Roman numerals. Publications I and II are included in Mikko Siurala's thesis (Improving Adenovirus-based Immunotherapies for Treatment of Solid Tumours, University of Helsinki, 2017). R. Havunen contributed to all publications by participating in planning and conducting experiments, analysing the results, and writing or reviewing the manuscripts.

ABBREVIATIONS

ACK	Ammonium-Chloride-Potassium
ACT	Adoptive cell therapy
Ad5	Adenovirus type 5
Ad5/3	Adenovirus type 5 with type 3 fiber knob
ANOVA	Analysis of variance
bp	Base pair
BTLA	B and T lymphocyte attenuator
CAR-T	Chimeric antigen receptor T cells
CAR	Coxackie-adenovirus receptor
CBA	Cytometric bead array
CCD	Charge-coupled device
CMV	Cytomegalovirus
CT	Computed tomography
CTLA-4	Cytotoxic T-Lymphocyte Associated Protein 4
DMEM	Dulbecco's Modified Eagle medium
DMSO	Dimethyl sulfoxide
ELISA	Enzyme-linked immunosorbent assay
FBS	Foetal bovine serum
FDA	Food and Drug Administration
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HLA	Human leukocyte antigen
hTERT	Human telomerase reverse transcriptase
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
IL	Interleukin
IRES	Internal ribosome entry site
MDSC	Myeloid-derived suppressor cell
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt
NK	Natural killer
OVA	Chicken ovalbumin
PBS	Phosphate-buffered saline

PCR	Polymerase chain reaction
PD-1	Programmed death 1
PGE2	Prostaglandin E2
qPCR	Quantitative polymerase chain reaction
Rb	Retinoblastoma
RPMI	Roswell Park Memorial Institute medium
SPECT	Single-photon emission computed tomography
T-Vec	Talimogene laherparepvec; herpes simplex virus coding for GM-CSF
TCR	T cell receptor
TDO	Tryptophan-2,3-dioxygenase 2
TGFβ	Transforming growth factor beta
TIL	Tumour infiltrating lymphocyte
TNFα	Tumour necrosis factor alpha
TNFR	Tumour necrosis factor receptor
Treg	Regulatory T cell
VEGF	Vascular endothelial growth factor
VP	Viral particles
VSV	Vesicular stomatitis virus

1 INTRODUCTION

Cancer is the leading cause of mortality worldwide. The latest global statistics from 2012 reported the number of new cases to be 14.1 million (Globocan 2012). Locally, cancer prevalence in Finland was 33 000 new diagnoses in 2015, with a mortality rate of 12 000 (Syöpärekisteri 2017). The mortality rates are staying stable, however, despite the constantly growing cancer incidence (Globocan 2012). A great amount of research is performed to develop new treatments and to improve conventional therapies.

Conventional cancer treatments, such as surgery, chemotherapy, and radiation therapy, are still first-line treatments for most cancer patients and often effective against local tumours. However, immunotherapies appear to be an emerging field along with the traditional treatments (Qiao et al. 2016). The roots of immunotherapies are usually dated in the late 19th century, when oncologist William Coley employed a mixture of inactivated bacteria (so called "Coley's toxins") to treat patients (Bickels et al. 2002). A mycobacterium-based tuberculosis vaccine, called Bacillus Calmette-Guérin, was successfully used in the 1970s to treat superficial bladder cancer (Morales et al. 1976). The treatment is now considered the standard of care and the mechanism-of-action is strongly related to immune reactions such as the release of immunostimulatory cytokines and chemokines from the tumour (Redelman-Sidi et al. 2014). Today, the immunotherapy concept includes a variety of treatment approaches from adoptive cell therapies to antibodies, cytokines, and oncolytic viruses (Farkona et al. 2016).

The 2010s have been a golden decade for the emerging immunotherapies. Treatments such as Sipuleucel-T (a method to program a patient's dendritic cells to act against prostate cancer) and monoclonal antibodies against checkpoint receptors Cytotoxic T-lymphocyte associated protein 4 (CTLA-4) and Programmed death 1 (PD-1) were approved for clinical use (Farkona et al. 2016). The first oncolytic virus for cancer treatment in the USA and Europe, herpes simplex virus Talimogene laherparepvec (T-Vec), was also approved

at the end of 2015 (Grigg et al. 2016). Altogether, 26 immunotherapies have been approved to date, the latest being chimeric antigen receptor T cells (CAR-T) targeting CD19 on B-cell acute lymphoblastic leukemia patients in 2017 (Tang et al. 2018).

New therapies are accepted one by one, but they are mainly effective against haematological cancer types and immunologically active melanomas that bear a high mutational load (Alexandrov et al. 2013). Solid tumours often have an immunosuppressive microenvironment and are heterogeneous in nature, which is why one treatment approach is usually not enough. Immunotherapies, however, are able to counteract the immunosuppressive environment by stimulating the so-called cancer-immunity cycle described by Chen and Mellman (2013).

The cancer-immunity cycle begins with immunogenic cell death and the release of cancer cell antigens. Next, dendritic cells and other antigen-presenting cells capture the tumour antigens and present them to cytotoxic T cells. T cells then traffic to and infiltrate tumours, recognize the malignant cells, and attack them (Chen & Mellman 2013). Immunotherapeutics can induce each of these steps.

Cancer immunotherapies aim to awaken the immune system and to enable the attack against escaped tumours. Conventional treatments, such as chemotherapy and radiation therapy, induce immunogenic cell death, but oncolytic viruses have similar yet more potent effects (Inoue & Tani 2014, Apetoh et al. 2007). Cytokines like granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin 2 (IL2) enhance antigen presentation and T-cell activation. Monoclonal antibodies against CTLA-4 and PD-1 enhance T-cell activation and cytotoxicity, and adoptive T-cell therapies (ACT) increase the recognition of cancer cells (Chen, Mellman 2013). This study combined ACTs with cytokine-armed oncolytic adenoviruses to make solid tumours less immunosuppressive and to create strong but safe immune reactions against solid tumours by inducing the cancer-immunity cycle.

REVIEW OF THE LITERATURE

1.1 TUMOUR ESCAPE FROM THE IMMUNE SYSTEM

The first suggestions that the immune system might prevent tumour formation date back to the beginning of the 20th century. The statements were formulated into a cancer immunosurveillance hypothesis in the late 1950s (Dunn et al. 2002, Burnet 1957). More data supporting the hypothesis accumulated later on, and it was acknowledged that the interaction between a tumour and the immune system could even promote development of tumours invisible from the immune system (Dunn et al. 2002). Today, avoiding immune system destruction counts as one of the cancer hallmarks, referring to the universality of tumour cells having developed a mechanism to escape from the immune system (Hanahan, Weinberg 2011).

1.1.1 CANCER IMMUNOEDITING

The theory of cancer immunoediting explains the phenomenon of tumours escaping the immune system (Dunn et al. 2002). The theory, which takes the concept of immunosurveillance one step forward, comprises three phases: elimination, equilibrium, and escape. Innate immunity cells, such as natural killer (NK) cells, NK T cells, and γδ T cells, recognise malignant cells in the elimination phase and start producing interferon (IFN) γ. IFNγ then recruits more NK cells and antigen-presenting cells, such as dendritic cells and macrophages, into the cancerous tissue. Simultaneously, IFNγ promotes apoptosis in cancer cells and prevents angiogenesis (Dunn et al. 2002).

CD4+ and CD8+ T cells activate and traffic towards the tumour due to antigen presentation by the dendritic cells. Cytotoxic T cells kill sensitive cancer cells but, according to Darwinian selection, the cancer cell population shifts towards resistance during the equilibrium phase. Finally, the clones that are resistant to

elimination start to dominate during the escape phase. As a result, an observable tumour mass invisible from the immune system starts to form (Dunn et al. 2002).

1.1.2 CANCER IMMUNE EVASION

The escaped cancer cells enhance the invisibility by developing an immunosuppressive microenvironment. The cells in a tumour microenvironment secrete soluble factors that create favourable conditions for immunosuppressive cells and directly suppress immune cell activation. The changes in the immune cell milieu comprise a dominance of helper T cell phenotype Th2 over Th1, the dysfunction of antigen-presenting cells, impaired cytotoxicity of CD8+ T cells and NK cells, and induction of immunosuppressive regulatory T cells (Treg), M2 macrophages, and myeloid-derived suppressor cells (MDSC). Moreover, cancer cells can directly inhibit cytotoxicity and proliferation of CD8+ effector cells by activating checkpoint inhibitors, such as PD-1 or CTLA-4 (Wang & DuBois 2015).

1.1.2.1 Soluble factors

Cancer cells often secrete soluble factors that suppress the differentiation and maturation of immune cells or induce immunosuppressive cell phenotypes (Kim et al. 2007). Such factors include, for example, Vascular Endothelial Growth Factor (VEGF), IL10, Transforming Growth Factor (TGF) β, Prostaglandin E2 (PGE2), FasL, and CCL21 (Shields et al. 2010, Kim et al. 2007). Moreover, factors like indoleamine 2,3-dioxygenase (IDO) and tryptophan-2,3-dioxygenase 2 (TDO) deplete tryptophan, an essential amino acid for T-cell activity, from the tumour micro-environment (Pilotte et al. 2012).

VEGF, IL10, and PGE2 together induce the expression of apoptosis mediator FasL, which leads to induction of Tregs and suppression of cytotoxic CD8+ T cells (Motz et al. 2014). As a single factor, in addition to stimulating angiogenesis and in that way promoting tumour development, VEGF inhibits dendritic cell maturation, which is a crucial promoter for T cell activation

(Gabrilovich et al. 1998). IL10 has a dual role as immune suppressor and activator. IL10 traditionally relates to inhibition of helper and cytotoxic T cells, but it also stimulates T cells and NK cells (Dennis et al. 2013). High IL10 levels in serum, however, relate to poor prognosis in cancer patients, indicating the dominance of suppressive functions (Zhao et al. 2015). Like IL10, PGE2 inhibits proliferation and activation of CD4+ helper-type T cells (He & Stuart 1999).

Regarding immunosuppression, FasL has similar functions to TGFβ, which promotes Treg induction and suppresses CD8+ effector cells but also downregulates NK-cell and B-cell functions and shifts macrophage population phenotype towards immunosuppressive M2 (Beck et al. 2001). CCL21 additionally induces Tregs by altering the cytokine profile in tumours and promotes MDSC tumour infiltration (Shields et al. 2010). These two cell types are the major regulatory cell types in immunological reactions.

1.1.2.2 Suppressive immune cells

Immunosuppressive cells are essential in maintaining self-tolerance and preventing autoimmunity by suppressing immune responses. Regarding cancer, the presence of these cells, however, prevents immune reactions against tumours. Such cells include Tregs, MDSCs, and M2 phenotype macrophages.

Among the population of T cells, Tregs are distinguished by the expression of cell markers CD4, FoxP3, and CD25. They suppress the activity of effector T cells, NK cells, and dendritic cells via direct cell-to-cell interaction and by secreting IL10 and TGFβ (Wang & DuBois 2015). Patients with ovarian, esophageal, gastric, or colorectal cancer show elevated numbers of Tregs in their blood and tumours, and their presence in tumours correlates with poor prognosis (Mizukami et al. 2008, Kono et al. 2006, Curiel et al. 2004, Ichihara et al. 2003, Wolf et al. 2003).

A high number of Tregs also correlates with a high number of MDSCs (Gabitass et al. 2011). MDSCs have a close relation to both Tregs and M2-like macrophages: MDSCs induce Treg activation and expansion and skew macrophage differentiation towards M2 phenotype (Gabrilovich et al. 2012). Elevated MDSC numbers in

tumours and in blood circulation correlate with increased metastatic load, clinical stage of a cancer, and poor prognosis in several cancer types, including breast, colorectal, esophageal, gastric, and pancreatic cancers (Sun et al. 2012, Gabitass et al. 2011, Diaz-Montero et al. 2009).

The immunosuppressive effects of MDSCs comprise four strategies, which mainly affect T-cell functionality (Gabrilovich et al. 2012). First, they drive T-cell growth arrest by depleting L-arginine and L-cysteine from the tumour microenvironment (Srivastava et al. 2010, Rodriguez et al. 2004). Second, they produce reactive oxygen and nitrogen species impairing T-cell signalling (Mazzoni et al. 2002). Third, the expression of ADAM17 and peroxynitrite by MDSCs limit the T-cell migration into lymph nodes and tumour stroma, respectively (Molon et al. 2011). Finally, the MDSCs express IFNγ, IL10, and TGFβ, which promote Treg differentiation and proliferation (Gabrilovich et al. 2012).

M2-like macrophages also express IL10 and TGFβ (Gabrilovich et al. 2012). Macrophages gain the M2 phenotype in the presence of IL4, IL10, IL13, and glucocorticoid hormones (Gabrilovich et al. 2012). They are associated with poor survival, at least among classic Hodgkin's lymphoma patients, and they promote metastasis formation in breast cancer (Steidl et al. 2010, Qian et al. 2009). The soluble immunosuppressive factors secreted by the M2-like macrophages are the main cause for the M2-mediated immunosuppression (Gabrilovich et al. 2012). In addition, M2-like macrophages express PD-L1, which impairs T-cell activity when binding to its receptor, checkpoint inhibitor PD-1 (Kuang et al. 2009).

1.1.2.3 Checkpoint inhibitors

Cytotoxic CD8+ cells express safe-switch receptors – checkpoint inhibitors – that inhibit the activity and proliferation of the cell. Cancer cells, however, are able to utilize these receptors when escaping from immunosurveillance (Wang & DuBois 2015). The two most investigated checkpoint inhibitors besides TIM-3 and LAG-3 are PD-1 and CTLA-4, against which there are approved blocking antibodies for treatment of melanoma (Farkona et al. 2016).

Antigen-presenting cells, including cancer cells, are able to inhibit T cells via PD-1 by expressing its ligands PD-L1 or PD-L2. Of the soluble immunosuppressive factors, at least PTEN and IFNγ induce the expression of PD-L1, and IL10 and FasL drive T-cell apoptosis upon checkpoint activation (Song et al. 2013, Dong et al. 2002). Elevated expression of the ligands has been detected broadly among different cancer types, and it usually associates with poor prognosis (Zhu et al. 2017, Song et al. 2013, Cao et al. 2011, Hamanishi et al. 2007, Ohigashi et al. 2005, Thompson et al. 2005).

Contrary to PD-1, CTLA-4 is usually activated by antigen-presenting cells other than cancer cells. Mainly dendritic cells express CD80 and CD86, which bind either to T-cell activating costimulatory receptor CD28 or to CTLA-4 causing T-cell inhibition (Walker & Sansom 2015). Hence, the competition between these two receptors determines the faith of a T cell: If CTLA-4 is blocked by an antibody, the cell will activate upon encountering an antigen-presenting cell (Walker & Sansom 2015).

CTLA-4 is also widely expressed on Tregs, which can inhibit the activation of conventional T cells via competition over CD80 and CD86. Moreover, Tregs are able to remove these ligands from antigen-presenting cells, preventing the activation of conventional T cell expressing CD28 (Walker & Sansom 2015). This complex interaction between checkpoint inhibitors, immunosuppressive cells, and soluble factors has an important function in preventing autoimmunity but also in protecting cancer. Immunotherapies, such as ACT, oncolytic viruses, and cytokine treatments, are crucial for resetting the immune system in tumour microenvironment.

1.2 ADOPTIVE T-CELL THERAPY

ACTs are based on patient-derived immune cells that are modified and expanded before administrating them back into the patient (Rosenberg & Restifo 2015). The cells derive from the patient's peripheral blood or straight from the tumour. If extracted from the blood, a population of T cells becomes tumour-specific when modified to express tumour-associated, antigen-specific T cell receptors (TCR) or chimeric antigen receptors (CAR-T). Tumour-infiltrating lymphocytes (TILs) have the ability to recognize the

tumour by nature and, thus, can also be used as such after purification and expansion. Traditionally, a patient receives the cells after preconditioning with chemotherapeutics and postconditioning with IL2 to optimize the cell graft function (Rosenberg & Restifo 2015). Both regimens, however, may cause toxic adverse events (Morgan et al. 2010, Brentjens et al. 2009, Schwartz et al. 2002). Especially TIL graft infusion itself is usually tolerable and does not cause severe adverse events (Cruz et al. 2010).

1.2.1 TUMOUR-INFILTRATING LYMPHOCYTES

The simplified idea behind TIL therapy is to extract effector T cells from a tumour, expand and activate them in a laboratory, and administer them back to the patient to provoke an attack against the tumour (**Figure 1**) (Lee & Margolin 2012). T cells derived from enzymatically or mechanically disrupted tumour extracts are expanded with IL2 for a maximum of five weeks, after which the cells are activated with a rapid expansion protocol. Anti-CD3 antibody and irradiated feeder cells induce the rapid expansion over two weeks. The cell number is expanded up to 2 000 fold before administration back to the patient (Dudley et al. 2003). The success rate for growing TILs from extracts seemed problematic in the past, but today the cells are extractable from nearly every sample (Besser et al. 2009).

A standard protocol has been shortened to produce so-called young TILs to avoid loss of T-cell activity during expansion (Itzhaki et al. 2011, Dudley et al. 2010, Tran et al. 2008). Nevertheless, the young TILs do not seem to be more efficient than the standard or CD8-enriched TILs, but they are easier to produce, have longer telomeres, and express more effector memory cell markers, such as CD27 and CD28 (Dudley et al. 2013, Donia et al. 2012, Itzhaki et al. 2011, Tran et al. 2008).

