Cancer Gene Therapy Group
Translation Immunology Research Program
Doctoral Programme in Clinical Research
University of Helsinki
Finland

Armed oncolytic immunotherapies for overcoming tumor induced immune suppression.

Sadia Zafar

ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Medicine of the University of Helsinki, for public examination in the Auditorium Sibelius of the HUS Psychiatry Center,

on 23rd April of 2021, at 13:15h.

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Image on cover: Electron microscopy image of TILT-123 (Ad5/3-E2F-d24-hTNF-α-IRES-hIL2) with human lymphocytes and erythrocytes. Image was taken at University of Helsinki, Electron and Advance Microscopy Unit by Sadia Zafar and modified by Ayesha Imran and Victor Cervera-Carrascon.

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Tu Shaheen Hai, Parwaz Hai Kaam Tera

Tere Samne Asman Aur Bhi Hain

Issi Roz-o-Shab Mein Ulajh Kar Na Reh Ja

Ke Tere Zaman-o-Makan Aur Bhi Hain

English Translation:

Your desire to change must be greater than your desire to stay the same

Allama Iqbal

National poet of Pakistan

To my papa

Abstract

Dendritic cells (DCs) are the sentinels of the immune system and are specialized in initiating adaptive immune responses by presenting foreign antigens to T cells. Thus, dendritic cells are critical regulators of immune responses and accordingly have been a focus of cancer immunotherapy research. DC therapy is considered as a promising approach in cancer immunotherapy. However, DC-based vaccines have shown limited efficacy in clinical trials. Oncolytic adenovirus replicates and lyses only cancer cells. Virus-mediated lysis of cancer cells also induces danger signals and exposes tumor epitopes that promote immune system activation against cancer. This study investigated the oncolytic adenovirus 3 coding for CD40 Ligand: Ad3-hTERT-CMV-CD40L (also known as TILT-234) as an enhancer of DC therapy.

In the first study, human cancer patient data suggested that intravenous adenovirus administration is able to transduce distant tumors and virally-produced CD40L can activate DCs *in situ*. Ad3-hTERT-CMV-CD40L was shown to efficiently kill tumor cells *in vitro.* Studies with immunodeficient mice bearing human xenografts suggested that the virus possesses potent antitumor activity. Syngeneic studies conducted in immunocompetent mice with replication-incompetent virus provided data on virally delivered transgene and DC therapy. Replication-incompetent virus in combination with DC therapy elicited potent antitumor activity and triggered antitumor immune responses.

In the second study, we evaluated the synergistic effects of Ad3-hTERT-CMV-CD40L and DCs in the presence of human peripheral blood mononuclear cells both *in vitro* and *in vivo.* This companion therapy showed 100% survival of humanized mice. Adenovirus-delivered CD40L induced DC activation, leading to the induction of Th1-type immune responses. This resulted in greater antitumor efficacy than either approach as monotherapy.

The third study focused on the treatment of prostate cancer. In this study, the Ad3-hTERT-CMV-CD40L and DC therapy companion effect was evaluated in a humanized mouse model bearing a human prostate xenograft and with *in vitro* prostate cancer histocultures. Treatment with companion therapy was shown to induce greater antitumor immune responses *in vivo* and to induce a robust increase in proinflammatory cytokines in addition to DC maturation in established histocultures.

In the fourth study, we focused on the interaction of a chimeric adenovirus Ad5/3 with human lymphocytes and erythrocytes. This study showed that the binding of Ad5/3 with human lymphocytes and erythrocytes occurs in a reversible manner, which enables the virus to transduce different tumors and to retain oncolytic potency both *in vitro* and *in vivo*, with or without neutralizing antibodies.

When bound to lymphocytes or erythrocytes, chimeric Ad5/3 adenovirus showed enhanced tumor transduction after systemic administration in immunodeficient mice bearing xenograft tumors (in the present study A549 and PC3-MM2 were studied).

In summary, the first three studies demonstrated the ability of Ad3-hTERT-CMV-CD40L to modulate the tumor microenvironment and that local delivery of CD40L is safe and efficient regarding DC therapy. In conclusion, Ad3-hTERT-CMV-CD40L was shown to be a potential enabler of DC therapy. The fourth study revealed the ability of a chimeric Ad5/3 adenovirus to transduce non-injected tumors through blood, even in the presence of neutralizing antibodies. Mechanistically, this happens through reversible binding to human lymphocytes and erythrocytes.

Contents

List of original publications

This thesis is based on the following publications

Publication I

Zafar S, Parviainen S, Siurala M, Hemminki O, Havunen R, Tähtinen S, Bramante S, Vassilev L, Wang H, Lieber A, Hemmi S, de Gruijl T, Kanerva A, Hemminki A. Intravenously usable fully serotype 3 oncolytic adenovirus coding for CD40L as an enabler of dendritic cell therapy. *Oncoimmunology* 2016;6(2):e1265717.

Publication II

Zafar S, Sorsa S, Siurala M, Hemminki O, Havunen R, Cervera-Carrascon V, Santos JM, Wang H, Lieber A, De Gruijl T, Kanerva A, Hemminki A. CD40L coding oncolytic adenovirus allows long-term survival of humanized mice receiving dendritic cell therapy. *Oncoimmunology* 2018;7(10):e1490856.

Publication III

Zafar S., Basnet S, Launonen I-M, Quixabeira DCA, Santos JM, Hemminki O, Malmstedt M, Cervera-Carrascon V, Aronen P, Kalliokoski R, Havunen R, Rannikko A, Mirtti T, Matikainen M, Kanerva A, Hemminki A. Oncolytic adenovirus type 3 coding for CD40L facilitates dendritic cell therapy of prostate cancer in humanized mice and patient samples. Human Gene Therapy, 2021:32(3-4):192-202.

Publication IV

Zafar S*, Quixabeira DCA*, Kudling TV, Cervera-Carrascon V, Santos JM, Grönberg-Vähä-Koskela S, Zhao F, Aronen P, Heiniö C, Havunen R, Sorsa S, Kanerva A, Hemminki A. Ad5/3 is able to avoid neutralization by binding to erythrocytes and lymphocytes. Cancer Gene Therapy, 2020:1-13.

*Equal contribution

List of Abbreviations

A549	Human lung cancer cells
ACK	Ammonium-Chloride-Potassium
AMU	Advance Microscopy Unit
APCs	Antigen presenting cells
ATAP	Advance Therapy Access Program
ATCC	American Type Culture Collection
BCG	Bacillus Calmette-Guerin
CAR	Chimeric antigen receptor
CBA	Cytometric Bead Array
CCL21	CC chemokine ligand 21
CD40L	CD40 Ligand
cDCs	Conventional DCs
CPD	Citrate-phosphate-dextrose
CRS	Cytokine release syndrome
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte-associated protein 4
DAMPs	Danger associated molecular patterns
DCs	Dendritic cell
DMEM	Dulbecco's modified Eagle's medium
EBV	Epstein-Barr virus
EJ	Bladder cancer
EMA	European Medicines Agency
FACS	Fluoresence-activated cell sorter
FDA	Food and Drug Administration
GM-CSF	Granulocyte–macrophage colony-stimulating factor
HOCI	Hypochlorous acid

HPV	Human papilloma virus
HSV	Herpes simplex virus
hTERT	Human telomerase reverse transcriptase
IL-12	Interleukin 12
IL-4	Interleukin 4
imDCs	Immature DCs
IMDM	Iscove's Modified Dulbecco's Medium
LPS	Lipopolysaccharide
Luc1	Luciferase
moDCs	Monocyte derived DCs
MAGE-A3	Melanoma-associated antigen-A3
MAPK	Mitogen activated protein kinases
MDSC	Myeloid-derived suppressor cells
MG1	Maraba virus
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NAbs	Neutralizing antibodies
NFkB	Nuclear factor kappa B
NMRI	Naval Medical Research Institute
NK	Natural killer cells
PAMPs	Pathogen-associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PC-3MM2	Prostate cancer cells
PD-1	Programmed cell death protein 1
PGE2	Prostaglandin E2
p-h	Post hoc
PI3K	Phosphatidylinositol-3 kinase
qPCR	Quantitative polymerase chain reaction.
Rb	Retinoblastoma
RPMI	Roswell Park Memorial Institute Medium

SCID	Severe combined immunodeficiency
SEAP	Secreted embryonic alkaline phosphatase
SEM	Scanning electron microscopy
TAA	Tumor associated antigen
TEM	Transmission electron microscopy
Tfh	T follicular helper cells
TIL	Tumor infiltrating leucocytes
TRAFs	TNFR-associated factors
Treg	Regulatory T cell
Th 1	T helper 1
TME	Tumor microenvironment
T-VEC	Talimogene laherparepvec
VEGF	Vascular Endothelial Growth Factor
TGF-β	Transforming Growth Factor

Introduction

Cancer is one of the leading causes of death worldwide. Cancer arises due to several genetic and epigenetic changes that causes cells to grow or divide uncontrollably. Hallmarks of cancer include sustained proliferative signaling, evasion of growth inhibitors, replicative immortality, invasive and metastatic ability, induction of angiogenesis, and resistance to cell death (**Figure 1)** (Gutschner and Diederichs 2012). Together with the immunosuppressive tumor microenvironment (TME), these properties collectively make tumors relatively difficult to eliminate.

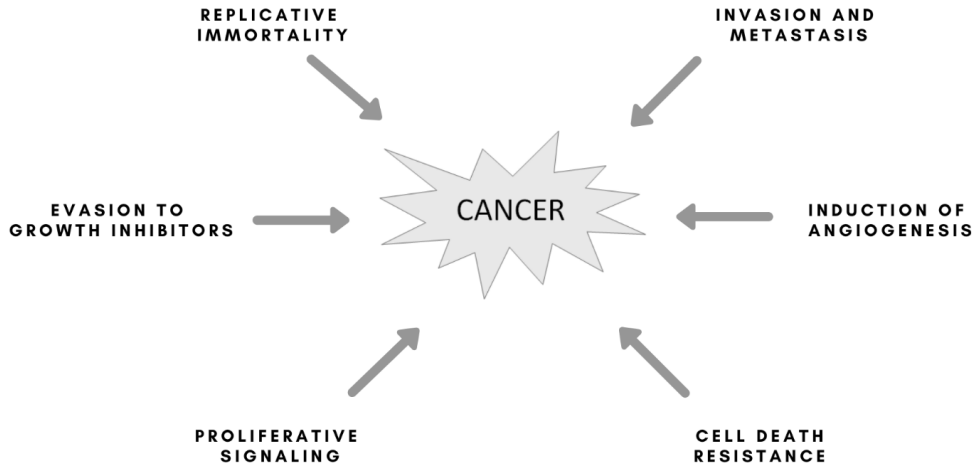

Figure 1: Hallmarks of cancer. Six hallmarks of cancer. Figure adapted from Gutschner and Diederichs 2012.

Despite improvements in the prevention and diagnosis of cancer, mortality due to cancer is still increasing. Global statistics from 2018 revealed 18.1 million new cancer cases and 9.5 million cancer-related deaths (Bray et al. 2018). First-line treatments for most cancers are still conventional cancer treatments such as chemotherapy, surgery, or radiation. Most cases of local cancer can be cured with surgery and adjuvant therapies. However, these therapies have shown limited durable responses for most patients with metastatic cancer (Hemminki et al. 2018). Immunotherapy is an emerging field and may have therapeutic effects along with conventional treatments (Qiao, Liu and Fu 2016). The

concept of immunotherapy dates back to when surgical oncologist William Coley treated cancer patients with a mixture of killed bacteria (Coley's toxin) (Coley 1893). The tuberculosis vaccine, Bacillus Calmette-Guerin (BCG), consists of attenuated *Mycobacterium* and is still used successfully to treat patients with superficial bladder cancer (Lamm et al. 1991). These treatments support the idea that antitumor immune responses play an important role in the treatment of cancer (Lizée et al. 2013). Immunotherapy includes a variety of approaches, ranging from adoptive cell therapies to cytokines, antibodies, and viruses (Farkona, Diamandis and Blasutig 2016). Immunotherapies (such as sipuleucel-T, a therapy that trains autologous dendritic cells for treatment of prostate cancer) (Topalian, Weiner and Pardoll 2011) and antibodies against checkpoint inhibitors (such as programmed cell death protein 1 [PD-1] and cytotoxic T lymphocyte-associated protein 4 [CTLA-4] (Sharma and Allison 2015) have been approved for clinical use. In 2005, the first oncolytic adenovirus, H101, was approved in China for the treatment of nasopharyngeal cancer. Talimogene laherparepvec (T-VEC), an oncolytic herpes simplex virus encoding for GM-CSF, was approved for treatment of cancer by the Food and Drug Administration (FDA) in 2015 followed by the European Medicines Agency (EMA) (Andtbacka et al. 2019).

Immunological research has improved understanding of the molecular mechanisms of the immune system, and immunotherapy has been implemented broadly in the treatment of cancers (Riley et al. 2019). Solid tumors are highly immunosuppressive and heterogeneous and therefore one treatment approach is usually not sufficient (Chen and Mellman 2013, Riley et al. 2019). Immunotherapies can counteract the immunosuppression of the TME. Effective antitumor immune responses occur through a series of steps, starting from the immunogenic cell death that leads to release of tumor antigens. Antigen-presenting cells (APCs) then capture these antigens and present them to T cells. Activated T cells traffic to and infiltrate the tumors and recognize and kill their target cancer cells (Motz and Coukos 2013). However, in cancer patients, each of these steps are hampered.

This study examined the use of cytokine-armed oncolytic adenovirus to treat solid tumors by creating safe and strong antitumor immune responses. In studies I, II, and III, CD40 ligand (CD40L)-armed oncolytic adenovirus enabled DC therapy and induced antitumor immune responses in different murine models *in vivo* and in cancer patient samples *in vitro*. Study IV addressed the mechanism of adenovirus tumor transduction through blood in an immunodeficient mouse model.