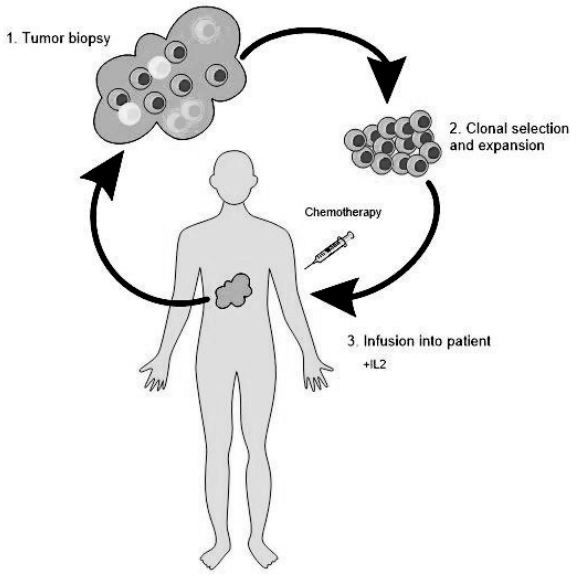


Figure 1. Simplified schematic presentation of TIL therapy. First, a tumour biopsy is acquired from a patient (1.). Next, cytotoxic CD8+ cells are extracted from the biopsy and expanded in a laboratory in the presence of IL2 (2.). After activation with IL2 and the anti-CD3 antibody, the cells are infused back into the patient often after lymphodepleting chemotherapy and IL2 administration (3.). Figure adopted from Lee & Margolin 2012.

The TIL-treatment protocol usually includes lympho-depleting pre-conditioning with chemotherapeutics like cyclophosphamide and fludarabine to clear the patient's body from endogenous T cells (Itzhaki et al. 2013). High-dose post-conditioning with IL2 improves the cell graft survival, but both of these regimens are toxic for the patients (Itzhaki et al. 2013, Schwartz et al. 2002). Meanwhile, TIL-treatment itself appears safe with mild adverse events (Jiang et al. 2015, Dudley et al. 2013, Radvanyi et al. 2012).

The first clinical trial with TILs, performed by Rosenberg et al. between the late 1980s and early 1990s, ended up with overall objective response rate of 34%, comprising complete responders and patients whose tumours shrank more than 50% (Rosenberg, Yannelli et al. 1994). Clinical trials on metastatic melanoma with different pre- and post-conditioning regimes have later shown overall response rates up to 72% (Pilon-Thomas et al. 2012, Radvanyi et al. 2012, Dudley et al. 2008). Moreover, complete responders seem to have long-term benefits from the treatment, since up to 93% of them

are alive after five years (Rosenberg et al. 2011). Importantly, TIL treatment also benefits patients who have failed other immunotherapies (Besser et al. 2013).

In addition to melanoma, TIL trials have been conducted in hepatocellular carcinoma, non-small cell lung carcinoma, ovarian cancer, and renal cell carcinoma (Jiang et al. 2015, Andersen et al. 2015, Ratto et al. 1996). TIL reactivity was increased in cervical cancer patients by selecting papillomavirus-reactive TILs for the treatment (Stevanovic et al. 2015). However, immunologically active melanoma shows currently the most promising results. In other indications, TIL therapy results in prolonged survival but complete responses are rare (Andersen et al. 2015).

The main problem for TIL therapy efficacy is poor trafficking of the cell graft into the tumour. Most importantly, tumours need to have established vasculature through which lymphocytes can arrive at the tumour site (Sackstein et al. 2017). Peritumoural blood vessels guide the lymphocytes to the tumour margins, but the intratumoural vasculature is often inadequately structured. The second important player in lymphocyte migration is the expression of chemokines that attract the cells to invade a tissue (Sackstein et al. 2017). Preclinical studies show that transducing TILs with a chemokine receptor, such as CXCR2, enhances the trafficking of the TILs into a tumour (Peng et al. 2010). Another approach is to stimulate chemokine expression in a tumour with immunostimulatory molecules, such as cytokines and pro-inflammatory agents (Atsumi et al. 2014).

In addition to improved trafficking, the enrichment of the right populations from a heterogenic T-cell pool enhances TIL functionality (Cohen et al. 2015). Only a fraction of epitopes recognized by TILs is tumour associated, and the activity of the cells can regress during expansion (Andersen et al. 2012). The rest of the TILs recognize neoantigens, viruses, or currently unknown targets (Cohen et al. 2015, Robbins et al. 2013, Andersen et al. 2012).

1.2.2 T CELL RECEPTOR-MODIFIED CELLS

As TILs are a polyclonal population of T cells recognizing different tumour associated antigens, genetic modifications turn peripheral T cells into a homogenous population of cells (Lagisetty & Morgan

2012). T cells are modified to express a specific TCR that recognises tumour antigens that human leukocyte antigen (HLA) complex presents on malignant cells. Retroviral or lentiviral vectors enable the genetic engineering and the personalisation of the treatment (Lagisetty & Morgan 2012).

The TCR engineering starts with the isolation of tumour-reactive T cells from a patient (Lagisetty & Morgan 2012). After receptor analysis, retroviral or lentiviral vectors deliver the receptor genes into unspecific T cells, for example, to blood mononuclear lymphocytes (Clay et al. 1999). Transduced T cells retain their natural abilities to act and proliferate and when the cells encounter their specific antigen, they respond by secreting cytokines and attacking the target cells (Zhao et al. 2007).

Some tumours lack TILs or a tumour might be difficult to reach, so it is not always possible to extract TCR genes from tumour-reactive cells. TCRs can be produced in gene-engineered mice or with phage display technology in these cases (Zhao et al. 2007, Stanislawski et al. 2001). The transgenic mice having HLA system can be immunised against known tumour-associated antigens and the genes for TCRs isolated (Lagisetty & Morgan 2012). The affinity of TCRs can be tested and optimised with the phage display method before transfer to T cells (Li et al. 2005).

The first TCR transfer to blood mononuclear lymphocytes resulting in reactivity against melanoma cell lines was successful in the late 1990s (Clay et al. 1999). Melanoma, having a high mutational load and well-characterised tumour-associated antigens, is one of the most popular targets for TCR therapy (Alexandrov et al. 2013). The first phase I trial against melanoma antigen MART-1, published in 2006, resulted in responses in 13% (two out of 15) of the patients (Morgan et al. 2006). Another trial targeting MART-1 concluded with objective response rate of 30% but also with on-target, off-tumour toxicities such as uveitis and hearing loss (Johnson et al. 2009).

Other frequent targets for melanoma TCR therapy are gp100 and NY-ESO-1. Targeting these antigens has resulted in response rates of 19% (three out of 16) and 45% (five out of 11), respectively (Robbins et al. 2011, Johnson et al. 2009). NY-ESO-1 TCR therapy also had an

effect without toxicity on four out of six patients with synovial cell sarcoma (Robbins et al. 2011).

In addition to melanoma and synovial cell carcinoma, TCR trials have been conducted on colorectal cancer against carcinoembryonic antigen for three patients, resulting in a progressive disease by six months in two patients that initially responded (Parkhurst et al. 2011). The treatment also caused inflammatory colitis in all patients. Targeting MAGE-A3 in nine patients with metastatic cancers resulted in neurological adverse events for four patients, killing two and benefiting five patients (Morgan et al. 2013). TCRs against MAGE-A4 in ten esophageal cancer patients resulted in short-term benefits for seven patients and long-term benefits for two without serious adverse events (Kageyama et al. 2015).

Key issues for an effective TCR therapy are a stable expression of the transduced gene, an abundant number of TCRs on a cell surface, and the affinity and specificity of the TCR against the antigen (Uttenthal et al. 2012). The affinity can be increased, for example, by substituting one or two amino acids in the antigen-binding region of a TCR (Dunn et al. 2006, Li et al. 2005). The murine versions of TCRs or hybrids with murine constant regions and human variable domains enhance the affinity in some cases (Johnson et al. 2009, Cohen et al. 2006). This might, however, raise a concern regarding neutralizing antibodies against foreign epitopes. Davis et al. (2010) studied serum samples from two different trials embodying murine TCRs against gp100 and p53 and discovered that 23% of the patients had developed neutralizing antibodies against these TCRs. The development of antibodies, however, did not correlate with the treatment outcome or cell persistence.

The transferred cells can persist in a patient for at least a month and even up to a year, if the patient receives preconditioning with lymphodepleting chemotherapy and postconditioning with IL2 (Johnson et al. 2009, Morgan et al. 2006). Moreover, the transferred T-cell phenotype influence the persistence. In mouse studies, for example, CD4+ cells persisted longer in the animals than CD8+ cells did with the same TCR modification (Engels et al. 2012). Furthermore, central memory or naïve T cells seem to persist longer *in vivo* than the effector cells (Hinrichs et al. 2009, Berger et al.

2008). The persistence of TCR-modified T cells is generally better than that of CAR-Ts (Uttenthal et al. 2012).

1.2.3 CHIMERIC ANTIGEN RECEPTOR T CELLS

CAR-Ts recognize antigens expressed on the cancer cell surface directly in contrast to TCRs that require activation by HLA complex-mediated antigen presentation (Rosenberg & Restifo 2015). In addition to tumour-specific proteins, CAR-Ts can recognize carbohydrates and glycolipids, giving the target selection more flexibility (Dotti et al. 2014). Thus, CAR-Ts have the characteristics of a monoclonal antibody combined with replication-competent T cells (Dotti et al. 2014).

The ectodomain of a CAR-T contains a single-chain variable fragment of an antibody that recognizes a tumour antigen, and one or more TCR signalling parts that activate the T cell (**Figure 2**) (Dotti et al. 2014, Eshhar et al. 1993). The number of intracellular signalling domains determines the classification of the CAR-Ts into first, second, or third generation (Dotti et al. 2014). CAR-Ts can also be modified, for example, to express immunostimulatory agents or receptors for cytokines that further activate the cells (Jackson et al. 2016).

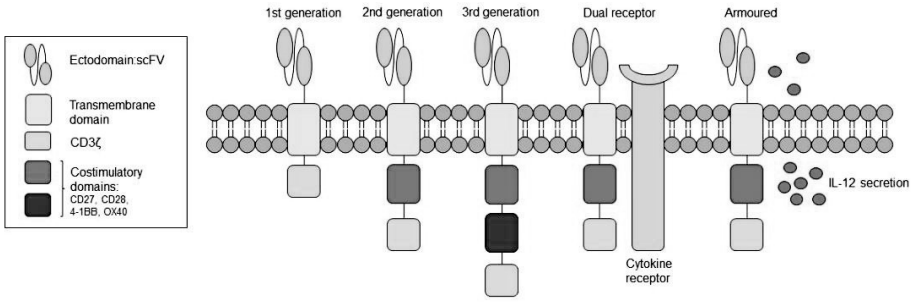


Figure 2. The structure of different generation CAR-T receptors. The extracellular domain of a CAR-T receptor has a single-chain variable fragment (scFV) of an antibody. Intracellular domains mediate the activation signals when the antibody fragment binds to its antigen. The cells can be modified to express cytokine receptors or secrete stimulatory cytokines to further enhance CAR-T functionality (Jackson et al. 2016).

Impressive response rates (up to 90%) to CAR-T therapies have been achieved especially in haematological malignancies, and the first products targeting CD19, Tisagenlecleucel and Axicabtagene ciloleucel, were approved in 2017 for B-cell lymphoma patients (Tang et al. 2018, Jackson et al. 2016). CAR-Ts targeting B-cell antigen CD19 have a proven efficacy against different types of leukemias and lymphomas, such as acute lymphoplastic leukemia, chronic lymhocytic leukemia, and non-Hodgin lymphoma (Brudno et al. 2016, Jackson et al. 2016, Porter et al. 2015, Brentjens et al. 2013). Treatment of solid tumours, however, is more challenging because of heterogeneity in the cell surface markers and more complex tumour microenvironment (Jackson et al. 2016).

Potential markers for solid tumours are, for example, HER2, EGFR, and GD2 (Newick et al. 2017); however, the best results from the conducted clinical trials are modest. Treatment of liver metastases with CEA-targeted CAR-Ts halted tumour progression in one out of six patients (Katz et al. 2015). Four out of 17 patients with HER2-positive sarcoma resulted in a stable disease when treated with HER2-specific CAR-Ts (Ahmed et al. 2015). Treatment of non-small-cell lung cancer targeting EGFR led to partial responses in two out of 11 patients (Feng et al. 2016). The only complete responses reported thus far occurred in neuroblastoma patients when targeting GD2: Three out of 11 patients obtained complete remissions (Louis et al. 2011). The treatment with CAR-Ts in these trials did not cause severe adverse events.

A common adverse event encountered with CAR-T therapy of haematological cancers is cytokine release syndrome (Brudno & Kochenderfer 2016). Cell infusion causes elevation in IL6, IFNγ, IL2, IL8, and IL10 serum levels, among others (Brudno & Kochenderfer 2016). The symptoms include fever, rash, and nausea, in addition to more severe cardiovascular and neurologic adverse events (Lee et al. 2014). The symptoms are controllable to some extent with corticosteroids or tocilizumab, an antibody against IL6 receptor, but in the worst cases, they threaten the patient's life (Maude et al. 2014). Off-target toxicities and on-target off-tumour toxicities additionally cause adverse events (Brudno & Kochenderfer 2016).

The challenge of CAR-T therapy is to select an antigen that is common solely on tumour to avoid on-target, off-tumour toxicities.

The prediction of off-tumour effects is difficult, because the preclinical studies are done in animals. The studies often employ species-specific CAR-Ts, since the ectodomains in human CAR-Ts rarely recognize the corresponding non-human molecule, or the expression of the target might differ between the species (Dotti et al. 2014). The target antigen should also be present on the majority of cancer cells to minimize the risk for immune escape. One way to lower the off-target toxicities and the risk for immune escape is to use a bispecific ectodomain that recognizes two different antigens (Zah et al. 2016, Hegde et al. 2016). The use of two different CAR-T populations might also prevent immune escape and increase efficacy (Feng et al. 2017).

The efficacy of a CAR-T depends on the affinity of the single chain variable fragment and the distance of the recognized epitope from the target cell surface (Haso et al. 2013, Hudecek et al. 2013). A limitation of the first generation CAR-Ts' efficacy has been short persistence and weak proliferation of the cells in a patient (Jensen et al. 2010). Second and third generation CAR-Ts have better efficacy and differences between these two are negligible (Jackson et al. 2016). To improve CAR-T efficacy, the cells could be administered, for example, with checkpoint inhibitor antibodies, oncolytic viruses, or cytokines (Newick et al. 2017).

1.3 CYTOKINES IN IMMUNOTHERAPY

Immune cells secrete cytokines to regulate and modify immunological reactions, cell proliferation and differentiation, angiogenesis, and cell death (Vacchelli et al. 2015). Regarding recombinant cytokines in the treatment of cancer, the Food and Drug Administration (FDA) approved IFNα for hairy cell leukemia in 1986 and IL2 for metastatic renal cell carcinoma in 1992 and later for melanoma (Floros & Tarhini 2015). In addition to the approved cytokines, clinical work is ongoing with several other cytokines, such as GM-CSF, IFNγ, IL8, IL12, IL15, IL18, and IL21 (Vacchelli et al. 2015, Floros & Tarhini 2015). The results have been modest and adverse events severe regarding monotherapies, which is why current trials often study cytokines in combination with other

immunotherapies, chemotherapy, or radiation therapy (Floros & Tarhini 2015).

1.3.1 INTERFERON BETA

IFNs were discovered in the 1950s and named after their ability to interfere with viruses in infected cells (Razaghi et al. 2016). IFNs fall into to three distinct groups: Type I, II, and III. IFNβ belongs to Type I IFNs, together with IFNα, IFNε, IFNκ, and IFNω (Zitvogel et al. 2015). Mainly fibroblasts and plasmacytoid dendritic cells produce IFNβ as a response to a virus, lipopolysaccharide, or double-stranded RNA (Farrar & Schreiber 1993).

Like all Type I IFNs, IFNβ binds to the heterodimeric IFNα/β receptor consisting of IFNAR1 and IFNAR2 and signals through the Jak-Stat pathway (Samuel 2001). Tyk2 and Jak1 interact with IFN receptors and phosphorylate Stat2 and Stat1, respectively. Next, Stats form a complex with IRF-9, which then acts as a transcription activator (Samuel 2001). The main function for all IFNs is to produce antiviral proteins and induce antigen presentation, but IFNβ uniquely affects, for example, Hypoxia-inducible factor-1 and Protein kinase R (Der et al. 1998). On the contrary, IFNβ is a less potent inducer of pro-apoptotic genes compared with IFNα and IFNγ (Der et al. 1998). Regarding effects on immune cells, Type I IFNs in general enhance T-cell and NK-cell cytotoxicity and induce dendritic cell maturation and migration (Zitvogel et al. 2015).

Defects in Type I IFN signalling or production have been associated with mammary carcinogenesis (Zitvogel et al. 2015). Despite its cell proliferation restricting and immune system stimulating functions, IFNβ has not been that promising against cancer. Several clinical trials have studied the efficacy of IFNβ in combination with standard treatment for glioblastoma, breast cancer, pancreatic cancer, and non-small-cell lung cancer (Utsuki et al. 2011, Recchia et al. 2009, Colman et al. 2006, Bradley et al. 2002, Recchia et al. 1998, Repetto et al. 1996). The results, however, show poor effects on survival and tumour growth control, even though the rationale behind the treatment is strong: Many conventional therapies, such as chemotherapy and radiation therapy, work

through Type I IFN induction (Zitvogel et al. 2015). IFNβ is currently an approved treatment for multiple sclerosis (Samuel 2001).

1.3.2 INTERFERON GAMMA

IFNγ is the only member of Type II IFNs. It is the most active among the interferon protein family, being up to 10 000 times stronger an immune-modulator than the other IFNs but less specific for antiviral activities (Pace et al. 1985). It was discovered in 1965 as a response to virus infection, and its structure was resolved with X-ray crystallography in 1991 (Ealick et al. 1991, Wheelock 1965). CD8+ T cells, Th1 helper cells, and NK cells produce IFNγ when stimulated with antigens and mitogens (Farrar & Schreiber 1993). IL12 also stimulates the production (Wolf et al. 1991).

IFNγ appears as a soluble 34-kDa homodimer (Farrar & Schreiber 1993). It binds to the heterodimeric IFNγ receptor consisting of IFNGR1 and IFNGR2 that are expressed on most cell types. Binding of IFNγ to its receptors activate Jak1 and Jak2, which phosphorylate Stat1. Stat1 forms a homodimer known as gamma activation factor that activates gene expression through GAS enhancer element (Samuel 2001).

All IFNs regulate HLA class I expression on most cells, but IFNγ can also induce class II expression on other than B cells, where it regulates immunoglobulin production (Samuel 2001, Snapper & Paul 1987, Mond et al. 1986). The most noticeable responders for IFNγ are monocytes and macrophages in which IFNγ promotes expression of nitric oxide synthase that catalyses the production of antimicrobial and antiviral nitric oxide (Samuel 2001). IFNγ also promotes antigen presentation and interaction between T cells and macrophages (Farrar & Schreiber 1993). Regarding other than immune cells, IFNγ stimulates cell death in apoptosis-resistant cell lines by upregulating caspase 8 (Fulda & Debatin 2002).

The clinical results against cancer are modest despite the positive effects on immune reactions and driving cells to apoptosis (Razaghi et al. 2016, Samuel 2001). IFNγ stimulates cytotoxic and helper T cells, NK cells, and B cells in non-invasive bladder cancer patients (Giannopoulos et al. 2003). In ovarian cancer patients, the addition of IFNγ to a standard treatment increases progressive free survival,

but the effect on overall responses is questionable (Alberts et al. 2008, Windbichler et al. 2000). IFNγ has no efficacy against renal cell carcinoma or colon cancer (Gleave et al. 1998, Wiesenfeld et al. 1995). Treatment with recombinant IFNγ causes flu-like symptoms and neutropenia as adverse events (Marth et al. 2006, Windbichler et al. 2000).

1.3.3 TUMOUR NECROSIS FACTOR ALPHA

As the name suggests, Tumour Necrosis Factor alpha (TNFα) first appeared as a substance causing tumour cell apoptosis and necrosis, but it is currently associated mainly with inflammation (Baud & Karin 2001, Carswell et al. 1975). TNFα belongs to a TNF superfamily consisting of transmembrane type II proteins with intracellular N-terminus. TNFα also acts as a trimeric soluble protein when proteolytically cleaved by ADAM17 (Black et al. 1997). Both soluble and transmembrane forms are active and mainly produced by activated macrophages, monocytes, lymphocytes, keratinocytes, and fibroblasts (Baud & Karin 2001). The crystal structure of the trimer was described in 1989; it consists of monomers 17 kDa in size (Eck & Sprang 1989).

TNFα signals through two members of the TNF receptor (TNFR) superfamily, TNFR1 and TNFR2. TNFR1 is present on most cell types, whereas TNFR2 is mainly on immune cells and endothelial cells (Brenner et al. 2015). The binding of TNFα to TNFR1 leads to the recruitment of TRADD (TNFR-associated death domain) to interact with the receptor. TRADD mediates the signalling for apoptosis, whereas in the presence of RIP1 or TRAF2/5, the signalling leads to proliferation or inflammation via activation of MAP kinase or NFκB signalling pathways, respectively (Brenner et al. 2015, Baud & Karin 2001). Activation of TNFR2 mostly leads to cell survival signalling via NFκB. TNFα also stimulates the production of chemokines, cytokines, and growth factors, such as CXCL9, CXCL10, CCL17, IL1α, IL8, GM-CSF, and TGFα (Liu et al. 2007, Janes et al. 2006, Balkwill 2006, Tessier et al. 1997). The outcome of the signalling depends on the presence of intracellular mediators and other cytokines and growth factors (Janes et al. 2006, Pimentel-Muinos & Seed 1999).

TNFα has a conflicting role as causing not only cancer-driving chronic inflammation but also tumour cell necrosis (Balkwill 2006). Because of its cancer-promoting features, trials have been conducted with anti-TNFα antibody. These treatments, however, might also increase cancer risk (Askling et al. 2011). Historically, TNFα appears to be an active component of Coley's toxin, William Coley's experimental treatment for cancer patients in the late 19th (Bickels et al. 2002). Recombinant TNFα was studied in the 1980s as a systemic treatment in clinical trials, but the major problems occurred with dose-limiting toxicities and lower doses led to modest responses (Wiedenmann et al. 1989). Low doses, however, induce positive changes in the activation status of patient PBMCs, indicating that TNFα might have an important role in inducing system-wide immune reactions against tumours (Logan et al. 1997). Intralesional application is safer than systemic, but the responses remain local (Bartsch et al. 1989).

Recombinant TNFα was later studied in an isolated limb perfusion setting for regional treatment of sarcomas and melanomas. Positive results led to the approval of TNFα for clinical use in 1998 (van Horssen et al. 2006). The overall response rates for limb perfusions are impressive: 76% for sarcomas and up to 100% for melanomas. Of melanoma patients, complete responders comprise over 70% (van Horssen et al. 2006). The mechanism behind antitumour efficacy lies in induced tumour necrosis, early infiltration of lymphocytes and macrophages, and in hampering the tumour metabolism by affecting its vasculature (van Horssen et al. 2006, Lejeune et al. 1998). The treatment, however, may cause severe adverse events, such as hypotension with tachycardia and kidney failure (Lienard et al. 1992). Improved safety of systemic delivery was achievable lately with modified TNFα and vectored delivery with even higher antitumour efficacy (Li et al. 2012, Li et al. 2010, McLoughlin et al. 2005).

1.3.4 INTERLEUKIN 2

First nominated as T-cell growth factor, IL2 is a strong stimulator for T-cell propagation and differentiation. The crystal structure of this 15 kDa monomeric glycoprotein was published in 1992 (Bazan 1992).

IL2 belongs to the Type I cytokine family, and it is secreted mainly by CD4+ and CD8+ T cells but also by B cells and dendritic cells to a lesser extent (Gaffen & Liu 2004). Binding of an antigen to its receptor in effector T cells rapidly initiates the synthesis of IL2, thus creating a positive loop for activated T-cell expansion (Lenardo et al. 1999).

IL2 affects cells by binding to a trimeric receptor complex consisting of IL2 receptor alpha, beta, and gamma (Gaffen & Liu 2004). At least the two latter components are necessary for signalling pathway activation (Nelson et al. 1994). The signalling cascade starts with tyrosine kinases Jak1 and Jak3, which leads to activation of transcription factor Stat5 or further signalling via MAPK or PI-3K pathways (Malek & Castro 2010).

The signalling cascade influences lymphocyte proliferation via c-Myc and c-Fos, in addition to inhibiting apoptosis via Bcl-2 (Miyazaki et al. 1995). Importantly, IL2 also controls immune reactions via a negative feedback loop by inducing the expression of pro-apoptotic FasL, thus preventing autoimmunity (Refaeli et al. 1998). Moreover, the half-life of recombinant IL2 in humans is only minutes (Lotze et al. 1985). In addition to T cells, IL2 stimulates B and NK cells (Gaffen & Liu 2004).

The FDA approved the use of recombinant IL2 as a treatment for metastatic melanoma and renal cell cancer in 1998. The overall response rate for melanoma patients, however, is only 16%, and the proportion of complete responders is 6% (Atkins et al. 1999). The overall objective response rate for patients with metastatic renal cell cancer is 20%, and 9% result in complete response (Klapper et al. 2008). Treatment with recombinant IL2 correlates with enhanced tumour infiltration of CD4+ and CD8+ T cells and macrophages (Rubin et al. 1989). A major downside of IL2, however, is induction of immunosuppressive Tregs (Ahmadzadeh & Rosenberg 2006).

In addition to low response rates, treatment with high doses often correlates with severe adverse events, such as cytokine release syndrome and vascular leak syndrome, leading to liver cell damage and renal failure (Panelli et al. 2004, Rosenberg et al. 1994, Rosenberg et al. 1987, Lotze et al. 1985). At worst, high-dose IL2 has caused mortality in 4% of the patients (Schwartz et al. 2002). Lowering the dose diminishes adverse events with a cost in efficacy

(Yang et al. 2003). Fortunately, the toxicities are transient in most cases (Atkins et al. 1999).

Histological evaluation of renal cancer patients before treatment can improve the response rate from 27% up to 52%, lowering the risk-to-benefit ratio (Shablak et al. 2011). Local administration also appears more efficient and safer than systemic delivery (Ray et al. 2016, Weide et al. 2011). Several genetically engineered variants of IL2 and fusion proteins are under development to maximize efficacy and to minimize adverse effects and Treg stimulation (Rosalia et al. 2014). Moreover, *in vivo* results and phase I clinical trials suggest that vectored delivery of IL2 is safer and more efficient than the administration of recombinant protein (Dummer et al. 2008, Trudel et al. 2003, Slos et al. 2001).

1.4 ONCOLYTIC VIRUSES IN IMMUNOTHERAPY

Oncolytic viruses selectively replicate only in cancer cells. The lytic effect self-amplifies at the tumour while normal cells remain intact (Chiocca & Rabkin 2014). Genetic modifications enable oncolytic viruses to take advantage of the abnormal functions of malignant cells, but some viruses have this character by nature. Natural oncolytic viruses include parvoviruses, myxoma virus, Newcastle disease virus, reovirus, Seneca Valley virus, and coxsackievirus (Dharmadhikari et al. 2015, Chiocca & Rabkin 2014). Measles virus, poliovirus, vaccinia virus, herpes simplex virus, vesicular stomatitis virus, and adenovirus can also be made cancer selective by genetic engineering (Chiocca & Rabkin 2014).

Currently, there are 69 oncolytic viruses in clinical trials and 95 in the preclinical stage (Tang et al. 2018). An unarmed oncolytic adenovirus, H101 by Sanghai Sunwaybio, was approved for treating nasopharyngeal cancer in China in 2005, and oncolytic herpes simplex virus by Amgen coding for GM-CSF, T-Vec, was approved ten years later in the USA and Europe (Grigg et al. 2016, Garber 2006). A phase III clinical trial with a combination of H101 and chemotherapy resulted in a 79% response rate, improving chemotherapy alone by 39 percentage units (Garber 2006). T-Vec yields an objective response rate of 26.4%, comprising complete responders (10.8%) and partial responders (15.6%) (Andtbacka et al.

2015). Other oncolytic viruses in phase III trials include an adenovirus coding for thymidine kinase (ProstAtak by Advantagene) and an adenovirus coding for GM-CSF (CG0070 by Cold Genesys) (Kaufman et al. 2015). Several other adenoviruses, herpesviruses, reovirus, Seneca Valley virus and coxsackievirus are also in phase II trials (Kaufman et al. 2015).

The responses to oncolytic viruses derive both from direct cancer cell lysis and from immune reactions (**Figure 3**). Oncolytic viruses induce immunogenic cell death, which is a stronger activator for antitumour immunity than apoptotic cell death (Inoue & Tani 2014, Kepp et al. 2009). The immunogenic cell death releases pathogen- and danger-associated molecular patterns into the tumour microenvironment, which helps the immune system to recognize the infected cancer cells (Tang et al. 2012). Moreover, infection enhances the release of tumour-associated antigens, novel cancer antigens (neo-antigens), and epitope spreading, which also enable immune reactions against uninfected cells (Kaufman et al. 2015, Chiocca & Rabkin 2014).

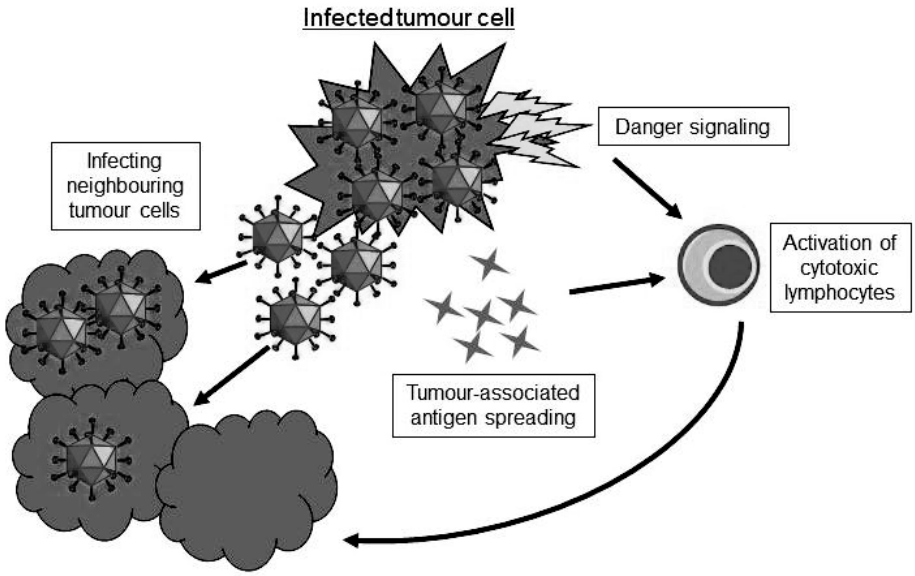


Figure 3. Oncolytic virus-mediated antitumour mechanisms. Infected tumour cell alerts immune system when expressing danger- and pathogen-associated molecular patterns. When the virus exits the cell, tumour-associated antigens are released to the tumour microenvironment, and the infection spreads to the neighbouring cells. Figure is constructed according to Kaufman et al. 2015.

The main problems with viral vectors are the pre-existing or emerging antivirus antibodies, tumour unavailability because of poor vasculature, and the virus spreading to unwanted organs (Chiocca & Rabkin 2014). Inside the tumour, hypoxia might restrict virus replication and a dense matrix prevents the virus from spreading (Mok et al. 2007, Shen & Hermiston 2005). Pre-existing neutralizing antibodies and circulating complement regulatory proteins hinder the virus delivery or persistence in a tumour (Biswas et al. 2012, Tomita et al. 2012). However, antiviral immune responses – even pre-existing – seem also to enhance antitumour immunity (Ricca et al. 2018, Li et al. 2017). In addition, cancer cells often overexpress proteins that viruses can use as receptors (Kaufman et al. 2015). For example, coxsackie-adenovirus receptor (CAR) is downregulated in many cancers, but adenovirus type 3 receptor desmoglein-2 is more commonly expressed in tumours (Biedermann et al. 2005, Sachs et al. 2002, Harada et al. 1996).

1.4.1 ADENOVIRUSES

Every fifth clinical trial focusing on gene therapy involves adenovirus, making it currently the most common virus vector (Edelstein 2017). The *Adeneviridae* family belonging to the *Mastadenovirus* genus comprises seven human adenovirus species (nominated alphabetically from A to G) and several animal adenovirus species (Hoeben & Uil 2013). Currently, different human adenovirus species include over 50 types or serotypes (Hoeben & Uil 2013, Nemerow et al. 2009).

1.4.1.1 Adenovirus life cycle

Adenovirus has a linear, circa 36 000 base pairs (bp) long, double-stranded DNA genome (Hoeben & Uil 2013). The naked icosahedral capsid is 70 to 90 nm in diameter. Trimeric hexons form the capsid with pentons at each vertex. Each penton holds a knobbed fibre protruding from the middle (Nemerow et al. 2009). The virus attaches to cells usually via a fibre knob, which most commonly has a high affinity for CAR, a glycoprotein located near tight junctions of epithelial cells (Bergelson et al. 1997). In addition, group B and D

adenoviruses use CD46 or desmoglein-2 as entry receptors (Wang et al. 2011, Wu et al. 2004, Gaggar et al. 2003).

Penton interaction with $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrin aids the adenovirus internalization into clathrin-coated vesicles (Wickham et al. 1993). The virus escapes from the early endosome and travels towards the nucleus along microtubules, gradually degrading the capsid structure (**Figure 4**) (Leopold et al. 1998, Greber et al. 1993). The viral DNA enters the nucleus and interacts with host-cell histones (Giberson et al. 2012).

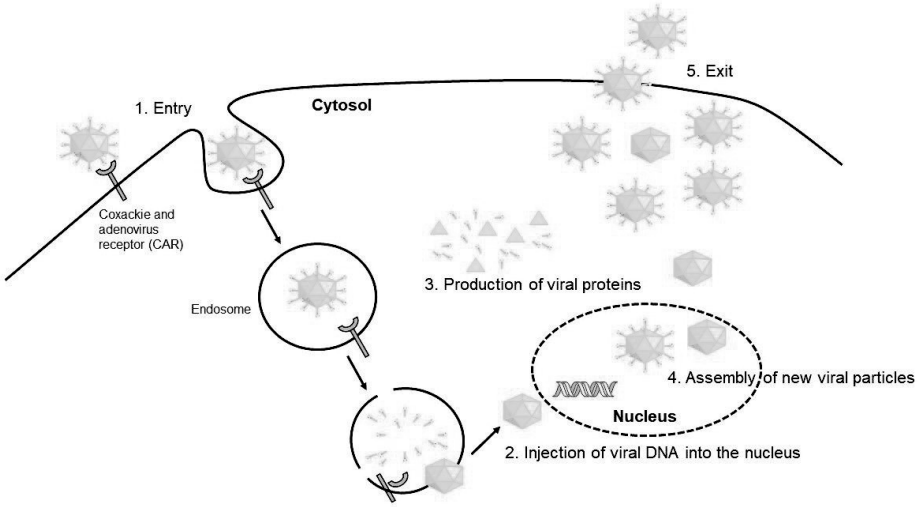


Figure 4. Adenovirus life cycle. Adenovirus enters a cell in clathrin-coated vesicles following attachment to CAR (1). The lowering pH inside an endosome starts disrupting the virus capsid and releases the virus into cytosol. The capsid delivers DNA into the nucleus, where the production of new virus particles starts with the transcription of early genes (2). Structural proteins accumulate in the cytosol (3), but the assembly occurs inside the nucleus (4). Upon exit, adenovirus disrupts the cell, releasing up to 10 000 new virus particles into the surroundings (5). Figure is constructed according to Giberson et al. 2012.

Adenovirus genome comprises one immediate-early unit (*E1A*), four early gene regions (*E1B*, *E2*, *E3*, and *E4*), and five late gene regions (*L1* to *L5*). The early gene products prepare the cell for virus DNA replication. Only E2 is necessary for the DNA replication, which occurs before starting the late gene transcription. The late genes mainly encode structural proteins from splice variants of a single transcript (Giberson et al. 2012).