1 Review of literature

1.1 Cancer

The first idea of using the immune system of the host to treat cancer dates back decades and relies on the premise that the immune system can eliminate malignant cells during initial transformation in a process termed immune surveillance (Sharma et al. 2011). Over time, more data supporting this hypothesis accumulated and it was acknowledged that the interaction between a tumor and the immune system could also promote tumor development by enabling the tumor to evade immune surveillance (Dunn et al. 2002). The ability of tumors to evade destruction by the immune system is considered one of the hallmarks of cancer. Immune evasion and immunoediting are the main mechanisms by which cancers escape immune surveillance (Hanahan et al. 2011).

1.1.1 Immune evasion

Cancer cells in a TME secrete soluble factors that induce immunosuppressive cell phenotypes and inhibit the activation of immune cells (Kim et al. 2007).

Examples of these soluble factors include IL-10, Vascular Endothelial Growth Factor (VEGF), Prostaglandin E2 (PGE2), Transforming Growth Factor (TGF-β), FasL, and CCL21 (Shields et al. 2010, Kim 2007). These factors are known to suppress cytotoxic CD8+ T cells, downregulate NK cells, induce regulatory T cells (Tregs), and promote myeloid-derived suppressor cells (MDSC) and M2 macrophages in the tumor microenvironment (Shields et al. 2010, Motz et al. 2014).

The presence of these suppressive immune cells subsequently prevents immune responses against the tumor. Tregs suppress effector T cells, dendritic cells, and natural killer (NK) cells either by producing immunosuppressive cytokines (such as IL-10 and TGF-β) or through direct cell-to-cell interactions (Wang and DuBois 2015). In tumors, an increase in the number of Tregs correlates with an increase in the number of MDSCs, which in turn are known to promote Treg activation and differentiation of macrophages towards the M2 phenotype (Gabitass et al. 2011, Gabrilovich, Ostrand-Rosenberg and Bronte 2012b). M2-like macrophages also express TGF-β and IL-10. They also express PD-L1, which binds to its receptor PD-1 on T cells and renders T cells inactive, thus promoting tumor progression (Gabrilovich et al. 2012b, Kuang et al. 2009). Moreover, APCs and cancer cells can inhibit T cells via PD-1 by expressing its ligands PD-L1 or PD-L-2. Another immune checkpoint molecule, CTLA-4, is expressed on T cells as a co-inhibitory molecule and regulates the extent of T-cell activation. CTLA-4 binds to its ligands B7-1 and B7-2 on APCs and inhibits T-cell activity (Webb et al. 2018). Collectively, these factors contribute to tumor progression. Cancer

immunotherapies, such as adoptive cell transfer, cytokine treatments, and oncolytic viruses all modulate the immunosuppressive TME.

1.1.2 Immunoediting

Cancer immunoediting refers to the ability of the immune system (both the innate and adaptive) to prevent the formation of tumors and to shape the immunogenicity of the developing tumors (Vesely et al. 2011). This process can be divided into three phases, namely elimination, equilibrium, and escape (**Figure 2)**. In the elimination phase, cells of the innate immune system, such as NK cells, NK T cells, and γδ T cells actively identify newly transformed cells and start producing interferon (IFN)-γ (Dunn et al. 2002, Girardi et al. 2001, Smyth, Godfrey and Trapani 2001, Matzinger 1994). IFN-γ then further enhances the recruitment of antigen-presenting cells, such as DC and macrophages, as well as more NK cells (Dunn et al. 2002, Yokoyama 2000, Cerwenka et al. 2000). Moreover, IFN-γ also contributes to the apoptosis of tumor cells (Kumar et al. 1997). DCs take up tumor antigens and present them in lymph nodes to CD4+ T cells and CD8+ T cells, which are then activated and traffic to the tumors where cytotoxic T cells kill sensitive tumor cells. According to Darwinian selection, due to mutations, cells develop resistance to immune attack during the equilibrium phase. These resistant tumor variants begin to expand in an uncontrolled way. This consequently leads to tumor progression (escape phase).

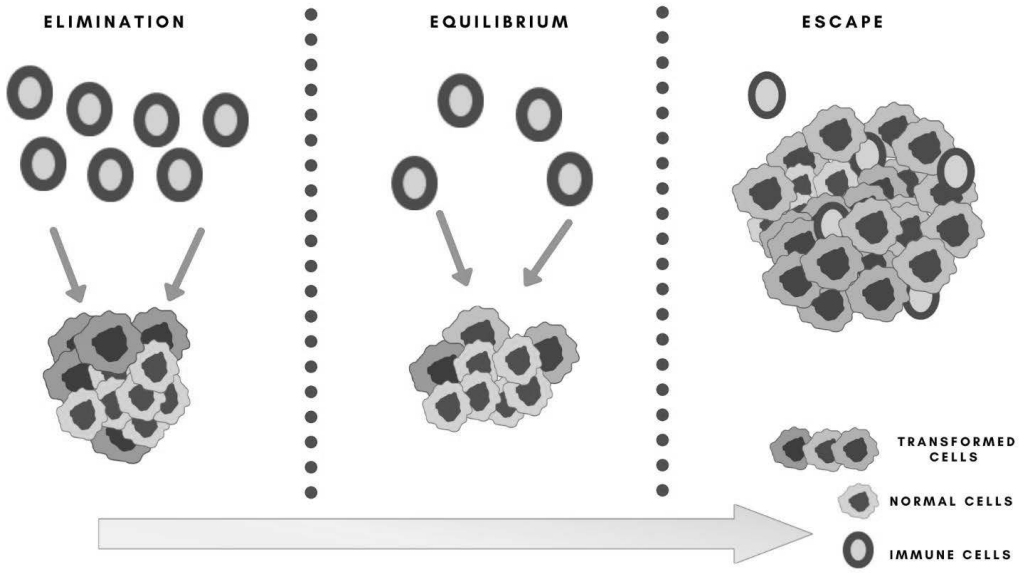

Figure 2: Cancer immunoediting. The three Es of cancer immunoediting include elimination, equilibrium, and escape. During the elimination phase, innate and adaptive immune cells recognize transformed cells and destroy them, thus resulting in a return to normal physiological tissue. However, if antitumor immunity is unable to completely destroy transformed cells, surviving tumor variants may enter into the equilibrium phase. During the equilibrium phase, cells of adaptive immunity prevent tumor outgrowth. These tumor variants may acquire further mutations that lead to evasion of tumor cell recognition and killing by immune cells. In the escape phase, transformed cells begin to grow in an immunologically unrestricted manner and emerge as clinically detectable malignancies.

1.2 Adoptive DC therapy

1.2.1 Dendritic cells

DCs are antigen-presenting cells with a unique ability to induce adaptive immune responses (Mastelic-Gavillet et al. 2019). They are characterized by their stellate morphology and their ability to migrate from peripheral tissues to lymphoid organs and to prime naïve T cells through antigen presentation, thus inducing immune responses (Palucka and Banchereau 2012). DCs act as a bridge between innate and adaptive immunity (**Figure 3**) and are considered as sentinels of the immune system (Aarntzen et al. 2008, Banchereau et al. 2000)

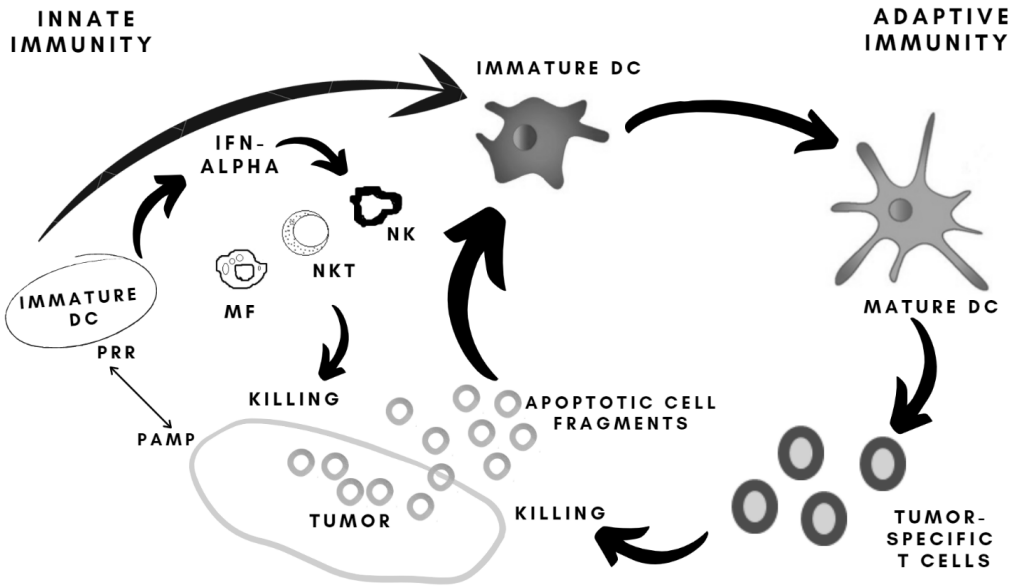

Figure 3: Dendritic cells as a bridge between the innate and adaptive immune systems. Immature DCs recognize tumor pathogen-associated molecular patterns (PAMPs) through their pattern recognition receptors. Immature DCs activate cells of the innate immune system through the release of IFN-alpha, which leads to the antitumor activity that results in release of apoptotic cell fragments. Immature DCs capture these apoptotic cell fragments, mature, and present the tumor antigens to T lymphocytes. Activated cytotoxic CD8+ T cells kill tumors directly and CD4+ T cells further activate other immune cells to induce tumor killing. Figure adapted from Jacques Banchereau et al. 2000

1.2.1.1 Maturation of Dendritic cells

Maturation of DCs is a complex process. Immature DCs (imDCs), which have high phagocytic capacity, patrol through tissues and collect antigens via pinocytosis. Upon exposure to PAMPs, imDCs undergo numerous phenotypic changes, including upregulation of costimulatory surface markers, such as CD86 (B7.2) and CD80 (B7.1), and secretion of pro-inflammatory cytokines. imDCs then migrate to lymphoid organs and mature to become mature DCs (mDCs). In lymphoid organs, mDCs initiate immune responses through antigen presentation to T lymphocytes. Upon interaction of

naïve CD4+ T cells and CD8+ T cells with DCs, they differentiate into antigen-specific effector T cells. Naïve CD8+ T cells can differentiate into cytotoxic T cells and CD4+ T cells can differentiate to T helper 1 (Th1), Th2 cells, Th17 cells, regulatory T cells (Treg), or T follicular helper cells (Tfh). These activated T lymphocytes expand, differentiate, and migrate to the target site (Garg et al. 2017). Therefore, DC maturation is an important prerequisite for the immunogenicity of DCs in humans. imDCs that have not matured can induce tolerance in T cells (Dhodapkar et al. 2001).

1.2.2 Dysfunction of tumor-infiltrating DCs

For the induction of protective antitumor immunity, optimal function of DCs is very important. However, since the TME is highly immunosuppressive, it can impair DC differentiation, maturation, and function (Bandola-Simon and Roche 2019, Pinzon-Charry, Maxwell and López 2005, Gabrilovich, Ostrand-Rosenberg and Bronte 2012a). The improper differentiation of DCs leads to inadequate antigen-presenting functionality, which then contributes to T-cell anergy or exhaustion (Gabrilovich et al. 2012a, Gabrilovich 2004). DCs derived from patients with advanced cancer exhibit a weak ability to stimulate T cells (Almand et al. 2000), and high levels of intratumoral DCs correlate with poor clinical outcome (Conrad et al. 2012). Tumor-infiltrating DCs undergo phenotype switching from an immunostimulatory to a regulatory phenotype (Scarlett et al. 2012). This correlates with enhanced upregulation of costimulatory molecules such as PD-L1 (Krempski et al. 2011) and TIM-3 (Chiba et al. 2012), along with increased L-arginase production (Norian et al. 2009). DCs with immunosuppressive properties are associated with impaired T cell activity (Karyampudi et al. 2016). Therefore, immunosuppressive DCs in the TME contribute to tumor progression and probably limit the clinic efficacy of DC therapy.

1.2.3 DC-based vaccines

Different clinical trials using DC-based vaccines have been conducted. These trials have included patients with more than two dozen tumor types. Most trials have studied patients with malignant melanoma, prostate cancer, colorectal carcinoma, or multiple myeloma using autologous DCs pulsed with synthetic antigens. DC vaccines can also be prepared by pulsing DCs with tumor lysates or RNA, or by transfection with tumor DNA. Various approaches to vaccination have been tested, for example frozen preservation of vaccines, a maturation step for DCs, and different cell numbers, length of vaccination program, or site of vaccination. The process of DC vaccine generation is shown in **Figure 4**. Adverse effects associated with DC vaccination are uncommon; most have been mild and self-limiting and none have been serious. Clinical responses have been observed in approximately

half of trials. DC vaccination may therefore provide a safe approach to cancer immunotherapy that can overcome the limited reach and immunogenicity of peptide vaccines (Ridgway 2003). Different dendritic cell therapies are listed in **Table 1.**

DC-based vaccines were initially prepared from DCs directly isolated from peripheral blood *in vitro*. However, this method yields low numbers of DCs (Hsu et al. 1996b). DC vaccines currently use exogenously matured and/or expanded monocyte-derived DCs (moDCs), or conventional DC (cDC) precursors. Most trials use moDCs, due to the relative ease in obtaining sufficient cell numbers from the blood (Granot et al. 2017).