After genome replication and translation, adenovirus assembly occurs sequentially: Hexons and pentons form empty capsids with non-structural proteins in the first phase. Next, a viral genome associates with packaging proteins and enters the capsids. Finally, a viral protease cleaves immature precursor proteins, enabling virus particle maturation (Ahi & Mittal 2016). The adenovirus replication cycle takes 24 to 36 hours, after which over 10 000 virions lyse the cell and escape (Giberson et al. 2012).

Adenovirus infections are common worldwide throughout the year. It can spread via direct contact, droplet transmission, or faecal-oral route with food and water as possible vectors (Echavarria 2008). The infection may remain asymptomatic, but it generally causes mild respiratory tract infection and symptoms like fever, eye inflammation, or diarrhea (Echavarria 2008). Latent adenoviruses, especially of species C types 1, 2, and 5, often persist in tonsils (Garnett et al. 2007). The virus is also able to remain stable outside a host for several weeks (Echavarria 2008).

1.4.1.2 Modifications

Because adenoviruses are not oncolytic by nature, modifications restricting infection and replication to cancer cells are necessary (Dharmadhikari et al. 2015). One of the most crucial adenovirus proteins regarding initiation of replication is E1A (Radko et al. 2015). Adding a cancer-specific promoter prior to this gene takes advantage of proteins commonly overexpressed in certain cancer types. For example, oncolytic adenovirus CV706 has *E1A* under prostate-specific antigen promoter and has showed efficacy in prostate cancer patients in a phase I trial (DeWeese et al. 2001). More commonly overexpressed in various cancer types is hTERT (human telomerase reverse transcriptase), which has been utilized, for example, in adenovirus KH901 where hTERT promoter regulates *E1A* (Chang et al. 2009).

E1A drives the host cell to S-phase and enables adenovirus replication by interfering with the retinoblastoma (Rb) signalling pathway (Whyte et al. 1988). The Rb pathway is commonly disabled in cancer cells, leading to excess E2F expression, which allows adenovirus to replicate in cancer cells without E1A binding Rb (Heise

et al. 2000). This appears as a safety mechanism in many adenovirus designs: 24-bp disabling deletion of *E1A* restricts adenovirus replication to cancer cells and prevents replication in non-replicating normal cells (**Figure 5**) (Heise et al. 2000). In addition, incorporation of E2F promoter before *E1A* improves the selectivity further (Rojas et al. 2009).

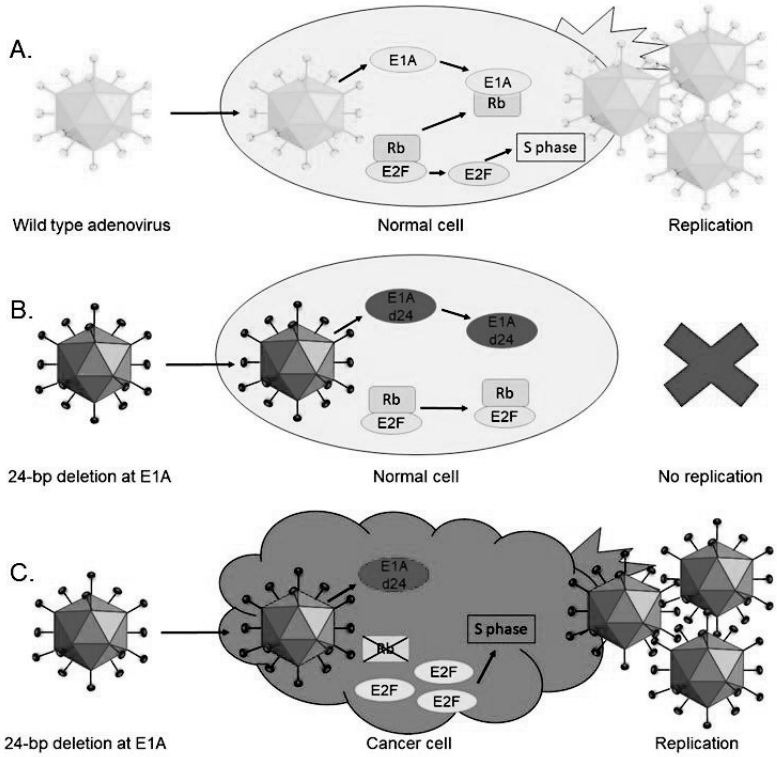


Figure 5. Selective replication of oncolytic adenovirus bearing 24-bp deletion at *E1A*. Wild type adenovirus E1A binds to cellular retinoblastoma protein, enabling accumulation of free E2F, which drives the cell to S-phase (A). Deletion of 24 bp at the region binding to retinoblastoma prevents virus replication in normal cells (B), whereas in cancer cells, the defects in the retinoblastoma pathway do not affect virus replication (C). Figure is constructed according to Heise et al. 2000.

One of the most common mutations in cancer cells, in addition to the Rb pathway, relates to apoptosis-inducing protein p53. Adenovirus protein E1B inhibits host cell apoptosis by binding and inactivating p53. A deletion in this gene region makes infected normal cells susceptible to apoptosis, whereas cancer cells are more prone to virus

infection and replication (Cheng et al. 2015). For example, the first published, conditionally replicating oncolytic adenovirus, ONYX-015, and the approved H101, rely on deletion in *E1B 55K* as a selection mechanism (Cheng et al. 2015, Heise et al. 1997).

1.4.2 ONCOLYTIC VIRUSES AS VECTORS

Modifications for selective replication direct the replication and immunological effects to tumours, but the addition of a transgene, such as a cytokine, usually enhances the efficacy (Liu et al. 2012). Moreover, because a high dosage of recombinant cytokines commonly induces severe adverse events, vectored delivery has also become of interest from a safety perspective (Kaufman et al. 2015). Herpes simplex virus and vaccinia virus can carry the largest transgenes, but adenoviruses can also accommodate several thousand base pairs of extragenomic DNA (Kaufman et al. 2015).

Currently, one of the most studied transgenes inserted in a virus genome is GM-CSF. T-Vec encodes this cytokine and clinical trials are ongoing with GM-CSF-armed vaccinia virus and adenovirus (Ranki et al. 2016, Breitbach et al. 2015, Andtbacka et al. 2015). In addition to GM-CSF, several other cytokines, such as interleukins, TNFα, IFNα/β, soluble CD80, CD40L, Flt3L, and some chemokines alone or in different combinations, have been investigated as arming devices in preclinical and some in clinical studies (Hirvinen et al. 2015, Kaufman et al. 2015, Chiocca & Rabkin 2014, Liu et al. 2012). In addition to arming viruses with cytokines, preclinical data shows much promise with modified viruses expressing soluble, tumour-associated antigens or carrying them on their surfaces (Capasso et al. 2015, Pulido et al. 2012). Moreover, viruses delivering genes to replace malfunctioning genes in target cells, for example, pro-apoptotic *p53* in cancer cells, represent the traditional approach in gene therapy (Zhang et al. 2013).

Oncolytic viruses are an intriguing option for transgene delivery, as they enable local transgene expression on tumour sites with reduced systemic adverse events (Larocca & Schlom 2011). The virus itself simultaneously induces immunological reactions and exposes the immune-escaped tumour for destruction by the patient's own immune system.

2 AIMS OF THE STUDY

The aims of this study are

1. to evaluate the mechanisms behind antitumour efficacy of adenovirally delivered IL2 and TNFα combined with adoptive cell transfers (I, II);
2. to evaluate the potential antitumour effects on distant tumours with local administration of viruses (III); and
3. to study if adenovirally delivered IL2 is equal to systemic administration of IL2 in terms of improving adoptive cell transfers (IV).

3 MATERIALS AND METHODS

3.1 CELL LINES (I–IV)

All the cell lines originate from ATCC (Manassas, VA, USA) unless otherwise stated in **Table 1**. The cells were cultured in RPMI-1640 or DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (all from Sigma-Aldrich, St Louis, MO, USA). **Table 1** presents the additional supplements. The growth conditions for all cell lines were +37°C and 5% CO_2. The cell lines used in animal experiments (B16.OVA, SKOV3-Luc, HapT1, and DDT1-MF2) were tested to be pathogen free at Surrey Diagnostics Ltd (Granleigh, UK).

Table 1. Cell lines used in the study. Growth media included 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin, unless otherwise stated.

Cell line	Origin	Growth media	Source	Study
Human cell lines				
293	Embryonic kidney	DMEM	ATCC	I
A549	Lung carcinoma	DMEM	ATCC	II
OVCAR-3	Ovarian adeno-carcinoma	RPMI-1640 20% FBS, 10 µg/ml human insulin (Sigma-Aldrich)	ATCC	II
Panc1	Pancreatic carcinoma	DMEM	ATCC	II
SK-MEL-28	Melanoma	DMEM 5% FBS	ATCC	II

SKOV3-Luc	Firefly luciferase-expressing ovarian carcinoma	DMEM	Dr Negrin, Stanford Medical School, CA, USA	II
Hamster cell lines				
DDT1-MF2	Leiomyo-sarcoma	DMEM	Prof. Wold, St. Louis, MO, USA	II
HapT1	Pancreatic carcinoma	RPMI-1640	DSMZ, Braunschweig, Germany	II, III, IV
Mouse cell lines				
B16. OVA	Chicken ovalbumin-expressing melanoma	DMEM 5 mg/ml G418 (Roche, Basel, Switzerland)	Prof. Vile, Mayo Clinic, Rochester, MN, USA	I, III, IV
B16-Blue IFN-α/β	Interferon-sensitive melanoma	RPMI-1640 100 µg/ml Normocin (Invivogen)	InvivoGen, San Diego, CA, USA	I
CTLL-2	Lymphocytes	RPMI-1640 10% T-Stim (Corning, Corning, NY, USA)	ATCC	I, II
L929	Fibroblasts	DMEM	ATCC	I, II

3.2 GENERATION OF VIRAL CONSTRUCTS (I–IV)

The construction of non-replicating control viruses Ad5-Luc1 and Ad5/3-Luc1 has been described by Krasnykh et al. (2001, 1996). Adenoviruses coding for murine cytokines were constructed by inserting expression cassettes of murine IFNγ, IL2, IFNβ, and TNFα into a shuttle plasmid pDC315 (AdMax, Microbix Biosystems, Mississauga, Canada). The shuttle plasmids were transfected into 293 cells together with a plasmid pBHGlox-delE13vre (AdMax) containing adenovirus genome with cytomegalovirus (CMV)

promoter and deleted *E1* and *E3* regions. The transgenes were introduced under the CMV promoter to replace the deleted *E1* region by site-specific recombination. The viruses were purified with caesium chloride gradient centrifugation (Luo et al. 2007), and the yield of viral particles (VP) was determined with spectrophotometer by reading at 260 nm wavelength. Spectrophotometer measures the amount of DNA in a sample, which is converted to VP titer using an artificially determined extinction coefficient (Mittereder et al. 1996). Infectious units were calculated according to Tissue Culture Infectious Dose (TCID50) assay.

The oncolytic adenoviruses were generated to a backbone of Ad5/3-E2F-d24 bearing fibre knob from adenovirus serotype 3, E2F promoter for *E1A*, a 24-bp deletion at the Rb protein-binding region at *E1A*, and deleted *E1B 19K*. The expression of the transgenes (human IL2 and/or TNFα) was linked to virus replication by replacing *Gp19k* and *6.7K* at *E3* region with the transgenes. The insertion was done using the BAC-recombineering strategy based on the selection marker galK (Muck-Hausl et al. 2015, Ruzsics et al. 2006, Warming et al. 2005). The internal ribosome entry site (IRES) between TNFα and IL2 acts as a translation initiation site.

The oncolytic viruses were produced with A549 cells and purified by caesium chloride gradient centrifugation. The critical parts of viral genomes (Knob3, d24, and E3) were verified with polymerase chain reaction (PCR). **Table 2** lists the primer sequences. Ad5/3-E2F-d24-hTNFα-IRES-hIL2 was additionally sequenced at the Biomedicum Functional Genomics Unit (FuGU, Helsinki, Finland). **0** presents the viruses used in this study.

Table 2. Primers used for validating the produced viruses. PCR running conditions included initial denaturation at 98°C for 30 s and 30–35 times repeated cycles of denaturation at 98°C for 10 s, annealing at 55–57°C for 20 s, and elongation at 72°C for 20–60 s. The final elongation step lasted 7 min at 72°C.

Gene	Type	Sequence (5'-3')
E1A	Forward primer	GTCCGGTTTCTATGCCAA
	Reverse primer	TCACCCTCTTCATCCTCGTC
Knob3	Forward primer	GCACAGGTGCCATTACAGTAG
	Reverse primer	TCATTATGTGTTCCCGCATTA
E3	Forward primer	CCAGACGGAGTGAGTCTACG
	Reverse primer	TCGCTGTAGTTGGACTGGAAC

Table 3. List of viruses used in the study. All viruses have a backbone of human type 5 adenovirus.

Virus	Modifications	Trans-gene	Study	Reference
Replication-incompetent viruses				
Ad5-Luc1	Deleted E1	Firefly luciferase	I, III	(Krasnykh et al. 2001)
Ad5/3-Luc1	Deleted E1	Firefly luciferase	II	(Krasnykh et al. 1996)
Ad5-CMV-mIL2	Cytomegalo-virus promoter	Murine IL2	I, III, IV	
Ad5-CMV-mIFNb	Cytomegalo-virus promoter	Murine IFNb	I	
Ad5-CMV-mIFNg	Cytomegalo-virus promoter	Murine IFNg	I	
Ad5-CMV-mTNFα	Cytomegalo-virus promoter	Murine TNFα	I, III	

Oncolytic viruses				
Ad5/3-E2F-d24	Ad3 fiber knob, E2F promoter, 24-bp deletion in *E1A*, deleted *E1B 19K*	None	II, III, IV	
Ad5/3-E2F-d24-hTNFα	Ad3 fiber knob, E2F promoter, 24-bp deletion in *E1A*, deleted *E1B 19K*	Human TNFα	II, III	
Ad5/3-E2F-d24-hIL2	Ad3 fiber knob, E2F promoter, 24-bp deletion in *E1A*, deleted *E1B 19K*	Human IL2	II, III, IV	
Ad5/3-E2F-d24-hTNFα-IRES-hIL2	Ad3 fiber knob, E2F promoter, 24-bp deletion in *E1A*, deleted *E1B 19K*	Human TNFα and IL2	II, III	

3.3 FUNCTIONALITY OF THE VIRUSES (I AND II)

The cell-killing ability of oncolytic viruses was determined by infecting a panel of cancer cell lines with an increasing viral dose. The presence of the cytokines coded by the transgenes was measured from cell culture growth media supernatants after infection. In addition, the indicator cell lines proved the biological activity of the cytokines.

3.3.1 MTS CELL PROLIFERATION ASSAY (II)

A panel of human (A549, SKOV3-Luc, OVCAR-3, Panc1, and SK-MEL-28) and hamster (HapT1 and DDT1-MF2) cancer cell lines were infected with 1–1 000 VP/cell or 1-10 000 VP/cell, respectively, in Study II. The cells were incubated with the viruses for 3–7 days until visible cell killing was achieved with the highest dose. Replication-incompetent Ad5/3-Luc1 virus served as a negative control.

Viable cells were detected with 10% MTS solution [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; CellTiter 96 AQueous One Solution, Promega, Madison, WI, USA] after a two-hour incubation. In the assay, metabolically active cells reduce the MTS reagent, generating a coloured, soluble formazan product. The amount of the product is relative to the amount of viable cells and is detectable with spectrophotometer at the wavelength of 490 nm.

3.3.2 CYTOKINE EXPRESSION (I AND II)

Murine cytokines were detected in Study I from virus-infected (100 VP/cell) 293 cell growth media supernatants 48 hours after infection. The supernatants were filtered through a 0.02 μm filter (Whatman, GE Healthcare Life Sciences, Chicago, IL, USA) to eradicate the viruses. The following enzyme-linked immunosorbent assays (ELISA) were used according to the manufacturer's protocol: mIFNβ (PBL InterferonSource, Piscataway, NJ, USA), mIFNγ (Abcam, Cambridge, UK), mIL2, and mTNFα (IBL International, Hamburg, Germany).

For human cytokine detection in Study II, two human (A549 and SKOV3-Luc) and hamster (HapT1 and DDT1-MF2) cancer cell lines were infected with 1 000 VP/cell and 5 000 VP/cell, respectively. The cell supernatant was collected 72 hours after infection and filtered through a 100-kDa filter unit (Amicon Ultra 4, Merk Millipore, Billerica, MA, USA). The cytokine concentrations of the diluted supernatants were determined with BD Cytometric Bead Array (CBA) Human Soluble Protein Master Buffer Kit together with Human IL2 and TNFα Felx Set kits (BD, Franklin Lakes, NJ, USA) according to the manufacturer's instructions.

Replication-incompetent viruses with murine cytokines were injected into subcutaneous B16.OVA tumours (1 x 10^9 VP) in C57BL/6 mice (Envigo, Cambridgeshire, UK) to measure whether the cytokines were also produced *in vivo* in Study I. In Study II, oncolytic viruses were injected into subcutaneous HapT1 tumours (1 x 10^8 VP) established in Golden Syrian hamsters (*Mesocricetus auratus*, Harlan laboratories/Envigo). The tumours and the blood were collected 72 hours after infection. Tumours were snap-frozen

and stored at -80°C before homogenization. The blood was incubated for one hour at room temperature before high-speed centrifugation to separate the serum from the red blood cells. The cytokine concentrations were measured with CBA and normalized to the total protein content of the sample.

3.3.3 BIOLOGICAL ACTIVITY OF THE CYTOKINES (I AND II)

The biological activity of the cytokines was investigated with indicator cell lines in Studies I and II. B16-Blue IFN-α/β murine type I IFNs sensor cells were used to evaluate the functionality of mIFNβ present in the cell culture supernatant according to the manufacturer´s instructions. The binding of mIFNβ to the receptors on these cells triggers the production of SEAP, which can be monitored using a specific detection medium.

The functionality of mIFNγ was determined by its ability to protect L929 cells from vesicular stomatitis virus (VSV) infection. The cells were seeded on a 96-well plate (3.5×10^4 cells/well) and incubated for six hours with the supernatants. The supernatant was then removed and the cells infected with VSV strain M51 (from Dr. Markus Vähä-Koskela, University of Helsinki) 1×10^4 VP/well. Cell viability was determined 96 hours later with MTS assay.