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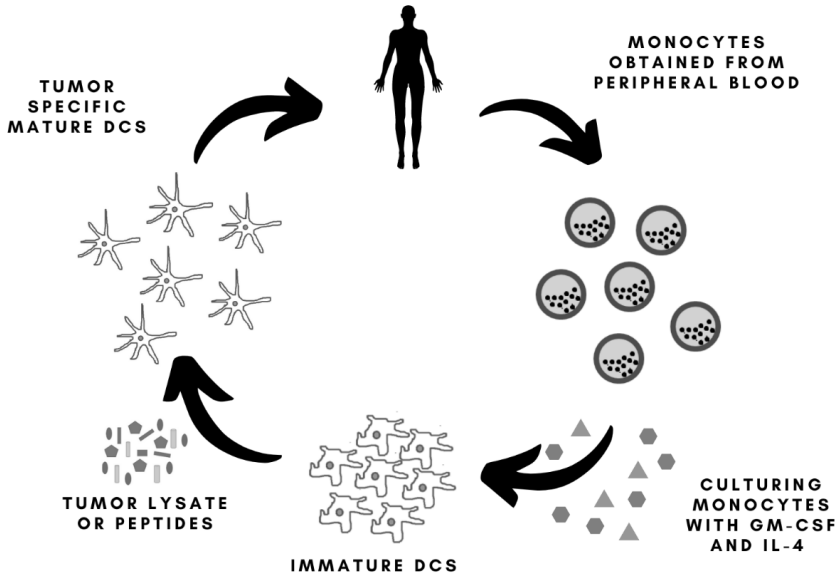

Figure 4: Process of DC vaccine generation. Monocytes are isolated from the patient's peripheral blood. Culturing monocytes in the presence of GM-CSF and IL-4 differentiates monocytes into immature DCs. Immature DCs are then cultured with cytokine cocktails and pulsed with tumor lysate, specific tumor antigens, or neo-antigens. Mature tumor-specific DCs are injected back into the patient.

1.2.3.1 DC-based vaccines using tumor-associated antigens

One of the most important components of DC vaccines is tumor-associated antigens (TAA), i.e. antigens that can be presented to and be recognized by tumor-specific T cells. TAAs are expressed on tumor cells but can also be present on normal cells. TAAs vary in their immunogenicity and may undergo immune editing to escape immune recognition (Escors, 2014). These antigens can be categorized into two groups depending upon their expression in healthy tissues. The first group consists of antigens overexpressed in tumors, i.e. the expression levels of TAAs are higher in tumor cells compared to normal cells. The second group consists of differentiation antigens, i.e. antigens specific for a cell lineage. For example, most melanoma tumors express melanocyte differentiation antigens (Kawakami et al. 1994). Most cancer vaccines use either a defined antigen or a mixture of defined antigens (98-103). One of the potential drawbacks of this approach is that tumors may escape through the loss of expression of these defined (or selected) antigens (Beatty and Gladney 2015, Mohme, Riethdorf and Pantel 2017). To overcome this challenge, the use of multiple antigens, either defined or undefined, may be important to achieve clinical efficacy.

1.2.3.2 DC-based vaccines using neoantigens

Tumor cells with high mutational rates express neoantigens, i.e. modified self-antigens. Neoantigens are generated by mutations in the tumor cell genome. They possess strong immunogenicity and are not expressed in normal tissues. Therefore, they can be considered as viable therapeutic targets for cancer immunotherapy (Lu and Robbins 2016). A neoantigen-targeted approach in cancer vaccines has shown some potential (Carreno et al. 2015, Ott et al. 2017). In phase I clinical trials, somatic mutations in tumors from three melanoma patients were identified and short peptides containing seven neoantigen epitopes were used to pulse DCs from these patients. Pulsed autologous DCs, in turn, can activate neoantigen-specific T cells (Carreno et al. 2015). However, the cost and time for the neoantigen epitope identification process (from tumor resection to vaccine administration) represent major challenges for this approach.

1.2.3.3 DC-based vaccines using whole tumor lysates

Autologous tumor lysates are a source of patient-specific TAAs (Palmer et al. 2009). Different tumor lysate preparation methods have been used, for example freeze-thawing, UV irradiation, oxidation of

resected tumors (e.g. use of hypochlorous acid [HOCI]), or combinations thereof (Chiang, Coukos and Kandalaft 2015, Courrèges et al. 2006, Benencia, Courrèges and Coukos 2008, Chiang et al. 2013). These preparation methods also increase the immunogenicity of the tumor lysate. DCs pulsed with tumor lysate are safe and well-tolerated in patients (Alfaro et al. 2011, Nestle et al. 1998). The main benefit of using whole tumor lysate as a source of TAAs is the reduced cost and development time when compared with neoantigen prediction strategies.

Table 1: Different dendritic cell -based vaccines

	Advantages	**Disadvantages**
Tumor-associated antigens	Prevalence in multiple patients is high, Economical	Variable tumor specificity Low to variable immunogenicity
Neoantigens	Tumor specificity is high High efficacy	Expensive Technology- and labor-intensive
Whole tumor lysate	Contains complete patient-specific tumor-associated antigens Identification and selection of neoantigens is not required Economical	Resected tumor tissue is needed as a source of autologous tumor cell lysate Variable cancer specificity

1.3 Clinical efficacy of DC therapy

The ability of DCs to reduce tumor growth was shown in a murine model over two decades ago (Zitvogel et al. 1996). In 1996, the first DC application was reported in humans (Hsu et al. 1996a). The first practical protocol for the generation of DCs was also reported in 1996 (Bender et al. 1996). This led to more opportunities for DC applications in the clinic. By 2001, the number of studies on DC-based vaccines reported at annual meetings and in different peer-reviewed international publications exceeded one thousand (Ridgway 2003).

The results of many of the phase I and II trials on DC therapy are variable; some have shown disappearance of some metastases, appearance of new metastases, or disease stabilization in a subset of patients. However, objective responses have been observed less consistently. According to a review of multiple clinical trials, DC therapy has led to tumor regression on average in only 7.1% of patients (Rosenberg, Yang and Restifo 2004, Timmerman and Levy 2004). In clinical trials with stage-IV melanoma patients, DCs pulsed with autologous tumor cells have shown an approximate 20% response rate (O'Rourke et al. 2007, O'Rourke et al. 2003). A summary of 98 published trials with DC treatment revealed that at least one or more subjects in 16 of the clinical trials had experienced complete responses and at least one subject in 48 trials demonstrated clinical responses (Ridgway 2003). Another study revealed 9% objective responses (complete response 3%, partial response 6%) for DC therapy in the treatment of melanoma (Engell-Noerregaard et al. 2008).

In 2010, the FDA approved an autologous moDC vaccine Sipuleucel-T (Provenge; Dendreon) for the treatment of castration-resistant prostate cancer. In this vaccine, DCs are pulsed with the tumor antigen prostatic acid phosphatase (PA2024) fused with cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF). Sipuleucel-T resulted in improved overall survival (Kantoff et al. 2010). However, its performance in the clinic as a monotherapy was ultimately disappointing (Saxena et al. 2018). More recently, Sipuleucel-T in combination with ipilimumab (anti-CTLA-4 antibody) has shown some clinical benefit (Scholz et al. 2017). Additional combination studies of Sipuleucel with other therapies are underway (Handy and Antonarakis 2018).

A phase I clinical trial investigated monocyte-derived DCs pulsed with HOCl-oxidized autologous tumor cell lysate to treat ovarian cancer patients who had previously undergone platinum treatment (Tanyi et al. 2018). Patients received either DC-based vaccine alone, in combination with bevacizumab (anti-VEGF antibody), or with bevacizumab and low-dose cyclophosphamide until disease progression. Treatment was shown to induce antitumor immune responses and to prolong overall survival. The combination of DC vaccine with bevacizumab and cyclophosphamide had the

best results. This study demonstrated that the clinical outcome of DC vaccines can be greatly enhanced via combination with other immunotherapies (Tanyi et al. 2018).

A phase III clinical trial investigated the use of monocyte-derived DCs pulsed with autologous tumor cell lysate for the treatment of patients with glioblastoma multiforme. Addition of a DC vaccine to the standard therapy for glioblastoma (i.e. surgery, radiotherapy, temozolimide) enhanced overall survival (Liau et al. 2018). Different ongoing clinical trials with DC vaccines are summarized in **Table 2**.

In summary, in clinical trials, DC vaccines have shown limited efficacy, possibly due to the highly immunosuppressive TME (especially at the advanced tumor stage), limited ability of DCs to migrate from site of administration to the draining lymph nodes, DC source, and frequency of DC administration. DC vaccines could be improved not only through the use of optimized DC subset selection and administration route but also through combining DC-based vaccination with other therapies.

Table 2 : List of different ongoing clinical trials with dendritic cell vaccines. The following trials were mentioned on the Clinicaltrials.gov website with active status. Search was conducted in November 2020.

Indication	Official Title of the trial	Intervention	Phase	Enrollment status	Clinical trial ID
Metastatic melanoma	Multi-center Phase I/IIa Trial of an Autologous Tumor Lysate (TL) + Yeast Cell Wall Particles (YCWP) + Dendritic Cells (DC) Vaccine in Addition to Standard of Care Checkpoint Inhibitor of Choice in Metastatic Melanoma Patients With Measurable Disease. (	Autologous tumor lysate, particle-loaded, dendritic cell (TLPLDC) vaccine in addition to standard of care checkpoint inhibitor of choice	Phase 1|Phase 2	45	NCT02678741
Newly diagnosed glioblastoma	Pilot Clinical Trial of Allogeneic Tumor Lysate-Pulsed Autologous Dendritic Cell Vaccination in Newly Diagnosed Glioblastoma	Malignant glioma tumor lysate-pulsed autologous dendritic cell vaccine + temozolomide	Early phase 1	21	NCT01957956
Malignant glioma|Glioblastoma	Dendritic Cell Vaccine For Malignant Glioma and Glioblastoma Multiforme in Adult and Pediatric Subjects	Dendritic cell vaccine|Tumor lysate|Imiquimod|Leukapheresis	Phase 1	20	NCT01808820
Sarcoma Soft Tissue Sarcoma Bone Sarcoma	A Phase I Trial of Dendritic Cell Vaccination for Children and Adults With Sarcoma	Dendritic Cells VaccineILysate of TumorIGemcitabineIImiquimod	Phase 1	56	NCT01803152
Malignant melanoma	A Prospective, Randomized, Blinded, Placebo-controlled, Phase IIb Trial of an Autologous Tumor Lysate (TL) + Yeast Cell Wall Particles (YCWP) +	Autologous Tumor Lysate (TL) + Yeast Cell Wall Particles (YCWP) + Autologous tumor lysate, particle-loaded,	Phase 2	120	NCT02301611

	Dendritic Cells (DC) Vaccine vs Unloaded YCWP + DC and Embedded Phase I/IIa Trial With Tumor Lysate Particle Only (TLPO) Vaccine in Stage III and Stage IV (Resected) Melanoma to Prevent Recurrence	dendritic cell (TLPLDC) |Placebo			
Multiple Myeloma	A Phase I Trial of Vaccination With CT7, MAGE-A3, and WT1 mRNA-electroporated Autologous Langerhans-type Dendritic cells as Consolidation for Multiple Myeloma Patients Undergoing Autologous Stem Cell Transplantation	CT7, MAGE-A3, and WT1 mRNA-electroporated Langerhans cells (LCs) plus standard of care.	Phase 1	28	NCT01995708
Newly-diagnosed Glioblastoma	Phase II Trial of Autologous Dendritic cells Loaded With Autologous Tumor Associated Antigens (AV-GBM-1) as an Adjunctive Therapy Following Primary Surgery Plus Concurrent Chemoradiation in Patients With Newly Diagnosed Glioblastoma	Autologous dendritic cells loaded with tumor-associated antigens from a short-term cell culture of autologous tumor cells. AV-GBM-1 is admixed with granulocyte-macrophage colony stimulating factor (GM-CSF) as an adjuvant, prior to injection	Phase 2	55	NCT03400917
Glioblastoma	Personalized Cellular Vaccine Therapy in Treating Patients With Newly Diagnosed Glioblastoma (PerCellVac)	Tumor antigen pulsed DC-based cellular vaccine. Subjects will undergo surgical resection and standard 6-week chemo/radiotherapy and cycles of TMZ treatment	Phase 1	20	NCT02709616
metastatic kidney cancer.	A Phase I, Open Label, Dose Escalation and Cohort Expansion Study to Evaluate the Safety and Immune Response to Autologous Dendritic	Dendritic cells transduced with AdGMCA9 expressing GM-CSF-carbonic anhydrase IX fusion protein	Phase 1	18	NCT01826877

	cells Transduced With Ad-GMCAIX in Patients With Metastatic Renal cell Carcinoma				
Glioblastoma, Malignant Glioma, Medulloblastoma Recurrent, Pediatric Glioblastoma Multiforme,Pediatric Brain Tumor, RecurrentPediatric Brain Tumor	A Phase 1 Trial of CMV RNA-Pulsed Dendritic cells With Tetanus-Diphtheria Toxoid Vaccine in Pediatric Patients and Young Adults With WHO Grade IV Glioma, Recurrent Malignant Glioma, or Recurrent Medulloblastoma	CMV RNA-pulsed dendritic cells (DCs), also known as CMV-DCs, with Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF)Itetanus toxoid (Td)	Phase 1	10	NCT03615404
Glioblastoma, Astrocytoma, Grade IV, Giant cell Glioblastoma, Glioblastoma Multiforme	Evaluation of Overcoming Limited Migration and Enhancing Cytomegalovirus-specific Dendritic cell Vaccines With Adjuvant Tetanus Pre-conditioning in Patients With Newly-diagnosed Glioblastoma	Unpulsed DCs|Td|Human CMV pp65-LAMP mRNA-pulsed autologous DCs|111In-labeled DCs|Temozolomide|Saline|Basiliximab	Phase 2	100	NCT02366728

1.4 Adenoviruses

Rowe and colleagues were the first to isolate adenovirus from adenoidal tissues (Rowe et al. 1953). Oncolytic adenoviruses are among the most stable and versatile group of oncolytic virus platforms to be used for cancer therapy (Kaufman et al. 2015, Cerullo et al. 2012). Human adenoviruses belong to the genus mastadenovirus, which contains seven human adenovirus species named A to G (Hoeben and Uil 2013). There are more than 50 serotypes (Rojas et al. 2016a). In gene-therapy studies, the most commonly used adenovirus vector is serotype 5 adenovirus (group C), as its structure and function has been studied extensively (Appaiahgari & Vrati 2015). However, the Ad5 receptor is known to be downregulated in advanced tumors. The use of adenovirus based on serotype 3 is an attractive alternative, as the receptor for serotype 3 is highly expressed on cancer cells (Hemminki et al. 2011).

1.4.1 Adenovirus structure and life cycle

Adenoviruses are non-enveloped viruses and have an icosahedral capsid 70 to 90 nm in diameter. The capsid contains the 36-kb genome consisting of double-stranded DNA (Davison, Benko and Harrach 2003, Nemerow et al. 2009). The capsid consists of 240 hexons with pentons at each vertex. The penton base is a pentameric molecule that associates with the fiber protein protruding from the middle (Nemerow et al. 2009). The fiber protein consists of shaft and knob parts that interact with the host cell for attachment (Law & Davidson 2005). For entry of adenovirus into the host cell, the fiber knob interacts with cellular receptors as the major attachment site. For serotype 5 adenovirus (group C), coxsackievirus and adenovirus receptor (CAR) is the high-affinity entry receptor (Bergelson et al. 1997); desmoglein-2, CD46, or CD86 are used as receptors for serotype 3 and 35 adenoviruses (group B and D) (Wang et al. 2011, Wu et al. 2004, Gaggar et al. 2003). The penton base is involved in secondary interactions that are required for virus entry into the cell. Interaction of penton with integrin $\alpha_v\beta_3$ or $\alpha_v\beta_5$ facilitates adenovirus internalization into the host cell via clathrin-coated vesicles (Wickham et al. 1993).

Upon entry into the host cell, the endosome acidifies, which causes destabilization of the capsid (Flint et al. 2020). These modifications result in the disruption of the endosome and the released virus is then transported through cellular microtubules to the nuclear pore complex. The virus is disassembled and viral DNA enters the nucleus through nuclear pores and interacts with host-cell histones (Meier and Greber 2004, Giberson, Davidson and Parks 2012).

The process of DNA replication consists of early and late phases. The adenovirus genome consists of early-phase gene regions (E1, E2, E3, E4) and late-phase gene regions (L1, L2, L3, L4, L5). The early-phase genes are mainly responsible for expressing non-structural, regulatory proteins that prepare the cell for viral DNA replication. The late-phase genes are responsible for expressing structural proteins to pack the produced DNA. For adenovirus assembly, hexons and pentons with non-structural proteins form empty capsids followed by interaction of the adenovirus genome with packaging proteins and then entry into the capsid. Lastly, the viral protease cleaves immature precursor proteins for virus maturation. Typically, the adenovirus replication cycle takes 24 to 36 hours and can produce viruses that continue to infect other cells when the infected cell is lysed (Figure 5) (Ahi and Mittal 2016, Jogler et al. 2006, Giberson et al. 2012).

1.4.2 Adenovirus modifications for cancer therapy

Modifications to adenoviruses that restrict infection and lysis to cancer cells are important. E1A is the most crucial protein for adenovirus replication, as it is firstly expressed protein and start the replication cycle (Radko et al. 2015). To restrict adenovirus replication to cancer cells, cancer-specific promoters are added before the E1A gene. For example, human telomerase reverse transcriptase (hTERT) has been used as a promoter as it is overexpressed in various cancer types. The conditionally replicating oncolytic adenovirus 5 armed with human GM-CSF (KH901) uses hTERT as a promoter for E1A regulation (Chang et al. 2009). Adenovirus CV706 uses a prostate specific antigen promoter and has been used in a phase I clinical trial that has shown efficacy in patients with prostate cancer (DeWeese et al. 2001).

Immediate early E1A protein drives cells into the S phase and enables adenovirus replication by interfering with the retinoblastoma (Rb) signaling pathway (Radko et al. 2015). In cancer cells, the Rb pathway is commonly disabled, which results in excess expression of E2F (family of DNA binding transcription factors). Thus, this allows the virus to replicate in cancer cells even in the absence of E1A and Rb binding (Heise et al. 2000).

Another modification that limits adenovirus replication to cancer cells and prevents replication in non-dividing normal cells is the 24 base-pair deletion of E1A (Heise et al. 2000). To further improve selectivity, the E2F promoter can be incorporated before E1A (Rojas et al. 2009). In addition to the Rb pathway, one of the most common mutations in cancer cells relates to the apoptosis-inducing protein p53. The adenovirus protein E1B inhibits apoptosis of host cells through binding and inactivating p53. A deletion in this gene makes infected normal cells susceptible to apoptosis.

Whereas, cancer cells are more prone to virus replication and infection, the first published ONYX-015 and the approved H101 are conditionally replicating oncolytic adenoviruses and have a deletion in E1B 55K as a selection mechanism (Cheng et al. 2015, Heise et al. 1997).

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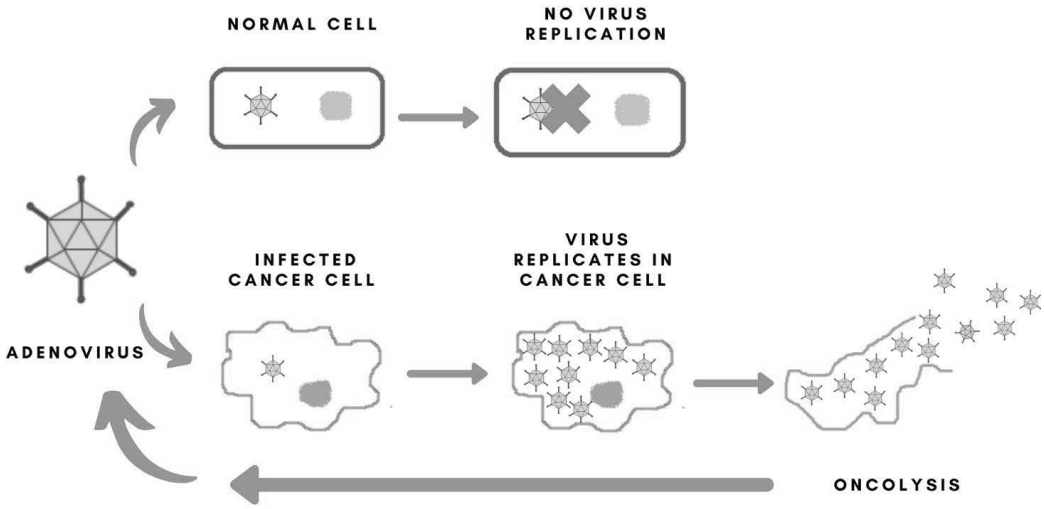

Figure 5: Mechanism of action of oncolytic adenoviruses. Oncolytic adenoviruses replicate and lyse only tumor cells. In normal cells, the virus does not replicate and the cell remains unharmed. In tumor cells, virus-mediated cell lysis releases virus in the TME and the virus can then infect other tumor cells.

1.5 Improving cancer immunotherapy by arming oncolytic viruses with CD40L

CD40L is a type II, 39-kDa membrane glycoprotein that belongs to the tumor necrosis factor superfamily. CD40L is primarily expressed on activated T cells and platelets. Its expression on the surface of activated T cells reaches a peak after 6 hours of activation and declines over the next 24 hours (Casamayor-Palleja, Khan and MacLennan 1995). CD40L binds to its receptor CD40, which was initially identified as a marker on B cells and bladder carcinoma cells (Paulie et al. 1989). Later it was found that CD40 is mainly expressed on APCs, such as monocytes, DCs, and B cells (van Kooten and Banchereau 1997) and also on activated CD8+ T cells (O'Sullivan and Thomas 2003). In

addition to immune cells, CD40L is also expressed on endothelial cells, fibroblasts, and on certain hematopoietic and epithelial tumor cells (Elgueta et al. 2009). In B cells, interaction of CD40 and CD40L is essential for the generation and survival of plasma cells and memory B cells. In T cells, the interaction of CD40 and CD40L is a pair of co-stimulatory molecules and is important for induction of adaptive immune responses (Ahmed et al. 2012).

The interaction of CD40 and CD40L induces intracellular signaling through the recruitment of TNFR-associated factors (TRAFs) in the inner membrane of cells. This then leads to the activation of mitogen-activated protein kinases (MAPK), phosphatidylinositol-3 kinase (PI3K), the phospholipase Cγ pathway, and the nuclear factor kappa B (NFκB) signaling pathway (Elgueta et al. 2009). DCs play an important role in priming T-cell responses through their T-cell receptors by presenting antigenic peptides through MHC-I and MHC-II, thus playing an essential role in the initiation of antitumor immune responses (Banchereau and Steinman 1998). The CD40L and CD40 interaction leads to DC activation and programs them to secrete IL-12 (a pro-inflammatory cytokine), supports CD4+ T-helper cell responses, and primes cytotoxic CD8+ T cells (Caux et al. 1994, Cella et al. 1996, Schoenberger et al. 1998).

Two models have been proposed for the role of CD40 signaling in generating cytotoxic T lymphocyte (CTL) responses (Ahmed et al. 2012). The first model proposes that the interaction of CD40L (expressed by CD4+ T helper cells) with CD40 (expressed by DCs) is important for DC maturation, which in turn is essential for triggering CTL responses (Ahmed et al. 2012). The second model proposes that interaction of CD40L (expressed by CD4+ T helper cells) with CD40 (expressed by CD8+ T cells) can also directly activate CD8+ T cells (Bourgeois, Rocha and Tanchot 2002). Thus, CD40L-CD40 signaling is important for inducing effective CTL responses.

In various studies, DC activation *in vivo* through CD40 agonist antibody binding along with chemotherapy induced T-cell-mediated antitumor immunity (Byrne and Vonderheide 2016). In a phase I study, patients with non-Hodgkin's lymphoma or advanced solid tumors were treated with recombinant CD40L and some experienced partial responses (2/32 patients) and at least 4 months without disease progression (4/32 patients) (Vonderheide et al. 2001). Many studies have investigated CD40 agonist antibodies. However, the first clinical trial with intravenous administration of a CD40 agonist antibody (CP-870,893) showed only a partial response (13.8% of patients) (Vonderheide et al. 2007). In another study, the same CD40 agonist antibody was used in combination with an immune checkpoint inhibitor (anti-CTLA-4) (Bajor et al. 2018). Adverse events with the use of CD40 agonist antibody (CP-870,893) included cytokine-release syndrome (CRS) (grade 1 or 2) and thromboembolic events (Vonderheide et al. 2007). In another clinical study for the treatment of solid

malignancies with a CD40 agonist antibody (ADC-1013), intravenous administration led to treatment-induced adverse events (Irenaeus et al. 2019). Adverse events included CRS, lower B cell count, and increased liver enzyme. However, intratumoral administration of CD40 agonist antibodies was well tolerated (Irenaeus et al. 2019).

1.6 Clinical trials with oncolytic viruses

Oncolytic viruses induce antitumor immunity through different mechanisms. For example, oncolytic viruses replicate in and lyse tumor cells, thereby releasing TAAs. APCs capture and process these antigens, eventually generating tumor-specific T-cell responses. Oncolytic virus-mediated cell lysis releases danger-associated molecular patterns (DAMPs) and PAMPs (Bartlett et al. 2013, Guo, Liu and Bartlett 2014). Virus replication in tumors helps to repolarize the immunosuppressive TME through induction of inflammatory responses and localized production of cytokines (De Graaf et al. 2018).

The China Food and Drug Administration licensed a recombinant unarmed oncolytic adenovirus (H101; Oncorine, Shanghai Sunway Biotech) in 2005 (Eissa et al. 2018). H101 is used in combination with chemotherapy for the treatment of refractory head and neck carcinoma. The combination resulted in a 79% response rate, compared with 40% for chemotherapy alone (Garber et al. 2006). In 2015, the FDA and EMA approved the oncolytic virus-based therapy talimogene laherparevec (T-VEC, Imlygic) (Greig et al. 2016). T-VEC is a herpes simplex virus (HSV) armed with human GM-CSF. A phase I clinical trial with T-VEC enrolled 30 patients and was conducted against different types of cancers. In this trial, no complete or partial responses were seen. However, two patients achieved stable disease (Hu et al. 2006). In a phase II trial, therapy was well tolerated with objective response observed in 26% of the patients (n=50) with stage III or IV melanoma (Senzer et al. 2009). In a phase III trial of T-VEC, 436 patients with stage III or IV melanoma were included and the trial showed improvement in the objective response rate and progression-free survival (Andtbacka et al. 2016).

There are two clinical trials evaluating Maraba virus (MG1) expressing human melanoma-associated antigen A3 (MAGE-A3) used in combination with adenovirus-expressing MAGE-A3 in patients with MAGE-A3-positive tumors (NCT02285816) and in non-small-cell lung cancer patients (NCT02879760) (Pol et al. 2019).

Patients with progressive advanced solid tumors have been treated with oncolytic adenovirus armed with CD40L (CGTG-401). Treatment revealed pronounced antitumor effects and 83% of patients exhibited disease control (Pesonen et al. 2012). Other clinical studies with oncolytic adenovirus encoding CD40L have also shown induction of tumor control and Th1-type immune responses (Loskog et al. 2016, Schiza et al. 2017, Malmström et al. 2010). A phase I/II clinical trial using oncolytic adenovirus armed with CD40L and 4-1BB ligand (LOAd703) is currently ongoing in patients with pancreatic cancer (NCT02705196) and in patients with ovarian, pancreatic, and colorectal cancers (NCT03225989).

A phase I trial evaluated oncolytic adenovirus DNX-2401 (Delta-24-RGD) in patients with malignant glioma. The trial was conducted in two groups (A and B). A single dose of virus (1 x 10e7 to 3 x 10e10 viral particles [VP]) in group A showed prolonged survival of more than 3 years in 20% of patients. In group B, patients received the virus two times at multiple sites. Tumor resection before the second injection of virus showed infiltration of CD8+ T cells (Lang et al. 2018, Lang et al. 2014). These results suggest that DNX-2401 can induce tumor-cell lysis and enhance immune responses.

Preclinical studies with an oncolytic adenovirus armed with OX40-ligand (Delta-24-RGDOX) have demonstrated activation and proliferation of tumor-specific lymphocytes in glioma models and within subcutaneous and intracranial melanomas (Jiang et al. 2017, Jiang et al. 2019). Based on preclinical studies, a phase I trial is ongoing in patients with glioblastoma (NCT03714334). Phase I studies with oncolytic HSV for the treatment of malignant glioma patients have also been performed to evaluate dose-escalation and safety (Patel et al. 2016).