The L929 cells are sensitive for both human and murine TNFα in the presence of actinomycin D. The cell supernatants and actinomycin D (2 μg/ml) were added to the cells and incubated for 24 hours before measuring the cell viability.

Functionality of human and murine IL2 was determined by measuring the viability of CTLL-2 lymphocytes (5×10^4 cells per well on a 96-well plate) after a 72-hour incubation with the cell supernatants. Commercial cytokine products were used as positive controls in all functionality assays: 1 ng/ml of mTNFα, mIL2, and mIFNγ; 1000 IU/ml mIFNβ; 0.5 ng/ml hTNFα; and 10 ng/ml hIL2 (all from R&D Systems, Minneapolis, MN, USA).

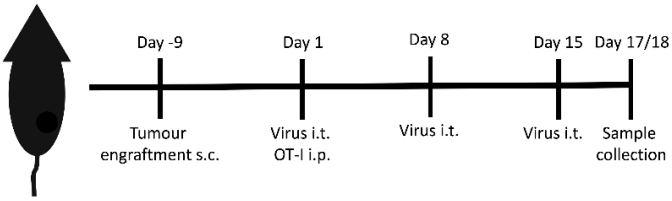
3.4 ANIMAL EXPERIMENTS (I–IV)

All animal protocols were reviewed and approved by the Experimental Animal Committee of the University of Helsinki and the Provincial Government of Southern Finland. In addition, the National Animal Experiment Board (Eläinkoelautakunta ELLA) of the Regional State Administrative Agency of Southern Finland granted permission for the experiments (ESAVI/7759/04.10.07/2013 and ESAVI/7755/04.10.07/2016). The animals were quarantined for at least a week before starting the experiments. All injections were given to animals anesthetised with isoflurane. The health status of the animals was monitored daily and the animals were sacrificed upon visible distress, tumour ulceration, or when the tumour diameter reached 18 mm or 22 mm for mice and hamsters, respectively.

3.4.1 IMMUNOCOMPETENT MICE (I, III, AND IV)

Mouse melanoma B16 tumours expressing chicken ovalbumin (OVA) were established subcutaneously with 2.5 x 10^5 cells to C57BL/6 mice (Envigo) in Studies I, III, and IV. The mice were randomized into groups of 5–11 when the average tumour diameter reached approximately 5 mm after 10 days. Tumour size was followed with a digital caliper and the volume estimated with a formula: 0.52 x (max dimension) x (min dimension)^2.

The tumours in Study I were treated with 1 x 10^9 VP (Ad5-CMV-mIFNβ, Ad5-CMV-mIFNγ, Ad5-CMV-mIL2, or Ad5-CMV-mTNFα) in 50 µl PBS with or without systemic administration of 1.5 x 10^6 CD8-enriched OVA-specific OT-I cells (described later) in 100 µl of plain RPMI-1640. The virus treatments were repeated every seven days and the tumour growth was followed with digital caliper every two to three days (**Figure 6**).

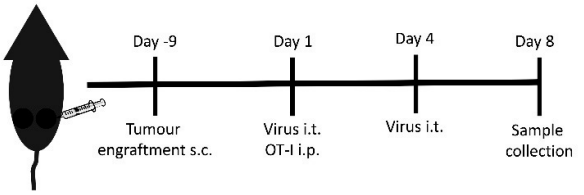


Group	VP/tumour	OT-I T cells/animal
Mock	-	-
OT-I	-	$1.5*10^6$
Ad5-CMV-mIFNg +/- OT-I	$1*10^9$	$1.5*10^6$
Ad5-CMV-mIFNb +/- OT-I	$1*10^9$	$1.5*10^6$
Ad5-CMV-mIL2 +/- OT-I	$1*10^9$	$1.5*10^6$
Ad5-CMV-mTNFa +/- OT-I	$1*10^9$	$1.5*10^6$
Ad5-CMV-mIL2 + Ad5-CMV-mTNFa +/- OT-I	$0.5*10^9 + 0.5*10^9$	$1.5*10^6$

Figure 6. Schedule and treatment groups for mouse experiment studying the efficacy of cytokine-armed adenoviruses. The viruses were administered alone or together with OVA-specific OT-I T cells to animals bearing B16-OVA melanoma tumours.

Two viruses with the best efficacy (Ad5-CMV-mIL2 and Ad5-CMV-mTNFα) were combined to achieve a superior antitumour efficacy and induction of T-cell transfer. Equal amounts of viruses (0.5 x 10^9 VP per virus) were administered intratumourally once a week with or without a single treatment with OT-I cells (**Figure 6**). Tumour growth was followed as in the first experiment.

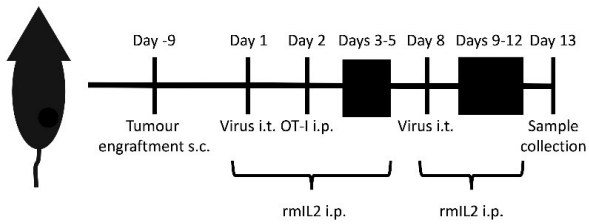
Two B16.OVA tumours were implanted on the flanks of C57BL/6 mice to assess the effects of a local treatment with armed adenoviruses on distant tumour growth in Study III (**Figure 7**). The animals received 1.4 x 10^6 OT-I cells intraperitoneally and a virus treatment with Ad5-CMV-mIL2, Ad5-CMV-mTNFα, or both, as previously described, only into one tumour. The tumours were collected seven days after the treatment, and the immune cell contents in the tumours were assessed with flow cytometry.



Group	VP/tumour	OT-I T cells/animal
Mock	PBS	-
OT-I	PBS	$1.4*10^6$
Ad5-Luc1 +/- OT-I	$1*10^9$	$1.4*10^6$
Ad5-CMV-mTNFa	$1*10^9$	-
Ad5-CMV-mIL2	$1*10^9$	-
Ad5-CMV-mIL2 + Ad5-CMV-mTNFa +/- OT-I	$0.5*10^9 + 0.5*10^9$	$1.4*10^6$

Figure 7. Schedule and treatment groups for mouse experiment studying the systemic antitumor efficacy of locally administered adenoviruses. Two B16-OVA melanoma tumours were engrafted subcutaneously and only one of them treated with viruses.

Local IL2 delivery within a virus genome was compared with systemic delivery of recombinant protein in Study IV. The animals received daily 1 x 10^5 IU of murine recombinant IL2 (PeproTech) intraperitoneally during 12 days with two days' rest in the middle of the treatment period (**Figure 8**). The virus group received 1 x 10^9 VP of Ad5-CMV-mIL2 on days 1 and 8. On day 2, 1 x 10^6 OT-I cells were administered for the animals intraperitoneally except for the mock control group. On day 13, 24 hours after the last recombinant protein treatment, the animals were sacrificed, and samples from tumours, bloods, hearts, lungs, livers, spleens, and kidneys were collected to evaluate the toxicity of the treatment and for the immune cell characterization.



Group	rmIL2/animal	VP/tumour	OT-I/animal
OT-I	-	PBS	$1*10^6$
Recombinant murine IL2 + OT-I	$1*10^5$ IU	-	$1*10^6$
Ad5-CMV-mIL2 + OT-I	-	$1*10^8$	$1*10^6$

Figure 8. Schedule and treatment groups for mouse experiment comparing systemic administration of recombinant IL2 with locally administered adenoviruses. The mice bearing B16-OVA melanoma received adenovirus coding for IL2 locally once a week and recombinant IL2 daily during ten days in total with two-day resting period in the middle of the experiment.

3.4.1.1 Extraction of OVA-specific OT-I T cells (I, III and IV)

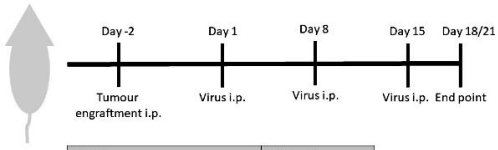
Spleens and inguinal lymph nodes from TCR-transgenic OT-I mice (strain C57BL/6-Tg(TcraTcrb)1100Mjb/J, The Jackson Laboratory, Bar Harbor, ME, USA) were collected to obtain OVA-specific CD8-positive T cells for Studies I, III, and IV. The organs were pressed through a 70 μm filter and the red blood cells lysed from splenocytes with Ammonium-Chloride-Potassium (ACK) buffer (150 mM NH4Cl, 10 mM KHCO3, and 110 mM Na2-EDTA). The remaining cells were washed twice by suspending the cells to RPMI-1640 growth media and centrifuging them at the speed of 500xg for four minutes. The immune cells were pooled and let settle in RPMI-1640 media supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 15 mM HEPES (Sigma-Aldrich), 50 μM 2-mercaptoethanol (Sigma-Aldrich), and 1 mM Na-pyruvate (Sigma-Aldrich). The CD8a positive cells were enriched after 48 hours using CD8a+ T Cell Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany), following the manufacturer's protocol. Enriched T cells were expanded in the presence of 160 ng/ml recombinant murine IL2 (R&D Systems), and 300 ng/ml soluble anti-mouse CD3e antibody clone 145-2C11 (Abcam, Cambridge, UK) for seven more days before adoptive transfer into B16.OVA tumour-bearing mice.

3.4.2 SEVERE COMBINED IMMUNODEFICIENT MICE (II)

The functionality of the oncolytic viruses was evaluated in Study II with female CB-17/Icr-Prkdc^{scid}/Rj mice (Janvier Labs, Saint Berthevin, France), aged 4—6 weeks upon arrival, bearing firefly luciferase-expressing otrhotopic ovarian carcinoma SKOV3-Luc. The cells (5 x 10^6) were injected intreperitoneally in 300 µl plain DMEM. The animals were randomized into groups three days later and treated weekly with intraperitoneal injections.

To establish an effective dose, the mice were split into groups of three receiving 1 x 10^5, 1 x 10^7, or 1 x 10^9 VP of Ad5/3-E2F-d24-TNFα-IRES-IL2 weekly in 300 µl of phosphate-buffered saline (PBS), or PBS only as a vehicle control (**Figure 9**). Tumour growth was followed once a week by imaging the animals with IVIS 100 (Xenogen, Alameda, CA). Eight minutes before imaging, the animals received 3 mg D-luciferin (Synchem, Felsberg, Germany) in 100 µl PBS administered to intraperitoneal cavity. D-luciferin is a substrate for luciferase initiating the bioluminescent reaction, which can be detected with a CCD camera. Bioluminescent imaging was performed with the following settings: 10 s exposure time, 1f/stop, medium binning and open filter. Photographic images were obtained using automatic exposure, 8 f/stop, small binning, and open filter. Photographic and bioluminescent images were overlaid with Living Image 3.2 (Xenogen) and total flux (photons/s) was measured from regions of interest bordering the peritoneal area.

To investigate whether the transgenes affect the infectivity of the viruses, the mice were randomized into groups of 5–7 and treated with the dose established in the dose-escalation experiment (1 x 10^9 VP, **Figure 9**). The virus was delivered once a week in 300 µl PBS. The vehicle control group received PBS alone. Imaging was performed twice a week for three weeks as previously described.



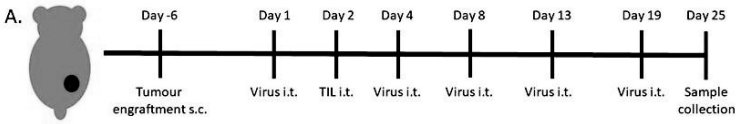
Group	VP/animal
Mock	PBS
Ad5/3-E2F-d24	$1*10^9$
Ad5/3-E2F-d24-hTNFa	$1*10^9$
Ad5/3-E2F-d24-hIL2	$1*10^9$
Ad5/3-E2F-d24-hTNFa-IRES-hIL2	$1*10^9/1*10^7/1*10^5$

Figure 9. Schedule and treatment groups for experiment with immunocompetent mice. When investigating the effect of dosing, the animals bearing orthotopic ovarian cancer SCOV3-Luc received 10^5, 10^7, or 10^9 VPs once a week, and the experiment was stopped on day 18. When comparing the oncolytic activity of the unarmed and armed viruses, 10^9 VPs were administered to the animals weekly until day 21. The animals were imaged twice a week.

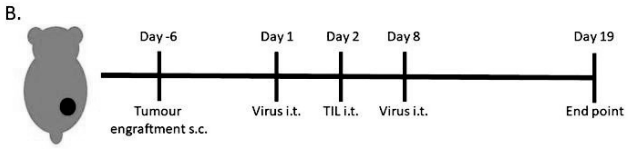
3.4.3 GOLDEN SYRIAN HAMSTERS (*MESOCRICETUS AURATUS*) (II–IV)

Golden Syrian hamsters (*Mesocricetus auratus*, Harlan Laboratories/Envigo) are semipermissive for human adenovirus infection unlike mice (Thomas et al. 2006), which makes them a useful model to investigate human oncolytic adenoviruses. Moreover, some of human cytokines are active in hamsters, including IL2 and TNFα. Subcutaneous tumours were established in Studies II–IV with 4 x 10^6 HapT1 cells to 5–6-week-old hamsters, and the tumour growth was followed with a digital caliper. The animals were randomized into groups of 5–7.

In Study II, the hamsters were treated intratumourally with 1 x 10^9 VP in 50 µl PBS or PBS alone. The animals received virus injections on days 1, 4, 8, 13, and 19 (**Figure 10A**). On day 2, tumours received 4 x 10^5 HapT1 TILs (extraction described later) in 50 µl of plain RPMI-1640, or media only. The tumour growth was followed every two to three days with a digital caliper until day 25, when the tumours and relevant organs (heart, lung, liver, spleen, kidney, and tumour-draining lymph node) were collected. The samples were used for histopathological evaluations and for the detection of immune cell subsets. The experiment was repeated with the critical virus (Ad5/3-E2F-d24-hTNFα-IRES-hIL2) using reduced virus dose (1 x 10^8 VP/tumour on days 1 and 8, **Figure 10B**).



Group	VP/tumour	TILs/tumour
Mock	PBS	-
TIL	PBS	$4*10^5$
Ad5/3-E2F-d24 +/- TIL	$1*10^9$	$4*10^5$
Ad5/3-E2F-d24-hTNFa +/- TIL	$1*10^9$	$4*10^5$
Ad5/3-E2F-d24-hIL2 +/- TIL	$1*10^9$	$4*10^5$
Ad5/3-E2F-d24-hTNFa-IRES-hIL2 +/- TIL	$1*10^9$	$4*10^5$

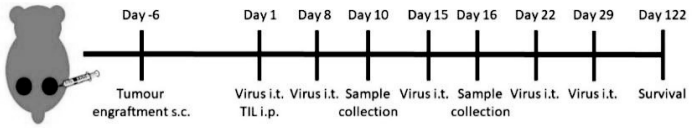


Group	VP/tumour	TILs/tumour
Mock	PBS	-
TIL	PBS	$4*10^5$
Ad5/3-E2F-d24-hTNFa-IRES-hIL2	$1*10^8$	-
Ad5/3-E2F-d24-hTNFa-IRES-hIL2+ TIL	$1*10^8$	$4*10^5$

Figure 10. Schedule and treatment groups for hamster experiments investigating the efficacy of oncolytic adenoviruses. The viruses were administered with or without TILs that were previously extracted and expanded from the same HapT1 tumour type. A) The $1*10^9$ VPs were administered to the animals five days in total. B) The experiment was repeated with reduced virus dose of $1*10^8$ VPs administered twice.

Cured animals had a two-week rest period before predisposing them for rechallenge. The same tumour cells (HapT1) or immunologically distinct (DDT1-MF2) cells were implanted on the flanks of the animals (4×10^6 cells/tumour). DDT1-MF2 control tumours reached the maximum tolerated diameter (22 mm) 18 days after implantation.

Systemic effects of the local treatment in Study III were evaluated by implanting two subcutaneous HapT1 tumours on the flanks of the hamsters and treating only one tumour with 1×10^8 VP once a week, five treatments in total (**Figure 11**). In addition, the animals received 5×10^7 TILs intraperitoneally in the beginning of the treatments. As a control, one group received PBS on the other tumour in addition to TILs, and one group was left untreated. Four animals per group were sacrificed on day 16 and the tumour sizes in the rest 5–6 animals per group were followed until they reached the maximum tolerated size (combined maximum dimension of 40 mm).



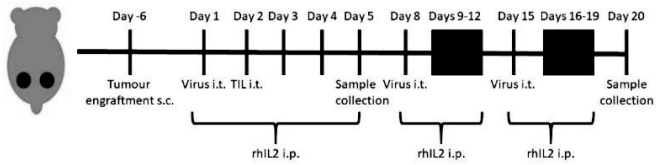
Group	VP/tumour	TILs/animal
Mock	-	-
TIL	PBS	$5*10^7$
Ad5/3-E2F-d24 + TIL	$1*10^8$	$5*10^7$
Ad5/3-E2F-d24-hTNFa + TIL	$1*10^8$	$5*10^7$
Ad5/3-E2F-d24-hIL2 + TIL	$1*10^8$	$5*10^7$
Ad5/3-E2F-d24-hTNFa-IRES-hIL2 + TIL	$1*10^8$	$5*10^7$

Figure 11. Schedule and treatment groups for hamster experiment studying the systemic antitumor efficacy of locally administered oncolytic adenoviruses. Two pancreatic HapT1 tumours were engrafted subcutaneously and only one of them treated with viruses. Samples for RNA sequencing were collected on day 10 and for virus biodistribution on day 16. The tumour growth on rest of the animals was followed until humane endpoint criteria were met.

Four subcutaneous HapT1 tumours were engrafted on hamster flanks to evaluate the mechanism behind systemic efficacy in non-injected tumours on gene-expression level. The animals received TIL treatment as previously described and 1 x 10^8 VP of Ad5/3-E2F-d24-hTNFα-IRES-hIL2 or the unarmed control virus Ad5/3-E2F-d24 on days 1 and 8 into two out of four tumours (**Figure 11**). A mock control group and a group treated only with TILs received PBS instead of viruses. Tumours were collected two days after the last virus treatment and stored in RNAlater (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA). RNA was extracted with an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol. The gene-expression levels were evaluated by sequencing the messenger RNAs from the samples (described later).