A phase I trial with Newcastle disease virus in patients with breast cancer revealed that the virus is well tolerated and led to 6-month prolonged stable disease in one of two patients studied (Laurie et al. 2006). A clinical trial with vaccinia virus that enrolled four breast cancer patients showed that the virus was well tolerated. One of the patients experienced adverse events, such as hemorrhage (Zeh et al. 2015). Many oncolytic viruses have demonstrated safety in clinical trials. However, as a monotherapy, the outcomes in clinical trials appear insufficient.

Many oncolytic viruses have been investigated preclinically for the treatment of pancreatic cancer either as monotherapy or together with chemotherapeutic drugs, such as gemcitabine. Some of these are in phase I and II clinical trials. Oncolytic adenoviruses, such as ONYX-105, are well-tolerated in patients, with the exception of one patient who experienced transient pancreatitis. However, no objective response was seen in this phase I trial (Mulvihill et al. 2001). The lack of virus replication observed within the tumor samples was due to the presence of few viable cells in the samples or due

to physical barriers of tumors that limit viral replication. ONYX-105 was used in a phase II trial in combination with gemicitabine in patients with pancreatic cancer. Two patients exhibited a minor response, two exhibited partial regression, and six had stable disease with no adverse events observed. The results of this trial showed that combination treatment appeared to have some efficacy (Hecht et al. 2003). Currently, oncolytic adenoviruses LOAD703 and VCN-01 either alone or with gemcitabine/nab-paclitaxel are in phase I clinical trials with pancreatic cancer patients (NCT02045602, NCT02045589, NCT02705196). A phase I clinical trial using HF10 as monotherapy revealed enhanced tumor infiltration of immune cells, such as T cells and macrophages, activation of NK cells, and progression-free survival of 6 months (Nakao et al. 2011, Kasuya et al. 2014). Currently, a HF10 phase I trial (NCT03252808) in combination with nab-paclitaxel, gemcitabine, and S-1 (gimeracil, oteracil, tegafur, TS-1) is ongoing to evaluate the safety and efficacy of these combinations.

In summary, oncolytic viruses induce immunologic responses by selectively replicating and lysing cancer cells. Arming the viruses with co-stimulatory transgenes ensures local expression of the transgene in the TME. Thus, this approach also reduces the risk of adverse events related to the systemic administration of transgenes. Oncolytic viruses are well-tolerated and have a favorable safety profile in humans. However, additional therapeutic benefits can be obtained by combining viruses with chemotherapeutic or immunotherapeutic drugs.

1.7 Combination immunotherapies: preclinical and clinical data

Immunotherapeutics, such as checkpoint inhibitors anti-PD-1/PD-L1 and anti-CTLA-4, have shown promising efficacy albeit with major adverse events (Postow, Callahan and Wolchok 2015). Moreover, some tumors are resistant to these immunotherapies (Kelderman, Schumacher and Haanen 2014). Combining oncolytic virus therapy with immunotherapeutics can potentially overcome the problems observed when these approaches are used as monotherapy.

Blocking CTLA-4 prevents inhibition of T-cell activation and reduces intratumoral Tregs (Khalil et al. 2016). Preclinical studies using oncolytic viruses, such as vaccinia virus (Rojas et al. 2015, Foy et al. 2016), poxvirus (Foy et al. 2016), and vesicular stomatitis virus (Gao et al. 2009) along with anti-CTLA-4 have shown long-term survival in different tumor models, such as lung (Foy et al. 2016), renal, (Rojas et al. 2015) and mammary (Gao et al. 2009). These combinations induced systemic protection upon rechallenge (Rojas et al. 2015, Foy et al. 2016) and cured mice (Gao et al. 2009).

Treatment of a murine melanoma model with Newcastle disease virus along with ipilimumab had an enhanced antitumor effect (Zamarin et al. 2014). Arming the adenovirus with anti-CTLA-4 increased the expression of anti-CTLA-4 within tumors with no adverse events in murine models (Dias et al. 2012).

In contrast to CTLA-4, PD-1 inhibits T-cell activation in tumors and tissues at later stages of the immune responses (Postow et al. 2015). Anti-PD-1 has been used in combination with different oncolytic viruses, such as adenoviruses (Cervera-Carrascon et al. 2018), vesicular stomatitis virus (Shen et al. 2016), measles virus (Hardcastle et al. 2017), and reovirus (Rajani et al. 2016, Ilett et al. 2017) for the treatment of acute myeloid leukemia (Shen et al. 2016), glioblastoma (Hardcastle et al. 2017), and melanoma (Rajani et al. 2016, Ilett et al. 2017). These combination therapies enhanced antitumor responses and led to prolonged survival in mice. Studies in murine models demonstrated that the use of unarmed vaccinia virus with systemic administration of anti-PD-1/anti-PD-L1 or use of anti-PD-1/anti-PD-L1-armed measles virus and vaccinia virus had the same level of antitumor efficacy (Engeland et al. 2014, Kleinpeter et al. 2016).

Use of either armed or unarmed oncolytic viruses together with various checkpoint inhibitors have shown synergy in murine models.- In a phase I study, treatment of melanoma patients with T-VEC and ipilimumab demonstrated enhanced tumor control along with a 50% objective response rate; 44% of patients had durable responses of ≥6 months (Puzanov et al. 2016). Overall, the treatment was well tolerated. However, in this study, some observed adverse events were related to systemically administered ipilimumab (Puzanov et al. 2016, Postow et al. 2015). A phase II study of combination therapy has further confirmed these results; 39% of patients had an objective response with T-VEC and ipilimumab as compared to a 18% objective response with ipilimumab alone (Chesney et al. 2018).

A phase Ib clinical trial with T-VEC and anti-PD-1 (pembrolizumab) revealed an overall response rate of 62% and a complete response rate in 33% of patients with advanced melanoma (Ribas et al. 2018). Treatment of 10 unresectable melanoma patients with a combination of T-VEC and pembrolizumab, nivolumab (anti-PD-1), or ipilimumab plus nivolumab revealed a 60% complete response rate and a 90% overall response rate for the injected lesions. This study also showed induction of a systemic immune response against the tumors, as indicated by the resolution of uninjected lesions (Sun et al. 2018). Currently, T-VEC along with pembrolizumab, nivolumab, atezolizumab (anti-PD-L1), ipilimumab or nivolumab is under evaluation in different clinical trials of patients with breast cancer, lung cancer, colorectal cancer, melanoma, sarcoma, malignant pleural effusion, carcinoma of the head and neck, and hepatocellular carcinoma (www.clinicaltrials.gov).

Oncolytic viruses also improve chimeric antigen receptor (CAR) (Nishio et al. 2014, Moon et al. 2018) and adoptive T cell therapies in murine models (Siurala et al. 2016, Santos et al. 2018). An oncolytic adenovirus armed with IL-2 and TNF-α (TILT-123) has been used together with *in vitro* expanded tumor-infiltrating leucocytes (TILs) in hamsters to treat pancreatic cancer. This treatment cured all hamsters and induced a memory response, as treated hamsters rejected reintroduced tumors (Havunen et al. 2017). Currently, TILT-123 is in a clinical trial with cancer patients receiving TIL therapy (NCT04217473).

Oncolytic viruses have also been used in combination with DC therapy. Preclinical studies have demonstrated the synergistic effect of oncolytic viruses and DC therapy. This combination may modulate the immunosuppressive TME and control tumor growth along with the induction of tumor-specific T cells (Komorowski et al. 2018, Koske et al. 2019). Oncolytic viruses, such as vaccinia virus armed with a CXCR4 antagonist (OVV-CXCR4-A.Fc) and an adenovirus armed with CD40L (TILT-234) enhanced the efficacy of DC therapy (Komorowski et al. 2018, Zafar et al. 2018, Zafar et al. 2017). The use of oncolytic viruses as enablers of DC therapy is entering early clinical trials. T-VEC and autologous myeloid DCs are being evaluated in metastatic melanoma patients (NCT03747744). Moreover, chimeric adenovirus 3/5 encoding GM-CSF (ONCOS-102) and DCs are being evaluated in patients with castration-resistant prostate cancer (NCT03514836).

2 Aims of the study

The aims of this study were

1. to examine the antitumor effects of adenovirus serotype 3 armed with CD40L (Ad3-hTERT-CMV-hCD40L)

2. to evaluate the antitumor effects of Ad3-hTERT-CMV-hCD40L in combination with DC therapy

3. to characterize Ad3-hTERT-CMV-hCD40L as an enhancer of DC therapy in prostate cancer

4. to investigate the mechanism of adenovirus-mediated tumor transduction through blood

3 MATERIALS AND METHODS

3.1 CELL LINES (I-IV)

Cell lines used during the study were obtained from American Type Culture Collection (ATCC), unless stated otherwise. All the cell lines were maintained either in Dulbecco's modified Eagle's medium (DMEM) or Roswell Park Memorial institute medium (RPMI) at +37°C and 5% CO_2 (**Table 3**). Culture media for all the cell lines were supplemented with 10% FBS, 1% L-glutamine, 1% Pen/Strep solution, except that for B16.OVA which also contained 5 mg/mL G418 (Roche, Basel, Switzerland).

Table 3: List of cell lines used in the study

Cell line	Origin	Growth media	Source	Study
Human cell lines				
A549	Lung carcinoma	DMEM	American Type Culture Collection (ATCC)	I, II, IV
LNCaP	Prostate carcinoma	RPMI	ATCC	I, II
SKOV3	Ovarian adenocarcinoma	DMEM	ATCC	II
EJ	Bladder carcinoma	DMEM	A.G. Eliopoulos (University of Crete Medical School and Laboratory of Cancer Biology, Heraklion, Crete,Greece).	I, II
293	Embryonic Kidney	DMEM	ATCC	I
PC-3	Prostate adenocarcinoma, castration resistance	RPMI	ATCC	III
PC-3MM2	Prostate adenocarcinoma, castration resistance	RPMI	Isiah J. Fidler, M.D. Anderson Cancer Center	III, IV
Ramos Blue	B- lymphocyte cell line stably expressing NFkB/AP-1-inducible SEAP (secreted embryonic alkaline phosphatase) reporter gene.	IMDM		I
Mouse cell lines				
B16.F10	Skin melanoma	DMEM	ATCC	I
B16.OVA	Chicken ovalbumin expressing melanoma	DMEM 5 mg/ml G418 (Roche, Basel, Switzerland)	Prof. Richard Vile (Mayo Clinic, Rochester, MN, USA)	I

3.2 Adenoviruses used in the study

Adenoviruses used in studies I, II and III are type 3 adenovirus Ad3-hTERT-E1A and Ad3-hTERT-CMV-hCD40L. For tumor selectivity, TATA box was replaced with an hTERT promoter. To construct adenovirus 3 coding human CD40L, CD40L transgene was inserted in the E3 region under CMV promoter. In study IV, chimeric replication-competent adenoviruses Ad5/3-E2F-d24 and Ad5/3-E2F-d24-hTNF-α-IRES-hIL2 were used. The viruses composed of adenovirus 5 backbone and fiber knob from adenovirus 3. E2F promoter was inserted in front of the adenoviral E1A gene that contains d24 deletion. Study IV also included experiments with the replication-incompetent adenovirus Ad5/3-Luc1 featuring adenovirus 5 backbone with a knob domain from serotype 3 and containing firefly luciferase (Luc1) in a deleted E1 region. The adenoviruses used in the study are summarized in **Table 4.**

Table 4: List of viruses used.

Adenoviruses	**Modifications**	**Trnasgene**	**Study**	**References**
Replication competent				
Ad3-hTERT-E1A	Human telomerase reverse transcriptase promoter	-	I, II, III	Hemminki O. et al., 2011
Ad3-hTERT-CMV-hCD40L	Human telomerase reverse transcriptase promoter, cytomegalovirus promoter	Human CD40L	I,II,III	Zafar S. et al., 2016
Ad5/3-E2F-D24-hTNF-α-IRES-hIL2	Ad3 fiber knob, E2F promoter, 24 bp deletion in E1A, deleted E1B 19K	human TNF-α and human IL-2	IV	Havunen R. et al., 2017
Replication-incompetent viruses				
Ad5/3-Luc1	Deleted E1	Firefly luciferase	IV	Krasnykh V.N. et al. 1996
Ad5/3-CMV-mCD40L	Cytomegalovirus promoter	Murine CD40L	I	Diaconu I et al. 2012

3.3 *In vitro* studies

3.3.1 Isolation of peripheral blood mononuclear cells (Study I-IV)

Buffy coat fractions from healthy blood donors were obtained from the Red Cross Blood Service (Helsinki, Finland). Peripheral blood mononuclear cells (PBMCs) and erythrocytes were isolated from blood through density gradient centrifugation using Lymphoprep (StemCell Technologies), according to the manufacturer's instructions. This density gradient centrifugation results in the formation of four layers. The second layer, below the uppermost (plasma) layer, which is characteristically white and cloudy, contains PBMCs. PBMCs were isolated and washed with PBS twice. To remove erythrocytes, isolated PBMCs were treated with Ammonium-Chloride-Potassium (ACK) red blood cell lysis buffer (Sigma, St Louis, MO). PBMCs were then washed again with PBS, counted and were either frozen or used fresh.

To isolate CD14+ monocytes and lymphocytes (CD14 – cells) from PBMCs, CD14+ magnetic beads (Miltenyi Biotec) were used according to the manufacturer's instructions. CD14+ cells were washed and used for the generation of dendritic cells (DCs) in studies I, II and III while lymphocytes were used in study IV.

In study IV, erythrocytes were collected from the bottom as they sediment through the gradient medium after density gradient centrifugation. Erythrocytes were treated with 10 % citrate-phosphate-dextrose (CPD, Sigma-Aldrich, USA) and stored at +4°C.

3.3.2 Generation of dendritic cells

3.3.2.1 Monocyte-derived DCs

4.5 x 10e6 CD14+ monocytes isolated from human PBMCs were cultured for 5–7 days in 10 ml of DC culture media. This media consist of 10% RPMI supplemented with 20ng/ml interleukin 4 (IL4, Peprotech) and 1000U/ml granulocyte-macrophage colony-stimulating factor (GM-CSF, Peprotech). The immature human dendritic cells generated were used in study I. For studies II,III immature DCs were pulsed with tumor cell lysate (50 µg/ml) for 24h, followed by 17-24h stimulation with lipopolysaccharide (LPS, 100ng) (Sigma). DC maturation markers (CD83, CD80, CD86) were then analysed with flow cytometry. These matured DCs were used in studies II and III.

3.3.2.2 Bone marrow-derived murine DCs

Mice were euthanized and tibia and femur were harvested. Bone marrow was collected by cutting the ends of the bones and flushing the bones with media under sterile conditions. Suspension of bone marrow cells was filtered and treated with the ACK red blood cell lysis buffer (Sigma, St Louis, MO) to remove red blood cells. Cells were washed and resuspended in 10% RPMI supplemented with 40 ng/mL murine GM-CSF (Peprotech). 2.5 x 10e5 cells per ml were seeded in 24-well plates for 5 days. Cells were then incubated with tumor cell lysate (50 µg/ml) for 24h, followed by 17-24h stimulation with lipopolysaccharide (LPS, 100ng) (Sigma). DC maturation was confirmed with flow cytometry. Matured murine DCs were used in study I.

3.3.3 CD40L functionality assays

In study I, to determine the functionality of virally - expressed CD40L, Ramos-Blue cells were used. Ramos-Blue is a B-lymphocyte cell line which stably expresses the NFkB/AP-1-inducible SEAP (secreted embryonic alkaline phosphatase) reporter gene. In order to have virally expressed CD40L only, A549 were infected with either Ad3-hTERT-E1A or Ad3-hTERT-CMV-hCD40L, or were left uninfected. After 48h, supernatant was collected and filtered (0.02 µm, Whatman). CD40L concentration in the filtered supernatant was determined with BD Cytometric Bead Array (CBA) flex set. Filtered supernatants were then used to stimulate Ramos-Blue cells. Upon CD40L stimulation, Ramos-Blue cells produce SEAP in the supernatant. SEAP is detectable through the reagent QUANTI-Blue (InvivoGen), which in the presence of SEAP turns purple/blue. Levels of activation were determined at the wavelength of 450 nm with a microplate reader. Recombinant hCD40L protein (Abcam) at a concentration of 2 ng/ml served as a positive control.

In study II, the ability of Ad3-hTERT-CMV-hCD40L to induce dendritic cell maturation was studied. A549 cells were infected with either Ad3-hTERT-E1A or Ad3-hTERT-CMV-hCD40L. Uninfected cells were used as a negative control. After 18 h, media was removed and cells were washed with PBS and then monocyte-derived immature DCs in fresh media were added. DC maturation marker status was studied through flow cytometry after 48h. In addition to this, to evaluate the functional consequence of DC stimulation, human T cells were isolated from PBMCs with the Pan T cell Isolation kit (Miltenyi Biotec), and were added to the DC and virus-infected tumor cell mixture. Following this addition, T cell activation status was determined after 24h by flow cytometry.

To evaluate the ability of Ad3-hTERT-CMV-hCD40L expressed CD40L to induce DC maturation, a similar experiment was performed but instead of viruses, supernatant from virus-infected A549 cells was used. Supernatants were collected, filtered and added to fresh A549 cells along with monocyte-derived immature DCs. After 48h, DC maturation status was assayed, followed by addition of T cells into the co-culture to evaluate the functional consequence of DC stimulation. Activation of T cells was determined after 24h by flow cytometry.

Both of the assays were done in triplicates, and as positive controls, LPS (100 ng) (Sigma) and recombinant hCD40L (500 ng) (Abcam) were used.

3.3.4 MTS cell cytotoxicity assay

This assay was used to determine viable cells. Cultured cells were incubated with MTS solution [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium for 2h. and absorbance was measured at 490 nm with a spectrophotometer. Viable cells reduce the MTS solution and generate formazan, which is soluble and coloured product. The amount of formazan and cell viability is directly proportional.

10,000 A549 and EJ cells (study I), and PC-3 and PC-3MM2 cells (study III) were seeded into 96-well plates. The next day (i.e. 24h later) cells were infected with Ad3, Ad3-hTERT-E1A or Ad3-hTERT-CMV-hCD40L at 1–1,000 VP/cell. Uninfected tumor cells served as controls. The number of living cells was determined on day 6 (study I) and on day 3, 5, 6 (study III) with the MTS solution (Cell Titer 96 AQueous One Solution Cell Proliferation Assay, Promega, Madison, WI).

A549, EJ, SKOV3 or LNCaP cells were infected with Ad3-hTERT-E1A or Ad3-hTERT-CMV-hCD40L at 1–1000 VP/cell (as mentioned above), or left uninfected. In this experiment, DCs and human PBMCs were introduced into the assay, 48h after viral infection. DCs, PBMCs and tumorcells alone served as controls. Viability of cells was determined 24h to 96h after adding DCs and PBMCs.

In study IV, A549 cells (10,000 cells per well) were used. Ad5/3-E2F-D24-hTNF-α -IRES-hIL2 was incubated for 30 minutes either with erythrocytes (0.036 VP/cell) or with lymphocytes (10 VP/cell) at 37°C. Samples were centrifuged for 10 min at 2000 g and re-suspended in 1ml of assay medium. Then, dilutions of cell-adenovirus mixture (at ratios of 1:1.7, 1:2.7 and 1:6.7) were added to A549 cells, which were seeded 24h before starting the experiment. In one condition, cell-adenovirus

mixture was washed with PBS three times and centrifuged, as mentioned above. The pellet was resuspended in 1 ml of assay medium and then from there a 1:2.7 dilution was used. Viruses at 0.1-100 VP/cell concentrations were used as positive controls. Erythrocytes and lymphocytes with virus but without A549 cells, and erythrocytes and lymphocytes without virus but with A549 cells served as negative controls. Viability of the cells was determined on day 3 with the MTS solution (Cell Titer 96 AQueous One Solution Cell Proliferation Assay, Promega, Madison, WI).

NOTE: In all the studies, cell viability was normalized against the viability of controls

3.3.5 Luciferase assay

In study IV, luciferase assay was performed using Ad5/3-Luc1 virus, which expresses firefly luciferase, with the same experimental settings as mentioned above. It is a highly sensitive and rapid method for the luciferase quantitation. In this experiment, 48h after the infection, media was removed and lysis buffer (Promega, USA) was used to lyse cells followed by freeze-thawing once and centrifugation. Luciferase activity of the supernatant was measured through the Luciferase assay reagent (Promega, USA) with a luminometer (Hidex).

3.3.6 Electron microscopy

For electron microscopy in study IV, freshly isolated human lymphocytes and erythrocytes were incubated with Ad5/3-E2F-D24-hTNF-α-IRES-hIL2 in 1 ml of PBS for 30 minutes at 37°C. Following incubation, cells were centrifuged and the cellular fraction was fixed in 5% (for erythrocytes) and 2.5 % (for lymphocytes) glutaraldehyde, according to the instructions from University of Helsinki Electron Microscopy Unit. Sample preparation for both Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) was conducted in the Electron Microscopy Unit at the University of Helsinki. Analysis of samples was carried out using SEM in Electron Microscopy Unit, and TEM in Advance Microscopy Unit (AMU) at the University of Helsinki, Finland.

3.3.7 Microarray from human tumor biopsies treated with CD40L and GM-CSF

Advance Therapy Access Program (ATAP) was a personalized therapy program during 2007-2012. All patients participating in this program had given written informed consent. All the participants were refractory to standard treatments and had solid tumors and were treated with oncolytic adenoviruses. The Helsinki University Central Hospital Operative Ethics Committee (HUS 62/13/03/02/2013) has approved the analysis of the data reported in study I.

mRNA expression levels from tumor biopsies collected from patients treated with oncolytic adenovirus encoding either human CD40L (n = 3) or another immunostimulatory molecule, granulocyte macrophage colony-stimulation factor (GM-CSF) (n = 11) were analyzed as reported previously (Taipale et al. 2016). Briefly, to evaluate gene-expression levels, tumor biopsies were collected and stored in RNALater (Life Technologies, Carlsbad, CA). RNA was extracted from the samples using TRIZOL Reagent (Life Technologies).

Genome-wide gene expression profiling of RNA samples was done by hybridizing the RNA to the Illumina HumanHT-12 v4 Expression Bead Chips arrays (Illumina, San Diego, CA). Total Prep RNA Labelling Kit (Illumina) according to manufacturer´s instructions was used for the labelling and hybridization. Bead Chips were washed, blocked and stained with streptavidin-Cy3 and scanned with Illumina iScan (Illumina) by using manufacturer provided protocols. To control the quality of the data Genome Studio software (Illumina) was used.

3.3.8 Quantitative polymerase chain reaction qPCR (IV)

In study I, a breast cancer patient was participating in ATAP. The patient was treated intravenously with 4 x 10e12 VP Ad3-hTERT-E1A (also known as CGTG-201). After 6 days, tumor biopsies were collected to detect the presence of virus through qPCR.

In study IV, adenoviruses were incubated either with erythrocytes (0.036 VP/cell) (Rojas et al. 2016b) or with lymphocytes (10 VP/cell) in 1 ml of PBS for 30 minutes at 37°C. Following incubation, cells were centrifuged at 2000g for 10 minutes and the supernatant and cellular fractions were collected. Cellular fractions were washed with PBS five times. During each wash a portion of the supernatant and pellet were collected. DNA was extracted from the samples using the QIAamp kit (Qiagen). For animal experiments in study IV, tumors were collected from treated and untreated mice, cut into small pieces and 25mg of these was used for DNA extraction through the QIAamp kit (Qiagen). The

presence of virus was then evaluated by detecting viral E4 copy numbers in samples from *in vitro* experiments (from supernatants and pellets (cellular fraction)) and *in vivo* experiments (tumors). Probes and primers used in qPCR are summarized in **Table 5.** The E4 copy number was normalized against human beta-actin.

Table 5: Probes and primers used in qPCR

Gene region		Sequence (5'-3')	Reference
Adenoviral E4	**Probe**	TGGCATGACACTACGACCAACACGATCT	Kanerva et al. 2002
	Forward primer	GGAGTGCGCCGAGACAAC	
	Reverse primer	ACTACGTCCGGCGTTCCAT	
Human beta-actin	**Probe**	ATG CCC TCC CCC ATG CCA TCC TGC GT	
	Forward primer	TCA CCC ACA CTG TGC CCA TCT	
	Reverse primer	GTG AGG ATC TTC ATG AGG TAG TCA GTC	

3.3.9 Neutralizing antibody titers

In study IV, Neutralizing antibodies (Nabs) were measured from serum samples of adenovirus-treated mice. To separate serum from red blood cells, the blood from adenovirus-treated mice was incubated for an hour at room temperature and centrifuged at high-speed. Serum samples were incubated at +56ºC for 90 min to inactivate the complement system. This was followed by serial dilutions, which were then mixed with Ad5/3-Luc (100 VP/cell) at room temperature. After 30 minutes, the serum-virus mix was added on A549 cells (after removing the media), which were seeded into 96-well plates (1×10e4 cells/well) 24 h before the experiments. After an hour, fresh media was added and the cells

were then incubated overnight (at +37°C). Finally, the media was removed and lysis buffer (Promega, USA) was used to lyse cells followed by freeze-thawing once and centrifugation. Luciferase activity of the supernatant was measured through the Luciferase assay reagent (Promega, USA) with a luminometer (Hidex).

3.4 *In vivo* studies

The Experimental Animal Committee of the University of Helsinki and the Provincial Government of Southern Finland reviewed and approved all the animal protocols. The National Animal Experiment Board (Eläinkoelautakunta ELLA) of the Regional State Administrative Agency of Southern Finland has also approved all the experiments (ESAVI/7759/04.10.07/2013, ESAVI/7755/04.10.07/2016, and ESAVI/28404/2019).

4-5 week old mice were ordered from Taconic and quarantined in a BSL-2 level facility for at least one week before beginning the experiments. All the intratumoral injections and tumor measurements were performed when the animals were anesthetised with isoflurane. For intravenous injections, animals were anesthetised with the mixture of Domitor (1x) and Ketalar (2x) in sodium chloride solution (7x). The health status of the animals was examined daily. Animals were euthanized if there was any visible sign of distress, tumor ulceration, or when the tumor diameter reached the maximum limit i.e. 18 mm (for mice).

3.4.1 Immunocompetent Mouse Models

In study I, immunocompetent female C57BL/6 mice were administered subcutaneously with 2.5 x 10e5 mouse melanoma B16 tumors expressing chicken ovalbumin (B16.OVA cells). Mice were divided into groups (n=8 mice/group) according to the tumor size. The groups are summarized in **Table 6**. When tumors became injectable, mice were treated intratumorally with 1 x 10e9 VP/tumor of Ad5/3-luc alone or Ad5/3-CMV-mCD40L alone, or only PBS (50 ul) on days 1, 3, and 7, or with 1.5 x 10e6 CD8-enriched OT-1 T-cells (described later) on day 1, intraperitoneally.

Table 6: Treatment groups for the mouse experiment to study the effect of virally - expressed CD40L on OT-1 T cells

Groups	VP per tumor	OT-1 T cells per animal
Mock	-	-
Mock + OT-1	-	1.5X10e6
Ad5/3-luc	1X10e9	-
Ad5/3-luc + OT-1	1X10e9	1.5X10e6
Ad5/3-CMV-mCD40L	1X10e9	-
Ad5/3-CMV-mCD40L + OT-1	1X10e9	1.5X10e6

Also in study I, 2.5 x 10e5 murine B16.F10 cells were implanted subcutaneously into both flanks of immunocompetent female C57BL/6 mice. Mice were treated with either Ad5/3-CMV-mCD40L virus alone (at 2 x 10e8 VP/tumor), DCs (1 x 10e6 cells) alone, or with both i.e. virus and DCs on alternative days. The groups are summarized in **Table 7.** Tumor growth was measured with a digital caliper every other day. At the end of the experiment, tumors and lymph nodes were collected for flow cytometry.