Local delivery of IL2 within an oncolytic virus genome was compared with systemic delivery of the recombinant protein in Study IV. The hamsters with two established subcutaneous HapT1 tumours were treated for three weeks with 5 x 10^5 IU of animal-free recombinant human IL2 (PeproTech) in a series of five days with two days recovery period in between the treatment periods (**Figure 12**). Alternatively, the animals received once a week oncolytic Ad5/3-E2F-d24-hIL2. Additionally, 24 hours after the first treatment with the recombinant protein or the virus, 4 x 10^6 TILs were injected into

the tumours. The control groups received intratumoural PBS injections with or without TIL treatment. The animals were sacrificed 24 hours after the last recombinant protein treatment and tumours, bloods, hearts, lungs, livers, kidneys, and spleens were collected to evaluate the toxic effects of the treatments, in addition to immune cell contents of the tumours. Tumour samples were collected also on day 5 from untreated animals or animals treated with Ad5/3-E2F-d24 or Ad5/3-E2F-d24-hIL2 to evaluate the effect of the treatment on endogenous hamster IL2 production with reverse transcriptase quantitative PCR (qPCR).



Group	rhIL2/animal	VP/tumour	TILs/tumour
Mock	-	PBS	-
TIL	-	PBS	$4*10^6$
Recombinant human IL2 + TIL	$5*10^5$ IU	-	$4*10^6$
Ad5/3-E2F-d24-hIL2 + TIL	-	$1*10^8$	$4*10^6$

Figure 12. Schedule and treatment groups for hamster experiment comparing systemic administration of recombinant IL2 with locally administered adenoviruses. Hamsters bearing two HapT1 pancreatic tumours received oncolytic adenovirus coding for human IL2 into both tumours once a week. Recombinant IL2 was administered systemically on five consecutive days followed by a pause for two days. The treatments continued for three weeks. Endogenous IL2 expression was examined from samples collected on day 5. Toxicity of the treatment and immune cell compartments in tumours were investigated with day 20 samples.

3.4.3.1 Extraction of TILs (II–IV)

HapT1 tumours grown for ten days were collected and cut to fragments of 1–3 mm^3 in size. The fragments were placed into a G-Rex 10 lymphocyte growth chamber (Wilson Wolf, New Brighton, MN, USA), 10-15 fragments per chamber, in RPMI-1640 media supplemented with 20% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 15 mM HEPES, 50 µM 2-mercaptoethanol, 1 mM Na-pyruvate, and 3000 IU/ml human IL2 (PeproTech, NJ, USA). Half of the media (15 ml) was changed after five days and every two days after that.

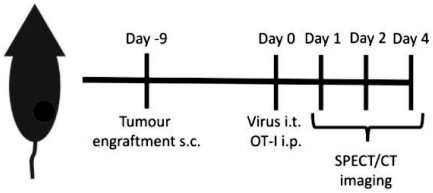
Concanavalin A (Sigma-Aldrich, MO, USA) was added with the fresh media to yield the concentration of 0.5 ug/ml. The cells were collected after ten days in culture and used for the experiments in Studies II, III, and IV.

3.4.4 CELL GRAFT TRAFFICKING (I AND III)

OVA-specific CD8a+ T cells and the TILs from HapT1 tumours were obtained as previously described. The cell grafts were labelled with ^{111}In-oxine (half-life of 68 h) for 15 min in room temperature and washed twice with saline. The mice (n=3/group) received 6 x 10^6 OT-I cells (5.98 ± 0.53 MBq per animal) intraperitoneally and 1 x 10^9 VP of Ad5-CMV-mIL2, Ad5-CMV-mTNFα, or a combination of both into B16.OVA tumours (**Figure 13A**). The control group received PBS. During days 1, 2, and 4 after the treatments, computed tomography images were obtained together with single-photon, emission-computed tomography images (nanoSPECT/CT, Bioscan Inc., USA). The hamsters (n=4/group) received 5 x 10^7 cells intraperitoneally (5.82 ± 0.73 MBq) and 1 x 10^8 VP of Ad5/3-E2F-d24 or Ad5/3-E2F-d24-hTNFα-IRES-hIL2 into one of the two HapT1 tumours on day 0 (**Figure 13B**). The control group received PBS. The animals were imaged on days 2, 3, and 4. The images were aligned with HiSPECT NG software (Scivis GmbG, Göttingen, Germany) and analysed with InVivo Scope image analysis software (Bioscan Inc., USA). The percentage of radioactivity in the tumours was determined by dividing the injected dose by the tumour volume (mm^3).

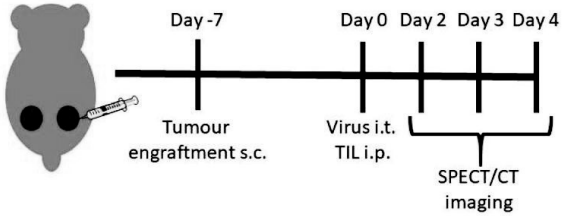
To obtain complementary data on T-cell trafficking into injected and non-injected tumours from mice, OT-I cells were labelled with nanoparticles carrying FITC fluorescent label (Qtracker™ 565 Cell Labeling Kit, Thermo Fisher Scientific). B16-OVA tumours were treated twice before collection on day 6 (**Figure 13C**). Collected tumours were pressed through a 70-µm strainer and the yielded cell suspension labelled fresh with anti-CD8 antibody. FITC positive CD8 cells were analysed by flow cytometry (see section 3.6).

A.



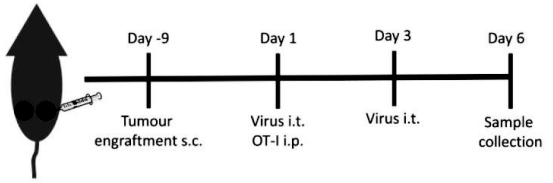
Group	VP/tumour	OT-I T cells/animal
OT-I	PBS	$6*10^6$
Ad5-CMV-mIL2 + OT-I	$1*10^9$	$6*10^6$
Ad5-CMV-mTNFa + OT-I	$1*10^9$	$6*10^6$
Ad5-CMV-mIL2 + Ad5-CMV-mTNFa + OT-I	$0.5*10^9 + 0.5*10^9$	$6*10^6$

B.



Group	VP/tumour	TILs/animal
TIL	PBS	$5*10^7$
Ad5/3-E2F-d24 + TIL	$1*10^8$	$5*10^7$
Ad5/3-E2F-d24-hTNFa-IRES-hIL2 + TIL	$1*10^8$	$5*10^7$

C.



Group	VP/tumour	OT-I T cells/animal
OT-I	PBS	$7.6*10^5$
Ad5-Luc1 +/- OT-I	$1*10^9$	$7.6*10^5$
Ad5-CMV-mIL2 + Ad5-CMV-mTNFa +/- OT-I	$0.5*10^9 + 0.5*10^9$	$7.6*10^5$

Figure 13. Schedule and treatment groups for cell graft trafficking experiments. A) Mice bearing B16-OVA melanoma were treated with cytokine-armed adenoviruses and the trafficking of ^{111}Indium-oxane-labelled OT-I T cells was followed over four days. B) TILs were labelled similarly and followed over time in hamsters bearing two pancreatic HapT1 tumours. Only one of the tumours was treated with unarmed or armed oncolytic virus. C) Complementary to hamster experiment, OT-I cells were labelled with fluorescent nanoparticles and administered to animals receiving virus treatments to one out of two tumours. Tumours were collected on day 6 and the presence of fluorescent OT-I cells was analysed with flow cytometry.

3.5 CHEMOKINE AND CYTOKINE LEVELS IN TUMOURS (I AND IV)

B16.OVA tumours were established in Study I on C57BL/6 mice, as previously described, to investigate the effect of viral treatment on the expression of chemokines. The mice received 1 x 10^9 VP of Ad5-CMV-mIL2, Ad5-CMV-mTNFα, or a combination of both intratumourally in addition to intraperitoneal injection of OT-I cells (**Figure 6**). The tumours were snap-frozen and homogenized after a week. RANTES, MCP1, MIP-1a, MIG, and I-TAC were detected with CBA Flex Sets (BD) as guided by the producer and normalised against the total protein concentration of the sample. Similarly, endpoint B16.OVA tumours were collected in Study IV to screen the treatment-related changes in the cytokine profiles. CBA mouse Th1/Th2/Th17 kit (BD) was utilized to determine the levels of IL2, IL6, and TNFα.

3.6 FLOW CYTOMETRY (I–IV)

Tumours, spleens, and lymph nodes were collected into an RPMI medium with 10% FBS and pressed through a 70-µm strainer. The cells were incubated for 24 hours in regular cell culture conditions before freezing them in an RPMI medium containing 20% FBS and 10% DMSO. Erythrocytes were lysed from the splenocyte suspension with ACK treatment. The cells were stored at -80°C and analysed immediately after thawing. Approximately 1 x 10^6 cells were allocated per well on a 96-well plate and washed once with stain buffer (PBS containing 0.1% BSA) 500xg for 5 min at +4°C. Pro5 MHC Pentamers (10 µl per reaction) were incubated with the cells at room temperature in dark for 20 min (SIINFEKL for OVA, KVPRNQDWL for gp100, and SVYDFFVWL for Trp2), followed by a wash cycle. **Table 4** lists the antibodies that were used in concentrations recommended by the manufacturer and incubated for 30–60 min at +4°C. The cells were resuspended into stain buffer after a wash, and 50 000–100 000 events were collected and analysed with BD Accuri C6.

Table 4. Antibodies used for detecting immune cell subsets. The cells were stained with the recommended amounts of antibodies during maximum 60 minutes.

Antibody	Host	anti-	Supplier	Study
Asialo-GM1- Alexa Fluor-488	Rabbit	(Poly-clonal)	eBioscience	II
CD11b-PerCP-Cy5.5	Rat	mouse	BD	I, III, IV
CD11c-FITC	Armenian hamster	mouse	BD	I, III
CD19-PE	Rat	mouse	eBioscience	I, III, IV
CD25-PE	Rat	mouse	eBioscience	I, IV
CD206-FITC	Rat	mouse	Biolegend	III
CD3-APC	Armenian hamster	mouse	BD	I
CD3-PE-Cy7	Rat	mouse	BD	III, IV
CD4-PerCP-Cy5.5	Rat	mouse	eBioscience	I, IV
CD4-APC	Rat	mouse	eBioscience	II, IV
CD69-PeCy7	Armenian hamster	mouse	eBioscience	I
CD86-PE	Rat	mouse	BD	I, III, IV
CD8a-APC	Rat	mouse	eBioscience	I
CD8b-FITC	Rat	mouse	eBioscience	I, III, IV
CD8b-PE	Mouse	rat	eBioscience	II, IV
F4/80-APC	Rat	mouse	eBioscience	III, IV

FoxP3-APC	Rat	mouse	eBioscience	I, IV
Galectin-3 (Mac-2)-PE	Rat	human/mouse	eBioscience	II
Gr-1-FITC	Rat	mouse	BD	I
MHC Class II-FITC	Mouse	mouse/rat	eBioscience	II
NK1.1-FITC	Mouse	mouse	eBioscience	I, III, IV
PD-1-PeCy7	Armenian hamster	mouse	eBioscience	I

3.7 IMMUNOHISTOCHEMISTRY (II AND IV)

The formalin-fixed (48 hours) tissue samples were preserved in 70% ethanol until paraffin embedding. The samples were sectioned at 4-µm thickness and stained with hematoxylin and eosin. A trained veterinary pathologist evaluated and scored the treatment-related histological changes, such as blood vessel abnormalities, signs of inflammation, and necrosis in normal tissues.

3.8 SPLENOCYTE PROLIFERATION (II)

The spleens were collected from the hamsters on day 25 after the beginning of the treatments in Study II. Splenocytes from each group were pooled and divided into four replicates on a 96-well plate, 10 000 cells/well. Cells were counted from 20 µl of cell suspension with BD Accuri Flow Cytometer after a 72-hour incubation. The splenocyte count from each well was divided by the mean cell count in the mock wells to learn the relative proliferation rate.

3.9 QUANTITATIVE POLYMERASE CHAIN REACTION (III)

The spread of the virus was evaluated in Study III by detecting viral *E4* DNA copy numbers from treated and untreated mouse and hamster tumours, as well as from hamster hearts, lungs, livers, spleens, and kidneys, with qPCR (Koski et al. 2010, Kanerva et al. 2002). Tissue samples were cut into pieces and 25 mg was used for the DNA extraction with QIAamp Mini Kit (Qiagen) using a QIAcube machine according to the manufacturer's protocol. The single portion of master mix for the qPCR reaction contained 4.3 µl H_2O, 0.75 µM both primers, 0.1 µM probe and 10 µl enzyme mix from the kit (LightCycler 480 Probes Master, Roche). The *E4* copy number was normalized against mouse *beta-actin* or hamster *GAPDH*. **Table 5** summarizes the primers and probes used in the study and **Table 6** the conditions for the PCR run.

Table 5. Primers and probes used in qPCR (from Oligomer). The probes were labelled with 6'FAM

Gene	Type	Sequence (5'-3')	Ref.
Adeno-viral *E4*	Forward primer	GGAGTGCGCCGAGACAAC	Kanerva et al. 2002
	Reverse primer	ACTACGTCCGGCGTTCCAT	
	Probe	TGGCATGACACTACGACCAACACGATCT	
mouse *beta-actin*	Forward primer	CGAGCGGTTCCGATGC	Kanerva et al. 2002
	Reverse primer	TGGATGCCACAGGATTCCAT	
	Probe	GGCTCTTTTCCAGCCTTCCTTCTTGG	
Hamster *GAPDH*	Forward primer	CACCGAGGACCAGGTTGTCT	Koski et al. 2010

	Reverse primer	CATACCAGGAGATGAGCTTTACGA	
	Probe	CAAGAGTGACCCCACTCTTCCACCTTTGA	

Table 6. Running conditions for qPCR.

Temperature	Time (mm:ss)	Cycles
95°C	10:00	1
95°C **60°C**	00:15 01:00	50
40°C	10:00	1

3.10 MESSENGER RNA SEQUENCING (III)

The RNA samples for the evaluation of gene-expression levels on injected and non-injected tumours were prepared for messenger RNA sequencing by constructing a library with NEBNext Ultra Directional RNA Library Prep Kit 3 (New England Biolabs, Ipswich, MA, USA). The sequencing was performed by single-ended Illumina NextSeq High Output 1 x 75 bp sequencing (Illumina, San Diego, CA, USA). FastQ tool analysed the quality of the sequencing output, and the data were summarised and trimmed with MultiQC and Trimmomatic, respectively (Ewels et al. 2016, Bolger et al. 2014, Andrews 2010). Syrian hamster reference genome, against which the sample reads were aligned, was obtained from NCBI database (RefSeq GCF_000349665.1_MesAur1.0). The FeatureCounts tool was employed when quantifying the results (Liao et al. 2014). The expression profiles of virus-injected tumours were compared with saline-injected tumours. Likewise, the non-injected tumours in treatment groups were compared with the non-injected tumours in vehicle group. The statistical significance of the differences in gene-expression levels was evaluated with DESeq (Love et al. 2014). Genes showing over two-fold up- or downregulation and having $p<0.05$ were examined in more detail and their ontology analysed against human orthologs with WebGestalt (Zhang et al. 2005).

3.11 REVERSE TRASCRIPTION QUANTITATIVE PCR (III AND IV)

RNA was extracted from HapT1 tumours four days after treatment with Ad5/3-E2F-d24 or Ad5/3-E2F-d24-hIL2 to investigate whether treatment of hamster tumours with oncolytic virus induces endogenous IL2 expression. Tumour samples (30 mg) were stored in RNAlater (Qiagen) until disrupted as single-cell suspension. The RNA was extracted with an RNeasy Mini Kit (Qiagen) and transformed into complementary DNA with a QuantiTect Reverse Transcription Kit (Qiagen). Hamster IL2 was detected by qPCR with the following primers and probe: Forward primer GTGCACCCACTTCAAGCTCTAA, reverse primer AAGCTCCTGTAAGCTCAGCAGTAAC, and probe 6-Fam-AGGAAACCCAGCAGCACCTCGAGC-BHQ-1. Hamster *GAPDH* expression was used for normalisation.

Similarly, RNA samples analysed with messenger RNA sequencing were transcribed to cDNA with High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) in order to analyse more in detail the effect of the treatment on TGFβ and FoxP3 expression levels. The sequences for TGFβ primers and probe were TGTGTGCGGCAGCTGTACA and TGGGCTCGTGAATCCACTTC for forward and reverse primers, respectively, and 6FAM-CGACTTTCGCAAGGACCTGGGCT-BBQ for probe. Forward and reverse primers for FoxP3 were AAGCAGATCACCTCCTGGAT and AGCTGCTGCTCCAGAGAC, respectively and the probe sequence 6FAM-CACCACTTCTCTCTGGAGGAGGCAC-BBQ. The qPCR reactions and running conditions were identical to those previously described.

3.1 STATISTICAL ANALYSES (I–IV)

The results of *in vitro* and *ex vivo* experiments were analysed with Student's *t*-test, non-parametric Mann-Whitney test, one-way analysis of variance (ANOVA), or non-parametric Kruskal-Wallis test. The tumour growth curves were analysed over both treatment and time with log-linear mixed-effects model or with two-way ANOVA. The outcome of the treatment in Study II was evaluated by

the Wilcoxon signed rank test and the survival curves in Study III with log-rank test. The analyses were performed with GraphPad Prism 6 and 7 (GraphPad Software Inc., San Diego, CA, USA) or IBM SSP Statistics versions 22–24 (IBM, Armonk, NY, USA). The differences between the groups were considered statistically significant when $p \leq 0.05$.