Table 7: Treatment groups for the mouse experiment to study the effect of virally expressed CD40L on DCs.

Groups	VP per tumor	DCs per tumor
Mock	-	-
Mock + DCs	-	1 x 10e6
Ad5/3-CMV-mCD40L	2 x 10e8	-
Ad5/3-CMV-mCD40L + DCs	2 x10e8	1 x 10e6

In study IV, to generate neutralizing antibodies (Nabs), immunocompetent mice were immunized with chimeric adenovirus Ad5/3 three times i.e. on day 0, 3 and 6. On day 23, mice were euthanized and blood was collected to separate serum. Nab titer was confirmed with a Nab assay (Särkioja et al. 2008). Neutralizing titer that blocks more than 50% of the virus was used in the experiments.

3.4.1.1 Extraction of OVA-specific OT-1 T cells (study I)

In study I, spleen from C57BL/6-Tg (TcraTcrb) 1100Mjb/J (OT-1) mice were collected and mashed through a 70 µm filter. To remove red blood cells, splenocytes were treated with ACK red blood cell lysis buffer (Sigma, St Louis). Cells were washed with 10% RPMI-1640 growth media twice. The immune cells were then rested for 48 h in RPMI-1640 media supplemented with 10% FBS, 2 mM L-glutamine, 100 µg/ml streptomycin, 100 U/ml penicillin, 15 mM HEPES (Sigma-Aldrich), 50 µM 2-mercaptoethanol (Sigma-Aldrich), and 1 mM Na-pyruvate (Sigma-Aldrich).

CD8a+ T Cell Isolation Kit II (Miltenyi Biotec, Germany) was used according to the manufacturer's instructions to enrich CD8+ T cells. The cells were then expanded for a week in growth media supplemented with 300 ng/ml soluble anti-mouse CD3e antibody clone 145-2C11 (Abcam, Cambridge, UK) and 160 ng/ml recombinant murine IL-2 (R&D Systems).

3.4.2 Immunodeficient mouse models

In study I, immunodeficient nude Naval Medical Research Institute (NMRI) mice were used. 1 x 10e6 human A549 or EJ cells were subcutaneously implanted in the flank. When tumors reached approximately 5 mm, mice were divided into three groups (n=7 mice /group) according to the tumor size. To study the anti-tumor efficacy of Ad3-hTERT-CMV-hCD40L and Ad3-hTERTE1A upon intravenous administration, mice were treated with either 1 x 10e10 VP/100µl of Ad3-hTERT-CMV-hCD40L, Ad3-hTERTE1A, or PBS (100µl) intravenously (i.e. through tail vein injection). Tumor growth was measured with digital caliper every other day and the tumor volume was calculated using the formula: 0.52 x (max dimension) x (min dimension)^2.

In studies I and II, 5 x 10e6 human A549 cells and in study III, 2 x 10e6 PC-3MM2 cells were subcutaneously implanted in the severe combined immunodeficiency (SCID) mice. In study I, to assess the maturation of DCs *in vivo*, mice were treated intratumorally with either 1 x 10e8 VP/50µl Ad3-hTERTCMV-hCD40L, Ad3-hTERT-E1A or PBS (50µl) on days 0, 2, 4. And 1 x 10e6 immature DCs per tumor (prepared as described above) were administered intratumorally on days 1, 3, 5. Mice were euthanized two days after the last treatment. Tumors were collected for flow cytometry. In study II and III, to evaluate the ability of the adenovirus to enhance DC therapy, mice were divided into

eight groups (n=10 mice/group) according to the tumor size, when tumors reached approximately 5 mm in diameter. On day 0, mice received 10 x 10e6 HLA-matched PBMCs intravenously (through tail vein injection). Mice received intratumorally either 1 x 10e8 VP/50µl of Ad3-hTERT-CMV-hCD40L alone, Ad3-hTERT-E1A alone, or only PBS (50µl) on days 1, 3 and 5 or 1 x 10e6 DCs alone on days 2, 4, and 6, or combination of both i.e. virus and DCs on alternative days. Tumor growth was followed until day 25 (study III) and day 44 (study II) with digital caliper and the survival was followed until day 93 (study III) and day 112 (study II). Mice with tumor ulceration were excluded from the experiment and are shown as reversed triangles. Tumors were collected for flow cytometry and cytokine analysis.

In study IV, immunodeficient NMRI mice received subcutaneous injections of either 5 x 10e6 A549 cells or 2 x 10e6 PC-3MM2 cells. Mice were divided into 8 groups (n=5-7 mice/group) when tumors became injectable. Mice bearing PC-3MM2 tumors were treated intravenously with either 500 virus particles (VP) /cell of TILT-123 previously incubated with human lymphocytes or erythrocytes. Intravenous injections of 1.5 x 10e10 VP/100µl of TILT-123 and PBS (100µl) served as positive and negative controls, respectively. Mice bearing A549 tumors were treated similarly but with an increased virus dose, i.e. 2 x 10e9 VP/100µl as the experimental dose. In this experiment, mice were treated intravenously with 667 VP/cell (2 x 10e9 VP in total) of TILT-123 previously incubated with human lymphocytes or erythrocytes. 2 x 10e10 VP/100µl of TILT-123 was used as a positive control. In addition to this, immunodeficient mice bearing PC-3MM2 and A549 tumors received the same treatments but in the presence of heat-inactivated antiserum, i.e. TILT-123 or TILT-123-cell complex (TILT-123 previously incubated with human lymphocytes or erythrocytes) was incubated for at least 30 minutes with heat-inactivated antiserum at room temperature before treating mice intravenously. Mice were euthanized after 3 days and the tumors were collected to detect the presence of virus genome and for cytokine (TNF-α and IL-2) analyses.

3.4.3 Patient tumor histocultures

Prostate cancer samples (n=5) were collected from patients undergoing surgical resection. All patients signed an informed consent. A Pathologist at Helsinki University Hospital has confirmed all tumor histologies. The local ethics committee has evaluated and approved the project.

To established tumor histocultures, prostate cancer tumors were first cut into small pieces. These small pieces were then enzymatically digested overnight at +37°C in media (RPMI 1640, Sigma) supplemented with 1% L-glutamine, 1% Pen/strep, DNase I (25 mg/ml), elastase (25 mg/ml)

collagenase type I (170 mg/l) and collagenase type IV (170 mg/l) (Worthington Biochemical). To remove undigested fragments, cells were then filtered (100µm filter). The filtrate was treated with ACK lysis buffer (Sigma, St Louis) to remove red blood cells. Single cell suspensions were washed with PBS. 0.35x10e6 cells/well were seeded into 96-well plate and treated with 100 VP/cell of Ad3-hTERT-E1A and Ad3-hTERT-CMV-CD40L or medium (mock).

3.5 Cytokine analyses

For cytokine analyses in studies I, II, IV, small parts of tumor tissue were snap-frozen. In study III, supernatants from treated prostate cancer histocultures were collected and stored at -80C. In studies I, II and IV, with the help of tissue homogenizer (Tissue master 125 rotor), tumor tissues were homogenized in the presences of PBS (supplemented with a protease inhibitor cocktail (Sigma-Aldrich) and 0.1 % BSA). Cytokines were then analysed with CBA flex set cytokine beads (BD), according to the manufacturer's instructions. Samples were run with BD Accuri C6 and analyzed with the FCAP array software. Cytokine concentrations were normalised against the total protein concentration of the sample.

3.6 Flow cytometry

In studies I and II, tumors and lymph nodes were collected, minced and to create single cell suspensions passed through a 70µm strainer. Cells were then incubated at +37°C for 24 h. After incubation, cells were frozen in freezing media (growth medium containing 20% FBS and 10% DMSO) at -80 C.

For analysis of samples from animal experiments, cells were thawed and washed with staining buffer (FACS). For *in vitro* experiments, fresh cells were used and washed before staining. For staining, 1-2 x 10e6 cells/well were allocated in a 96 well plate (round bottom). For staining with pentamers detecting T cell receptors specific for residues TRP-2 (180-188) and gp100 (25-33) (Proimmune, Oxford, UK), cells were incubated for 20 minutes at room temperature in the dark. Cells were washed and incubated in staining buffer containing fluorochrome conjugated antibodies at +4°C. After 30

minutes, cells were washed with the staining buffer twice. Samples were analysed with flow cytometry (BD Accuri C6), acquiring 50,000-100,000 events.

In study III, treated prostate cancer histocultures were stained with fluorochrome-labelled antibodies according to the manufacturer's instructions and acquired with the LSR Fortessa flow cytometer (BD). software v10 was used to analyze the data.

3.7 Statistical analyses

In studies I and II, two-tailed Student's t-test, two-way ANOVA (Tukey's multiple comparisons test), and log-rank were performed with Graphpad Prism (Graphpad Software Inc. La Jolla, CA). For tumor growth analysis, SPSS version 21 was used. In studies III and IV, a non-parametric Kruskal-Wallis-test was used to compare groups. If the Kruskal-Wallis statistic was statistically significant, post hoc analyses were performed. P-values of post hoc (p-h) analyses were adjusted using the Holm multiple testing correction method. Statistical analyses and figures were made using Graphpad Prism 6 and R statistical software (R Core Team (2019)). In all studies, p values <0.05 were considered statistically significant.

4 Results and Discussion

4.1 Adenovirus serotype 3 transduces human tumor metastases upon intravenous administration (I)

Tumor biopsies from a breast cancer patient who was treated intravenously with Ad3-hTERT-E1A (CGTG-201) showed the presence of virus genome on day 6 after administration (Figure 1A, study I). This demonstrates the ability of adenovirus 3 to transduce tumors after intravenous administration.

4.2 Treatment with CD40L encoding oncolytic adenovirus activates DCs in patients (I)

Cancer patients participating in ATAP were treated with oncolytic adenovirus armed with either GM-CSF (n=11) or CD40L (n=3). Tumor biopsies from these patients showed upregulation of genes associated with DC maturation following treatment CD40L-encoding adenovirus (Figure 1B, study I).

4.3 Functionality of constructed viruses *in vitro* (I, II, III)

The virus analyzed in studies I, II, and III is a type 3 adenovirus (Ad3-hTERT-CMV-hCD40L). To construct the adenovirus 3 coding human CD40L, the CD40L transgene was inserted in the E3 region under the control of CMV promoter. For tumor selectivity, the TATA box was replaced with an hTERT promoter (Figure 2a, study I). In study IV, a chimeric adenovirus Ad5/3 with the backbone of adenovirus 5 and fiber knob is of adenovirus 3 was used.

The oncolytic adenovirus Ad3-hTERT-CMV-hCD40L was able to replicate and lyse *in vitro* the human cancer cell lines A549 (CD40-) and EJ (CD40+) cells (Figure 2C-D, study I) and PC-3 and PC-3MM2 (CD40-) cells (Figure 1 A-C, study III). In study II, we evaluated the oncolytic potency of the adenoviruses Ad3-hTERT-CMV-hCD40L or Ad3-hTERT-E1A in the presence of DCs, PBMCs, or both. In this study, two CD40-negative cell lines (SKOV3 and A549) and two CD40-positive cell lines (LNCaP and EJ) were used. Cell killing *in vitro* was more prominent with the triple combination than with viruses alone or double therapy (virus with T cells or DCs). In LNCaP (Figure

3A, study II) and EJ cells (Figure 3B, study II), complete cancer cell killing was observed with the triple combination containing CD40L-armed adenovirus at 1000 VP/cell 24 hours after adding DCs and PBMCs. In contrast, in SKOV3 (Figure 3C, study II) and A549 cells (Figure 3D, study II), killing was observed only 72 hours after adding DCs and PBMCs. This difference was probably due to the proapoptotic effect of CD40L on CD40-positive cancer cells (Diaconu et al. 2012). The combination of Ad3-hTERT-CMV-hCD40L, DCs, and PBMCs showed more pronounced cell killing than Ad3-hTERT-E1A, DCs, and PBMCs in all cell lines except Skov3 (Figure 3 E-H, study II). Collectively, our results showed that the oncolytic potency of Ad3-hTERT-CMV-hCD40L is comparable to unarmed backbone Ad3 virus, Ad3-hTERT-E1A. This indicates that the presence of the transgene does not affect the oncolytic potency of the virus. Moreover, Ad3-hTERT-CMV-hCD40L in the presence of DCs improved PBMCs-mediated cell killing *in vitro.*

4.4 Ad3-hTERT-CMV-hCD40L induces DC maturation and T-cell activation *in vitro* (I, II)

The functionality of virally produced CD40L was assessed with Ramos-Blue cells stably expressing an NF-κB/AP-1-inducible SEAP construct, since this cell line is responsive to human CD40L and upon activation expresses alkaline phosphatase. Supernatant was collected from Ad3-hTERT-CMV-CD40L-infected A549 cells and filtered to remove the virus from supernatant. Experiments with virally produced CD40L demonstrated that Ad3-hTERT-CMV-hCD40L induces expression of functional CD40L (Figure 2B, study I). Moreover, virally produced CD40L induced DC maturation *in vitro* (Figure 2E–F, study I). In study II, we evaluated the functional consequences of DC stimulation. This was studied in two different settings. In the first setting, immature DCs cultured with lung cancer cells (A549) were infected with either Ad3-hTERT-E1A or Ad3-hTERT-CMV-hCD40L. Cultures containing Ad3-hTERT-CMV-hCD40L-infected A549 cells induced pronounced upregulation of the DC maturation markers CD83, CD80, and CD86 (Figure 1A-C, study II). To study the ability of these matured DCs to activate T cells, T cells were added in the coculture. Significantly higher levels of T-cell activation in the cultures containing Ad3-hTERT-CMV-hCD40L-infected tumor cells were observed (Figures 1D and 1E, study II). In the second setting, immature DCs were cultured with filtered supernatant collected after infection of A549 cells with either Ad3-hTERT-E1A or Ad3-hTERT-CMV-hCD40L. After 48 hours, we observed pronounced upregulation of DC maturation markers in DCs cultured with filtered supernatant containing hCD40L (Figure 2A-C, study II). Following addition of T cells into the coculture to further study the ability of

these matured DCs to activate T cells, we observed high levels of the T-cell activation marker CD69 on both CD3+CD4+ T cells and CD3+CD8+ T cells (Figure 2E and 2D, study II). These results indicate that Ad3-hTERT-CMV-CD40L infection leads to expression of functional CD40L and can induce DC maturation and T-cell activation *in vitro*.