4 RESULTS AND DISCUSSION

T cell therapies show much promise against haematological malignancies, but efficacy on solid tumours has room for improvement. Solid tumours often develop resistance against T-cell therapies by altering antigen presentation or when tumour phenotype changes due to clonal selection, for example (Sharma et al. 2017). Oncolytic viruses enhance immunogenic cell death, epitope spreading, antigen presentation, and T-cell trafficking and infiltration, all of which are essential for successful ACT (Chen & Mellman 2013). Moreover, oncolytic viruses potentially enable treatment of tumours that developed resistance to ACT, as infection induces sudden and periodical antigen appearance (Pradeu et al. 2013). Oncolytic viruses were studied here as enablers of TIL and TCR therapy in solid tumours.

4.1 CONSTRUCTED VIRUSES ARE FUNCTIONAL *IN VITRO* (I, II)

The studied viruses have a backbone of type 5 adenovirus. The viruses coding for murine cytokines (Ad5-CMV-mIL2, Ad5-CMV-mTNFα, Ad5-CMV-mIFNγ, and Ad5-CMV-mIFNβ) have the transgenes under CMV promoter replacing *E1* region, thus making the virus replication-incompetent (Figure 1a, Study I). The human transgenes were inserted into chimeric viruses bearing adenovirus type 5 genome with fibre knob from type 3 (Ad5/3-E2F-d24-hIL2, Ad5/3-E2F-d24-hTNFα, and Ad5/3-E2F-d24-hTNFα-IRES-hIL2). The transgene expression is linked to virus replication as the genes replace parts of *E3* (Figure 1a, Study II). The replacement of adenovirus *gp19k/6.7k* in E3 region also makes the virus more immunogenic, as those proteins normally block the cell's mechanisms to present antigens on their surfaces (Bennett et al. 1999).

The oncolytic viruses were able to lyse a panel of human and hamster cell lines (Figure 1b and Supplementary Figure S1, Study II), and the effect was more pronounced when combined with TILs

(Figure 1c, Study II). Syrian hamsters are semipermissive for adenovirus replication (Thomas et al. 2006), and in this study, the cell line lysis required five to ten times more virus than human cell lines (Figure 1b, Study II).

Both human and mouse cytokines produced from adenoviruses were biologically active *in vitro* and *in vivo* (Figure 1 and Supplementary Figure S1, Study I; Figure 2 and Supplementary Figure S2, Study II). Importantly, the transgene expression remained local *in vivo* and only negligible levels of IFNs appeared in mouse sera (Figure 1c–f, Study I; Figure 2c, Study II). The control virus expressing luciferase induced IL2, TNFα, and IFNγ expression in mouse tumours, indicating that the virus infection itself promotes immunological reactions (Figure 1c–f, Study I). The cytokine levels were more pronounced, however, when delivered within a virus. Treatment with oncolytic viruses did not induce the expression of endogenous IL2 in hamsters (Supplementary Figure S1, Study IV). As the viruses carry cytokines that are extremely toxic when administered systemically in high doses, local production is an important safety aspect (Schwartz et al. 2002, Schiller et al. 1991).

4.2 ANTITUMOUR EFFICACY OF THE ONCOLYTIC VIRUS IS DOSE DEPENDENT (II)

To establish effective dose for Ad5/3-E2F-d24-hTNFα-IRES-hIL2, immunocompromised mice bearing orthotopic human ovarian cancer (SKOV3-Luc) received systematically 10^5, 10^7, or 10^9 VPs (Figure 3a, Study II). A statistical significance existed between the highest and the lowest dose and, as expected with immunocompromised animals, adenovirally-delivered cytokines did not add to antitumour efficacy (Figure 3b and c, Study II). Moreover, the result indicates that the arming device does not compromise the lytic capability of the virus *in vivo*.

The tumours escaped from the growth control over time (Figure 3b, Study II). The phenomenon is typical for this model: SKOV3-Luc cells upregulate IFN signalling pathways when generating resistance against oncolytic adenoviruses (Liikanen et al. 2011). In addition to IFN pathway, Cancer Upregulated Gene 2 relates to cancer cell

resistance against oncolytic viruses via STAT1 signalling (Moerdyk-Schauwecker et al. 2013, Malilas et al. 2013). The phenomenon describes the core problem with any cancer treatment: Eradication of vulnerable clones induces emergence of the resistant clones (Sharma et al. 2017). To overcome the problem, combinational treatments are used to attack the tumour from multiple angles. In this study, oncolytic viruses were combined with ACT.

4.3 ARMING AN ADENOVIRUS WITH A CYTOKINE IMPROVES ADOPTIVE CELL THERAPIES (I, II)

We employed black C57BL/6 mice bearing subcutaneous B16.OVA melanoma as an immunocompetent mouse model. The cell line expresses chicken ovalbumin, which enables the engagement of OT-I T cells against OVA extracted from a TCR transgenic mouse. Hence, the ACT approach in mice represents TCR therapy. As human adenovirus replicates poorly in mice (Blair et al. 1989), the experimental settings lack the effect of oncolysis. Oncolytic viruses were studied in Syrian hamsters, where human IL2 and TNFα are also active (Gowen et al. 2008, Memon et al. 1997). Moreover, we have established a method to extract TILs from various hamster tumours (Siurala et al. 2016).

Regarding replication-incompetent viruses coding for murine cytokines, IL2 and IFNγ improved TCR therapy the most (Figure 2, Study I). The difference between TNFα alone and TNFα together with OT-I failed to reach statistical significance even though the combination showed the most pronounced antitumour efficacy (Figure 2b, Study I). The only virus failing to improve TCR therapy was Ad5-CMV-mIFNβ (Figure 2d, Study I). TNFα and IL2 were chosen for closer investigations based on these and previous results with recombinant cytokines (Tahtinen et al. 2015).

TNFα has direct antitumour effects when inducing cancer cell apoptosis and necrosis, but it also induces expression of T-cell attracting chemokines (Mocellin et al. 2005). IL2 has more direct effects on T cell proliferation and differentiation (Liao et al. 2013). Separate adenoviral delivery of both cytokines has been studied in cancer patients. Local delivery of TNFα in replication-incompetent

adenoviruses (TNFerade) has been well tolerated and resulted in complete responses in four out of 16 cancer patients (McLoughlin et al. 2005). However, the treatment has not been enough to prolong survival of pancreatic cancer patients compared with the standard of care (Herman et al. 2013). Local injection of adenovirus coding for IL2 (TG1024 and AdCAIL-2) has also been safe and resulted in some complete responses (Dummer et al. 2008, Trudel et al. 2003, Stewart et al. 1999). Moreover, the local treatment increases the presence of CD8+ T cells in tumours, which is an important indicator for efficacy (Dummer et al. 2008, Trudel et al. 2003, Stewart et al. 1999).

The combination of Ad5-CMV-mTNFα and Ad5-CMV-mIL2 enhanced the OT-I TCR therapy the most when compared with both viruses administered alone (Figure 3, Study I). Without OT-I treatment, the combination of the viruses resulted in as good antitumour efficacy as Ad5-CMV-mIL2 alone or together with OT-I (Figure 3, Study I). Ad5-CMV-mTNFα did not have as good antitumour efficacy as did Ad5-CMV-mIL2 in this experiment.

Oncolytic viruses in hamsters did not reveal big differences in efficacy between viruses coding for only one cytokine and the virus coding for both human TNFα and IL2 (Figure 4b-c, Study II). TIL therapy alone did not have antitumour efficacy, but when combined with unarmed oncolytic adenovirus, some of the tumours were even cured (Figure 4a and e, Study II). Armed viruses were also effective as single agents, but combining TIL treatment with Ad5/3-E2F-d24-hIL2 or Ad5/3-E2F-d24hTNFα-IRES-hIL2 cured 100% of the animals (Figure 4e, Study II). The cure rate in groups treated with armed viruses only remained between 55% and 85% (Figure4e, Study II). The results were similar even with lowered virus dosages (Figure 4f, Study II).

The combination of recombinant TNFα and IL2 administered systemically to non-small-cell lung cancer patients has generated modest responses, even though preclinical data has shown much promise (Yang et al. 1990, McIntosh et al. 1988, Winkelhake et al. 1987). The biggest obstacle in using recombinant cytokines is the balance between efficacy and systemic toxicity, which is why tumour-targeted delivery is preferred today. The delivery of IL2 and TNFα as antibody fusion proteins has also resulted in higher antitumour efficacy in combination than as single agents (Pretto et al. 2014).

Moreover, *in vitro* and *in vivo* results show that in this setting, TNFα plays an important role in tumour eradication, as do both cytokines in immune reactions and T-cell stimulation (Menssen et al. 2018).

4.4 COMBINATION OF IL2 AND TNFA MODIFIES IMMUNE CELL PROFILES IN TUMOURS (I, II, III)

The presence of tumour-infiltrating T cells is an important prognostic factor in clinic, and the efficacy of many treatments relies on inducing the T-cell infiltration (Geng et al. 2015, Ibrahim et al. 2014). The infiltration is improvable to some extent with conventional treatments like chemotherapy, but immunotherapies are more potent in this regard (Oelkrug & Ramage 2014). Oncolytic adenoviruses induce infiltration of a variety of immune cells in clinical trials: Specifically, M1 macrophages, CD8+ effector T cells, Th1 helper T cells, and B cells are abundantly present in tumour samples after treatment with oncolytic adenovirus (Lang et al. 2018, Ranki et al. 2016, Vassilev et al. 2015).

4.4.1 VECTORED DELIVERY OF IL2 AND TNFA INDUCES T CELL, NK CELL, AND ANTIGEN PRESENTING CELL INFILTRATION AND ACTIVATION (I, III)

The results show that IL2-armed adenovirus modulates the immune cell populations in tumours the most when compared with an unarmed or TNFα-armed adenovirus (Figure 4, Study I; Figure 5a-e, Study II; Figure 3, Study III). The addition of a therapeutic cell graft did not alter the immune cell profiles (Figure 4, Study I; Figure 5a-e, Study II). Having IL2 in the treatment virus increased the proportion of CD4+ and CD8+ T cells, activated T cells, and B cells in mouse tumours on day 18, and also Tregs to some extent (Figure 4a-e, Study I). Even though cytotoxic CD8+ cells often receive more attention in regard to treatment efficacy, recent studies have shown that CD4+ cells might have even bigger role in patients responding to immunotherapies (Spitzer et al. 2017).

Unlike on day 18 samples, the levels of tumour-specific T cells were not statistically significantly increased on day 8 (Figure 3e, Study III). T cell and NK cell levels were similar in groups treated with IL2 alone or with both IL2 and TNFα, but the combination might induce lymphokine-activated killer cells and NK cells better than IL2 alone (Schultz et al. 1994, Favrot et al. 1990, Yang et al. 1989).

The level of NK cells was increased at both time points, on days 8 and 18 (Figure 4f, Study I; Figure 3a, Study III). NK cells have an important role in eradicating tumours with poor HLA class I expression, which is common in many cancers (Geller & Miller 2011). Moreover, both TNFα and IL2 are essential for NK cell activation, which highlights the importance of this cell population in regard to a therapy employing both of these cytokines (Balasa et al. 2015). NK cells are also essential for tumour-associated antigen presentation on dendritic cells and dendritic cells stimulate NK cell priming (Deauvieau et al. 2015, Lucas et al. 2007).

Importantly, we saw a positive correlation between the presence of NK cells and dendritic cells in mouse tumours (Supplementary Figure S2, Study III). IL2 especially induced the presence of mature dendritic cells at an earlier time point, but on day 18 the increased levels were observed in groups treated with both cytokines or the combination of viruses and OT-I cells (Figure 3b, Study III; Supplementary Figure S2b, Study I).

TNFα had a clear effect on macrophages: Treatment with any virus induced the presence of macrophages, but the effect was most prominent in tumours treated with TNFα-armed virus (Figure 3c, Study III). Importantly, even though TNFα increased the level of macrophages in tumours, it decreased the level of immunosuppressive M2 phenotype at an early time point (Figure 3d, Study III), a phenomenon also reported elsewhere (Kratochvill et al. 2015). On day 18, the level of M2-like macrophages was slightly increased in tumours treated with Ad5-CMV-mTNFα or Ad5-CMV-mIL2 (Supplementary Figure S2a, Study I). However, when these two viruses were administered together, the levels corresponded to the control group receiving TCR therapy alone.

Treating mice with TCRs increased the proportion of cells expressing PD-L1 in tumours, and the addition of armed viruses

further promoted that effect (Figure 5a, Study I). Closer investigation of PD-L1-expressing cells in mice developing spontaneous melanoma suggests that Ad5-CMV-mIL2 or Ad5-CMV-mTNFα induces PD-L1 especially on MDSCs and not on cancer cells (Tahtinen et al. 2016). Increased levels of PD-L1 might also relate to the increased levels of activated T cells producing IFNγ in tumours (Tumeh et al. 2014). In addition, we observed downregulation of PD-1 expressing CD8+ T cells after treatment with viruses coding for IL2, yet the difference was not statistically significant (Figure 5b, Study I). PD-1 is a contradictory marker, since it functions as part of a negative feedback loop on activated T cells (Shi et al. 2013). Hence, the cells expressing PD-1 are cytotoxic but more prone to PD-L1-mediated silencing. In the presence of constant IL2 expression, however, T cells might be more resistant to silencing (Sultan et al. 2018).

4.4.2 ARMED ONCOLYTIC VIRUS INDUCES IMMUNOLOGICAL CHANGES IN TUMOURS BOTH ON CELL AND GENE EXPRESSION LEVEL (II, III)

Similar changes in tumour-infiltrating immune cell populations were observed in hamsters treated with oncolytic viruses than were observed in mice treated with replication-incompetent viruses. Tumours treated with IL2-armed viruses had bigger portions of CD8+ and CD4+ T cells in addition to GM1+ cells that are mainly NK cells (Figure 5a, b, e, Study II). Studies investigating TNFα and IL2 fused to an antibody suggest that the cytokine combination induces mostly CD4 helper cells and cytotoxic CD8 cells (Balza et al. 2010), but the lack of reagents hindered a more detailed characterization of T cells in this study. Therefore, we evaluated treatment-induced immunological changes in tumours with messenger RNA sequencing from day 10 tumours treated with Ad5/3-E2F-d24-hTNFα-IRES-hIL2 or Ad5/3-E2F-d24.

Compared with vehicle injection, Ad5/3-E2F-d24-hTNFα-IRES-hIL2 and Ad5/3-E2F-d24 upregulated 114 and 404 genes over two fold, respectively (Figure 5 and Supplementary Figure S3, Study III). Over two-fold downregulation was rarer: Six genes with Ad5/3-E2F-d24-hTNFα-IRES-hIL2 and 28 with Ad5/3-E2F-d24 (Figure 6 and

Supplementary Figure S3, Study III). Similar to mouse tumours, virus injection upregulated genes belonging to NK cell activation markers such as *Mertk*, *Coro1a*, *Pglyrp2*, *Slamf6*, *Ptpn22*, *Gas6*, *Itgb2*, *Il21r*, *Cd244*, *Pik3cd*, *Rab27a*, *Prdm1*, *Slamf1*, *Havcr2*, and *Cd2* (Supplementary figure S5, Study III). Likewise, genes clustering to dendritic cell differentiation markers (*Dock2*, *Irf4*, *Cd244*, *Slamf1*, *Spi1*, *Havcr2*, *Cd2*) were upregulated upon virus treatment (Supplementary Fig. S5, Study III).

Treatment with viruses had little effect on antigen-presenting immune cells in hamster tumours, as there were negligible changes in the levels of MHC class II or Mac-2 expressing cells when analysed by flow cytometry (Figure 5c, d, Study II). On gene expression level, however, we saw upregulation of macrophage marker *Scara5* (Figure 5, Study III). Tumours treated with Ad5/3-E2F-d24-hTNFα-IRES-hIL2 also expressed a panel of M1 macrophage-related genes and lacked most of the signature genes for M2 macrophages (Kratochvill et al. 2015). Thus, the gene expression profiles reflect the results obtained from mice with flow cytometric analyses (Figure 3, Study III).

Patel et al. (2017) recently reported a panel of genes especially important for successful immunotherapies. Interestingly, the unarmed Ad5/3-E2F-d24 was able to upregulate many of these genes: We saw upregulation of T cell marker *Aplnr* and *B2m*, *Tap1*, *Tap2*, and *Tapbp* that are related to HLA class I antigen processing and presentation (Supplementary Figure S4, Study III). The lack of these specific genes counteracts the efficacy of immunotherapies (Patel et al. 2017). Upregulation of genes related to antigen presentation is especially important, because this pathway is often malfunctional in tumours (Geller & Miller 2011).

Compared with unarmed Ad5/3-E2F-d24, tumours treated with Ad5/3-E2F-d24-hTNFα-IRES-hIL2 uniquely upregulated cytokine genes *Csf3* and *Il1b*, macrophage marker *Marco* and atypical chemokine receptor gene *Ackr3* (Figure 5, Study III). IL1β is an important cytokine in regard to TILs, as it shifts the Th17 helper cell phenotype from immunosuppressive to effector (Chatterjee et al. 2014). Macrophage-derived MARCO has recently been identified as a receptor for adenovirus type 5 that binds the receptor with hexon protein (Stichling et al. 2018). Macrophages commonly allow

replication of many pathogens, which then enhances the pathogen eradication and guides further immune reactions (Price & Vance 2014).

4.5 CYTOKINE-ARMED ADENOVIRUSES INDUCE CELL GRAFT TRAFFICKING INTO TREATED TUMOURS (I, III)

Virus coding for TNFα was especially important for inducing chemokine expression in tumours (Figure 5c, Study I). For this reason, we sought to investigate systemically administered therapeutic cell graft trafficking into locally treated tumours. OT-I T cells or hamster TIL graft were radiolabelled with ^{111}In-oxine and the trafficking of the cells was followed over time. The combination of IL2 and TNFα was the most efficient in recruiting the cells into mouse tumours (Figure 6, Study I). At earlier time points, 24 and 48 hours after cell administration, tumours treated with TNFα-encoding virus had the highest levels of engrafted cells, but the combination of the cytokines was essential for persistent presence of the cells until 96 hours after treatment (Figure 6, Study I). The signal obtained from the vehicle-treated tumours weakened over the time of observation both in mouse and in hamster tumours (Figure 6a, Study I; Figure 4a, Study III).

The combination of TNFα and IL2 was compared with unarmed virus in the oncolytic setting with TILs. The results were similar to those obtained with replication-incompetent viruses in mice: The arming device was necessary for cell graft persistence in tumours (Figure 4a and c, Study III). Even though the unarmed virus was capable of recruiting TIL graft into a tumour, the levels decreased over time, similar to vehicle treatment (Figure 4a, Study III). Overall, both TNFα and IL2 have important roles regarding ACT graft trafficking: TNFα attracts T cells into tumours, while IL2 enables their persistence (Sultan et al. 2018, Calcinotto et al. 2012, Johansson et al. 2012).

4.6 ARMING AN ADENOVIRUS WITH CYTOKINES INDUCES SYSTEMIC ANTITUMOUR EFFECTS (II, III)

Even though positive changes in immune cell milieu in a tumour microenvironment are essential for successful immunotherapy, systemic changes are at least as important (Spitzer et al. 2017). In this study, tumour-draining lymph nodes had lower levels of CD8+ cells when the animals received cytokine-armed viruses (Supplementary Figure S3a–c, Study II). The spleens of these animals had a decreased level of CD4+ cells and a mild increase of MHC class II+ cells (Supplementary Figure S3d–e, Study II). The levels were the opposite of the immune cell populations in tumours, reflecting the possible trafficking of the cells from the reservoirs to the site of action. Moreover, the splenocytes extracted from animals treated with cytokine-armed viruses showed increased activity *ex vivo* as measured by the proliferation rate (Figure 5f, Study II). Interestingly, the unarmed Ad5/3-E2F-d24 did not induce the same effect. When animals were cured with cytokine-armed viruses, they had developed immunological memory against the same tumour cell type (HapT1) but not against a foreign cell type DDT1-MF2 (Figure 6, Study II). Treatment with an unarmed virus cured one animal that was only partially protected against new tumours.

Based on these results, we studied whether local treatment could induce the systemic antitumour effect known as the abscopal effect. Syrian hamsters bore two tumours on their flanks, only one of which received injections of Ad5/3-E2F-d24-hTNFα-IRES-hIL2 or the control viruses. All oncolytic viruses had a similar antitumour efficacy in injected tumours, as previously noted (Figure 1a, Study III). Interestingly, the cytokine-armed viruses also restricted the growth of non-injected tumours, whereas the unarmed virus was not as efficient (Figure 1b, Study III). Low levels of viral DNA were present in non-injected tumours, indicating the virus was trafficking from injected tumours via blood circulation (Figure 1c, Study III). Small quantities of virus DNA were also found in hamster lungs, heart, spleen, liver, and kidneys, and there were no differences between the viruses in this regard (Supplementary Figure S1A, Study III). Previous studies with analogous capsid construct indicate similar distribution of the virus in patients (Koski et al. 2015).

Despite the system-wide distribution of the virus, hamsters treated with Ad5/3-E2F-d24-hTNFα-IRES-hIL2 survived over 120 days – more than three times longer than the untreated animals (Supplementary figure S1b, Study III). The humane endpoint criteria (mainly tumour size and ulceration) determine survival in subcutaneous tumour models, but it is also an important indicator for the systemic toxicity of the treatment. We did not observe any major treatment-related histological changes, such as blood vessel abnormalities, signs of inflammation, or necrosis, in hamster lungs, hearts, spleens, livers, or kidneys on day 25 after five treatments with any of the viruses (Supplemental Materials and Methods, Study II). Hence, despite the fact that the virus is able to penetrate distant organs, it does not cause systemic toxicity in animals.

Partly because of virus spread, tumour growth reduction was equally good in injected and non-injected tumours (Figure 1d, Study III). Treatment with oncolytic herpes virus, T-vec, has induced a similar abscopal effect, but the virus does not spread to distant tumours (Moesta et al. 2017). The response rate to local treatment in patients reaches 26% in injected tumours and 15% in non-injected, which derives, thus, mainly from the immunological effects induced by oncolysis and the transgene GM-CSF (Andtbacka et al. 2015).

The transgenes also had a major effect on systemic efficacy in our study. One out of two B16.OVA tumours on immunocompetent mice received replication-incompetent Ad5-CMV-mTNFα, Ad5-CMV-mIL2, or the combination of these two. Tumour growth was followed until an early time point, day 8. Injected tumours were around 30% of the size of vehicle-treated tumours by this day, whereas the non-injected tumours in the group receiving the combination of TNFα- and IL2-armed viruses resulted in 50% growth reduction (Figure 2a and b, Study III). The addition of TCR treatment did not provide additional efficacy during this short experiment (Figure 2c and d, Study III). The experiment was repeated and again, even though the tumour growth control was not visible on the injected tumours due to inflammation on day six, the non-injected tumours in group receiving cytokine-coding viruses were clearly smaller than the control tumours (Figure 2e and f, Study III). The virus did not spread to distant tumours in this model, indicating the importance of the

transgenes (Figure 2g, Study III); hence, the efficacy derives from both the virus spread and the transgenes.

Interestingly, virally delivered transgenes induced immunological changes also in the non-injected tumours (Figure 3, Study III). Non-injected tumours had significantly increased levels of NK cells and macrophages compared with vehicle treated tumours (Figure 3a and c). IL2 especially induced the presence of mature dendritic cells in injected tumours, but the levels failed to achieve statistical significance in non-injected tumours (Figure 3B, Study III). Similarly, the level of immunosuppressive M2 macrophages was lowered in tumours injected with cytokine-armed viruses, but not significantly in non-injected tumours, despite a clear trend (Figure 3d, Study III). There were no differences in melanoma-specific T cells in either injected or non-injected tumours (Figure 3e, Study III).

In addition to inducing similar immune cell milieus in injected and non-injected tumours, treatment of one tumour induced TIL graft trafficking also to the non-injected tumour (Figure 4b and c, Study III). Small number of replicates, however, prevents the statistical evaluation of the results. Nevertheless, the signal obtained from the tumours in groups receiving vehicle or unarmed virus seemed to decrease over time, whereas the signal from the group treated with Ad5/3-E2F-d24-hTNFα-IRES-hIL2 remained the same (Figure 4a and b, Study III). Importantly, complementary data from mice treated with replication-incompetent viruses point to the same direction: Injection of one tumour induced T-cell graft trafficking also to the non-injected tumour (Figure 4d, Study III). This and other results suggest that besides oncolysis, the cytokine arming-device plays a crucial role in the treatment efficacy.

Because of limited amount of reagents for hamster studies, we sought to investigate the mechanisms behind the observed abscopal effect from gene-expression profiles. The results in mice indicated the importance of chemokines in TCR cell graft trafficking. In hamsters, we saw unique upregulation of chemokines *Cxcl5* and *Rnase2* in the group treated with Ad5/3-E2F-d24-hTNFα-IRES-hIL2 (Supplementary Table S2, Study III), but to current knowledge from humans, more than T cells they attract neutrophils and dendritic cells, respectively (Rosenberg 2015, Zhou et al. 2012).

4.6.1 LOCAL INJECTION WITH ARMED ONCOLYTIC VIRUSES UPREGULATES IMMUNITY-RELATED GENES IN DISTANT TUMOURS (III)

In addition to chemokines, unarmed Ad5/3-E2F-d24 and Ad5/3-E2F-d24-hTNFα-IRES-hIL2 induced expression of several immune-related genes in injected and non-injected tumours (Figure 5 and 6, Supplementary Figure S3–S6, Study III). The hamsters were treated on days 1 and 8, and the samples were collected on day 10. Control animals receiving saline injections to one tumour served as a baseline sample. The gene expression of saline-injected tumours was compared with that of non-injected tumours to resolve the effect of injection itself. Not surprisingly, the gene-expression profile revealed that a needle puncture and an injection with the vehicle also induced responses related to immune system regulation and downregulated cytoskeleton and cell-cell adhesion molecules (Supplementary Table S1, Study III).

TIL treatment alone upregulated 101 genes and downregulated 35 genes in tumours that were injected with saline (Figure 5 and 6, Supplementary Figure S3, Study III). When the tumour was not injected, 136 genes were upregulated and 53 downregulated (Figure 5 and 6, Supplementary Figure S3, Study III). TIL treatment upregulated 17 genes in both tumours compared with vehicle treatment, regardless of injection. The most interesting genes among these are chemokine *Ccl11*, T-cell specific *Slamf9*, complement *C6*, and macrophage surface marker *Siglec1*. The results offer hints of the immunological changes TIL treatment can achieve: T-cell recruitment and antigen-presentation enhancement by macrophages. Interestingly, TIL treatment upregulated immune checkpoint molecule genes *Pdl1* and *Lag3*, which was not observed in virus-treated animals.

In non-injected tumours, Ad5/3-E2F-d24-hTNFα-IRES-hIL2 and Ad5/3-E2F-d24 upregulated 17 and 61 genes and downregulated 12 and 21 genes, respectively (Figure 5 and 6, Supplementary Figure S3, Study III). Treatment with Ad5/3-E2F-d24-hTNFα-IRES-hIL2 resulted in more pronounced systemic antitumour efficacy, which is why the differentially expressed genes in non-injected tumours in this group are especially interesting. The tumours upregulated B-cell related genes *Pou2af1*, *Mzb1*, and *Jchain*, in addition to

immunoglobulin-like genes that have not yet been fully characterised (Figure 5, Study III). Thus, not surprisingly, B cells seem to have an importance in mediating systemic antitumour effects. Similarly, high B cell levels were observed in mouse tumours treated with IL2 encoding viruses (Figure 4e, Study I and Figure 3g, Study IV). Downregulated genes in non-injected tumours in the group treated with Ad5/3-E2F-d24-hTNFα-IRES-hIL2 included heat shock protein genes *Hspb1* and *Cryab*, more commonly muscle-related *Ckm*, *Ca3*, *Mylof*, *Myh2*, *Myh3*, *Casq2*, *Csrp3*, and *Xirp1*, Thrombospondin 4 (*Thbs4*), and T cell-attracting chemokine *Ccl21* (Figure 6, Study III). Thus, the downregulated genes do not shed much light on the mechanisms behind systemic efficacy.

4.7 SYSTEMIC ADMINISTRATION OF RECOMBINANT IL2 IS REPLACEABLE WITH VECTORED DELIVERY (IV)

Current ACT protocols usually include systemic administration of recombinant IL2 after the cell infusion (Besser et al. 2013). Unfortunately, the toxicity of the cytokine often hinders the treatment's efficacy (Schwartz et al. 2002). Vectored delivery, however, has usually been well-tolerated (Dummer et al. 2008, Trudel et al. 2003, Stewart et al. 1999); hence, we studied whether local delivery of IL2 in an adenovirus vector could replace the need for systemic IL2 in regard to TIL and TCR therapy.

Oncolytic virus coding for IL2 (Ad5/3-E2F-d24-hIL2) with TIL treatment restricted hamster tumour growth more than continuous systemic administration of recombinant cytokine (Figure 1, Study IV). Moreover, when the local delivery was studied in mice where the virus does not replicate, the effect was even more pronounced (Figure 2a, Study IV). The IL2 levels were higher in tumours and lower in serums when the cytokine was delivered in an adenovirus vector than they were with systemic administration of recombinant IL2, which is important for treatment safety (Figure 2b, Study IV).

Vectored delivery of IL2 stimulated immune cell populations in tumours as well as – or even more than – the systemically delivered IL2 (Figure 3, Study IV). Recombinant IL2 increased the level of CD4+ cells in hamster tumours, which was not seen with vectored

delivery on day 19 (Figure 3a, Study IV). The specific subtype of these CD4+ cells could not be investigated due to limitations in reagent availability. However, in mice the systemic administration of IL2 induced Treg population by day 9 (Figure 3h, Study IV), which is a well-known problem with IL2 (Ahmadzadeh & Rosenberg 2006). On the contrary, Ad5/3-E2F-d24-hIL2 promoted the presence of CD8+ cells in hamster tumours slightly better than the recombinant protein (Figure 3b, Study IV). Both ways of cytokine delivery induced the presence of CD8+ cells equally well in mice (Figure 3c, Study IV).

There were no statistical differences in the levels of T cell populations recognising tumour antigen OVA or gp100 between the treatments (Figure 3d and e, Study IV). However, the virus was essential for increased levels of M1 macrophages and B cells in tumours (Figure 3f, and g, Study IV). B cells are especially important for CD4+ and CD8+ T-cell functionality (DiLillo et al. 2010), which, together with lower levels of Tregs, might partly explain the superiority of vectored delivery of IL2 over systemic administration. Similar changes in tumour-infiltrating immune cell subsets were observed in patients treated with type 5/3 chimeric adenovirus, coding for GM-CSF correlating with survival (Ranki et al. 2016, Vassilev et al. 2015).

Overall, the virus coding for IL2 was able to induce more positive changes in immune cell populations in tumours than the recombinant protein. This might reflect the so-called bystander effect, in which the virus promotes the attack against both infected and uninfected cancer cells (Cassady et al. 2016).

4.7.1 LOCAL DELIVERY OF IL2 IN AN ADENOVIRUS IS SAFER THAN SYSTEMIC DELIVERY OF RECOMBINANT PROTEIN (IV)

The vectored delivery of IL2 was safer than the systemic treatment when evaluating the treatment-induced histological changes in vital organs (Figure 4 and 5a, Table 1, Study IV). Recombinant IL2 caused extramedullary hematopoiesis in livers and spleens, and it induced necrosis and histiocytosis in hamster splenic ligaments (Table 1, Study IV). The arteries in mouse lungs had thickened muscular walls and narrowed lumina because of reactive endothelial cells (Figure 5a,

Study IV). Importantly, these histological changes were not visible in animals treated with IL2-encoding viruses (Table 1, Study IV).

IL2 often damages human hearts, lungs, kidneys, and central nervous systems, and the cause of toxicity is usually lymphoid infiltration or vascular leakage (Schwartz et al. 2002). IL2 also induces production of other cytokines that cause further damage. We observed increased levels of inflammation-indicating cytokines (TNFα, IL6, and IFNγ) in serums of animals treated with recombinant IL2 but not with Ad5-CMV-mIL2 (Figure 5b, Study IV). All these cytokines have major effects on vasculature, explaining the changes seen on mouse lungs (Sivakumar et al. 2013). Overall, IL2 administration in a virus vector compared with systemic delivery of recombinant protein was not only more effective but also caused less systemic damage.

5 SUMMARY AND CONCLUSIONS

This study presents a novel oncolytic adenovirus Ad5/3-E2F-d24-hTNFα-IRES-hIL2 designed to improve a variety of T-cell related therapies, including ACT and checkpoint inhibitors. The results indicate that the virus induces both local and systemic antitumour effects, and the effect is even more pronounced with therapeutic cell grafts. The effect derives from both infection and the immunostimulatory transgenes. The oncolysis is necessary for the virus to spread to distant tumours, whereas the transgenes induce system-wide immunological changes. The armed virus attracts cell grafts into tumours but also boosts existing immunity. The virus induces dendritic cell maturation, directs macrophage polarisation towards inflammatory M1 phenotype, and promotes cytotoxic T cells.

Treatment with armed oncolytic virus did not induce histological changes in normal tissues or cause systemic toxicity. Moreover, oncolytic adenovirus carrying IL2 could make current ACT protocols safer by replacing the need for systemic administration of IL2. A phase I clinical trial investigating the efficacy of Ad5/3-E2F-d24-hTNFα-IRES-hIL2 with TIL treatment will be carried out in melanoma patients.

6 FUTURE PROSPECTS

The future of cancer therapies lies in combinational treatments. Since each individual tumour is unique and constantly evolving according to Darwinian selection, cancer is an especially difficult disease to overcome from one treatment angle (Sharma et al. 2017). Immunotherapies provide an intriguing option, as they not only target the tumour itself but they also stimulate the patient's own body to fight the disease.

The journal Science already selected cancer immunotherapy as "Breakthrough of the year" in 2013, and new strategies are emerging in the field annually (Couzin-Frankel 2013). For example, this year Sockolosky et al. reported a new design for engineered T cells: orthogonal IL2 receptor and a cytokine that communicates the native IL2 signal in engineered T cells but does not activate the signalling elsewhere (Sockolosky et al. 2018). This blocks the unwanted effects of IL2 yet provides activation where it is essential.

The hunt for novel checkpoint inhibitors is also active. For example, TIM-3, LAG-3, BTLA, TIGIT, VISTA, and B7-H3 have recently raised interest (Marin-Acevedo et al. 2018). Like PD1 and CTLA-4, TIM-3, LAG-3, BTLA, and TIGIT are expressed on T cells, whereas VISTA and B7-H3 are expressed mainly on antigen-presenting cells (Marin-Acevedo et al. 2018). However, the results from clinical trials investigating the blockade of these receptors are still pending.

Checkpoint inhibitors currently work well in immunologically active, hot tumours, but poorly in immunologically silent, cold tumours. Hence, they could benefit from the companionship of oncolytic viruses able to turn cold tumours hot. One of the emerging checkpoint inhibitors that could have synergistic effects with Ad5/3-E2F-d24-hTNFα-IRES-hIL2 is B and T lymphocyte attenuator, BTLA. Oncolytic adenoviruses stimulate B and T cells, but if the cells start expressing BTLA, their activity is impaired. Of note, BTLA is often overexpressed in tumour-associated immune cells (Paulos & June 2010).

Regarding cytotoxic cells, T cells obtain much attention but also NK cells form an intriguing subset. Allogenic NK cell transplant does not cause graft-versus-host disease, and the cells can recognise malignant cells without antigen presentation (Mehta & Rezvani 2018, Ames & Murphy 2014). Currently, CAR-NK cells are under development to make the treatment more targeted, and the approach is now studied in five independent trials (Mehta & Rezvani 2018). Transferred NK cells have a restricted life span; thus, they could also benefit from local constant expression of immunostimulatory cytokines, such as IL2 (Ames & Murphy 2014). In future, some of these emerging therapies could provide a solution for patients suffering from currently incurable cancer.

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