4.5 Ad3-hTERT-CMV-hCD40L enhances the oncolytic efficacy and DC maturation *in vivo* (I - III).

The oncolytic potency of constructed Ad3 viruses and their ability to transduce tumors following intravenous administration was examined in immunodeficient nude mice bearing human lung cancer (A549) and bladder cancer (EJ) xenografts. Both Ad3-hTERT-E1A and Ad3-hTERT-CMV-hCD40L were able to transduce tumors upon intravenous administration and to significantly control tumor growth when compared with the control group (injected with PBS) (Figure 3 A-B, study I). However, there were no significant differences between the virus-treated groups. This result shows that the arming device (CD40L) does not compromise the oncolytic ability of Ad3-hTERT-CMV-hCD40L *in vivo*. Expression of CD40L was confirmed from the tumors collected at the end of the experiment (Supplementary Figure 1). As an important safety aspect, expression of the transgene remained local (i.e. only detected in tumors). This is important for preventing the potential toxic effects of high-dose systemic administration (Sun et al. 2000).

Of note, human CD40L is not active in mice (Diaconu et al. 2012). Therefore, we also chose immunodeficient mice bearing human xenografts to study the ability of Ad3-hTERT-CMV-hCD40L to induce maturation of immature DCs *in vivo*. DC maturation *in vivo* was significantly enhanced in the mice treated with Ad3-hTERT-CMV-hCD40L (Figure 3C-E, Study I).

4.6 Ad3-hTERT-CMV-hCD40L improves the efficacy of adoptive dendritic cell therapy but not adoptive T-cell therapy in immunocompetent mice (I)

In study I, we used immunocompetent C57BL/6 mice bearing either subcutaneous murine B16.OVA or B16.F10 melanoma tumors. Ad5/3-CMV-mCD40L was used to assess the impact of adenovirus-expressing CD40L on adoptive T-cell therapy and DC therapy. Since human CD40L is not active in mice, this virus encodes murine CD40L. Human adenovirus replication is non-permissive in mice (Blair et al. 1989), so studies with immunocompetent mice do not have the effect of viral oncolysis.

Replication-deficient Ad5/3-CMV-mCD40L (coding for murine CD40L) virus did not improve adoptive T-cell therapy, as we did not observe significant differences between mice treated with Ad5/3-CMV-mCD40L with or without OT-1 T-cell adoptive therapy (Figure 4A, study I). To evaluate the ability of CD40L-armed adenovirus to enhance adoptive DC therapy, Ad5/3-CMV-mCD40L was next used to study the effect of adenovirus-expressing CD40L on adoptive DC therapy. These studies revealed that Ad5/3-CMV-mCD40L improved adoptive DC therapy the most, as the mice treated with the adenovirus-expressing CD40L together with DCs showed significantly enhanced antitumor efficacy when compared with mice treated with either Ad5/3-CMV-mCD40L alone, murine DCs alone, or PBS (Figure 4B).

4.7 Ad3-hTERT-CMV-hCD40L and human DCs enhance antitumor efficacy and survival of humanized mice (II)

To study the ability of Ad3-hTERT-CMV-hCD40L to enhance DC therapy in a situation that resembles the clinical setting, we used mice humanized by intravenous injection of human PBMCs. PBMCs and DCs alone and the combination of PBMCs and DCs had minimal inhibitory effects on tumor growth. Addition of viruses showed significant inhibition of tumor growth compared to mock-treated mice. However, the triple combination of virus, PBMCs, and DCs had more pronounced antitumor efficacy than double therapy. Significant antitumor efficacy was observed in the group of mice treated with Ad3-hTERT-CMV-hCD40L, PBMCs, and DCs when compared with Ad3-hTERT-E1A, PBMCs, and DCs (Figure 4 and Supplementary Figure 1A, Study II). Impressively, cancer-specific survival data showed that mice treated with the hCD40L-armed virus, PBMCs, and DCs had markedly improved survival (Figure 4B and Supplementary Figure 1B, study II). Collectively, these data indicate that Ad3-hTERT-CMV-hCD40L is a potent enhancer of DC therapy.

In study III, a similar experiment was performed with PC-3MM2 prostate cancer xenograft mice humanized by intravenous injection of human PBMCs. Here, the addition of adenovirus enhanced antitumor efficacy and survival of mice when compared with the mock and PBMCs only groups (Figure 2A-B, study III). Experiments with DCs along with adenovirus showed that Ad3-hTERT-E1A plus PBMCs plus DCs did not significantly enhance antitumor efficacy and survival when compared with the group in which mice received Ad3-hTERT-E1A plus PBMCs treatment. Thus, unarmed virus did not enhance DC therapy (Figure 3A, 3B, study III). Significant antitumor efficacy in mice treated with Ad3-hTERT-CMV-hCD40L, PBMCs, and DCs was observed when compared with other groups in which mice were either treated with PBS (mock group) or PBMCs plus DCs or

Ad3-hTERT-CMV-hCD40L plus PBMCs (Figure 3C, study III). We did not observe enhanced cancer-specific survival in the group treated with Ad3-hTERT-CMV-hCD40L plus PBMCs plus DCs when compared with control groups. However, we did observe significant differences between PBMCs plus Ad3-hTERT-CMV-hCD40L and PBMCs plus Ad3-hTERT-CMV-hCD40L plus DC groups (Figure 3D, study III). Thus, our results indicate that Ad3-hTERT-E1A and Ad3-hTERT-CMV-hCD40L both have oncolytic potency resulting in antitumor effects. However, unarmed Ad3-hTERT-E1A does not improve the antitumor efficacy of adoptively transferred DCs, whereas Ad3-hTERT-CMV-hCD40L enhances adoptive DC therapy.

4.8 Combination of CD40L-armed adenovirus and DC therapy modulates immune responses (I, II, III)

Our results suggested that CD40L-armed adenovirus and DC therapy modulate immune responses towards a Th1-type immune response. In study I, analysis of lymph nodes showed significantly higher levels of B and T lymphocytes and mature DCs in the group of mice treated with adenovirus and DCs compared with other groups (Figure 5, study I). In the same experiment, analysis of tumors revealed an increased proportion of tumor-specific CD8+ T cells, CD4+ T cells, mature DCs, and NK cells in the group treated with adenovirus and DCs (Figure 6, Study I). The presence of high levels of proinflammatory cytokines (such as TNF-α, IFN-γ, and RANTES) in tumors of mice treated with CD40L-armed adenovirus and DCs further suggests the induction of a Th1-type immune response (Supplementary figure 2, Study I). Collectively, the results suggest that the expression of CD40L induces DC maturation, which in turn is required for the activation of other immune cells. In study II with humanized mice, four mice from each group were euthanized a week after treatment to investigate the treatment mechanism of action. Tumor analysis showed that mice treated with Ad3-hTERT-CMV-hCD40L, DCs, and PBMCs had increased upregulation of DC maturation makers CD83, CD80, and CD86 (Figures 5A-C, study II) and significant infiltration of B and T lymphocytes. In the same group, high levels of TNF-α, IFN-γ, IL-2, IL-12, granzyme B, and IL-6 were observed in the tumors (Supplementary Figure 3, Study II).

In study III, five human patient prostate cancer samples were studied *in vitro*. Treatment of human prostate cancer samples with Ad3-hTERT-CMV-hCD40L induced significant upregulation of DC maturation markers (such as CD83, CD80, CD86) when compared with the mock (uninfected cells) or Ad3-hTERT-E1A-treated samples in 4 out of 5 patients studied (Figure 4; B-D, study III). In addition, cytokine analysis of the supernatant of treated samples showed significant production of

proinflammatory cytokines (such as IL-2, TNF-α, IL-12, granzyme B, and IFN-γ) and CD40L along with reduced production of immunosuppressive cytokines TGF-β1 and IL-10 (Figure 5 A-E, study III). Our results suggest that Ad3-hTERT-CMV-hCD40L infection in the TME leads to the expression of virally expressed CD40L, which in turn induces DC maturation and production of proinflammatory cytokines.

4.9 Ad5/3 adenoviruses are able to reversibly bind to the surface of human lymphocytes and erythrocytes and this does not inhibit the oncolytic capacity of the viruses *in vitro* (IV)

To evaluate the binding of a chimeric adenovirus Ad5/3 with blood cells (i.e. lymphocytes and erythrocytes), Ad5/3 virus was incubated with lymphocytes at 1, 10, and 100 VP/cell and with erythrocytes at 0.0036, 0.036, and 0.36 VP/cell for 30 minutes followed by centrifugation. Analysis of the cellular fractions at all ratios showed the presence of Ad5/3 virus (Supplementary figure 1, study IV). The binding of Ad5/3 adenovirus to lymphocytes and to erythrocytes was further determined in another experiment, in which Ad5/3 adenovirus with lymphocytes (at 10 VP/cell) (Figure 1A and 1B, study IV) and with erythrocytes (at 0.036 VP/cell) (Figure 1C and 1D, study IV) were incubated for 30 minutes at 37°C. After incubation, samples were centrifuged and the cellular fractions were washed with PBS five times. Samples were collected for viral DNA quantification after each wash. Although we observed unbound virus in the supernatant after every wash, a portion of the virus persisted in the cellular fraction. Thus, our results indicate that the virus can consistently bind to the selected blood cell types.

After studying virus binding ability with selected blood cell types, we next evaluated whether this binding is reversible and whether it inhibits adenovirus transduction. This was first studied through a luciferase assay using replication-deficient Ad5/3-Luc1 to detect if the virus can transduce cancer cells when delivered with the selected cell types, which would lead to luciferase expression. Cell-virus mixtures (lymphocytes or erythrocytes plus Ad5/3-Luc1) were incubated for 30 minutes at 37°C. After centrifugation, either different dilutions (i.e. 1:1.7, 1:2.7, 1:6.7) of cell-virus mixture were directly incubated with A549 cells or cell-virus mixtures were washed three times before incubating with A549 cells. We observed comparable Ad5/3-Luc1 transduction to the control conditions (i.e. virus only at 0.1-10 VP/cell). We did not observe luminescent signal from negative control samples, i.e. erythrocytes or lymphocytes incubated with Ad5/3-Luc1 only without A549 cells (Supplementary

Figure 2, study IV). Our results therefore demonstrated a clear transduction of Ad5/3-Luc1 virus regardless of the presence of lymphocytes or erythrocytes (Figure 2A and B, study IV).

Replication-competent TILT-123 adenovirus was used to study the oncolytic potency of cell-bound adenovirus. Similar conditions as in the previous experiment were used to examine the cell-killing efficacy of cell-bound adenovirus with the MTS assay. We observed cell killing comparable to the control conditions (i.e. virus only 0.1-10 VP/cell). This indicates that the binding of adenovirus to these cell types does not inhibit the oncolytic potency of the virus (Figure 2C and D, study IV).

The result was further confirmed through a migration assay, where we observed that cell-bound adenovirus (TILT-123) was able to kill tumor cells. This suggests that the virus can be released from the cells, migrate through the transwell, and kill tumor cells. Thus, our results showed that the adenovirus binds reversibly to lymphocytes or erythrocytes and retains oncolytic ability (Supplementary figure 3, study IV).

We also visualized the binding of adenovirus (i.e. TILT-123) to blood cells with electron microscopy (both SEM and TEM). Images from SEM confirmed that the adenovirus binds to the surface of lymphocytes (Figures 3A and B) and erythrocytes (Figures 3C, study IV). Erythrocytes are not able to internalize adenoviruses (Rojas et al. 2016b), but this has not been well studied for lymphocytes. We used TEM to analyze the binding of adenovirus Ad5/3 to lymphocytes. Analysis with TEM showed that TILT-123 bound only to the surface of lymphocytes, as we did not observe any internalized adenovirus (Figure 3D, study IV). Thus, these results further confirmed our finding that Ad5/3 adenovirus has a surface association with both lymphocytes and erythrocytes.

4.10 Adenovirus Ad5/3 binding with erythrocytes and lymphocytes is reversible and does not inhibit tumor transduction *in vivo* (IV)

Systemic administration of virus provides the possibility to treat the primary tumor and metastatic tumors simultaneously (Ferguson, Lemoine and Wang 2012). Therefore, we decided to study the ability of erythrocytes and lymphocytes to deliver Ad5/3 into tumors upon intravenous administration and whether binding to these cells could protect the virus from neutralization.

In this study, we used immunodeficient mice bearing human prostate tumors (PC-3MM2) subcutaneously. They were administered intravenously either TILT-123 alone (1.5 x 10e10 VP/100 µl as positive control or 1.5 x 10e9 VP/100 µl as experimental control) or bound to erythrocytes or lymphocytes at 500 VP/cell. Tumors were analyzed with qPCR for the presence of virus. The

presence of viral DNA was observed in all groups, indicating that binding of virus to erythrocytes or lymphocytes did not impede tumor transduction *in vivo* (Figure 4A, study IV).

In addition to the tumors, viral DNA was also found in different organs, such as the liver, spleen, and lungs (Figure 4B-D, study IV). Less viral DNA was found in the liver when TILT-123 was delivered with lymphocytes than when delivered with erythrocytes. When TILT-123 was bound to lymphocytes, significantly less viral DNA was found in the lungs than the positive control (10x, i.e. 10 times more virus). We also observed extended blood persistence of virus in the positive control group (10x) when compared with the other groups (Figure 4E, study IV). Thus, our results suggest that human erythrocytes and lymphocytes did not prevent adenovirus transduction in tumor or organs. Interestingly, analysis of tumor-to-liver ratios showed that when adenovirus was bound to either lymphocytes or erythrocytes a better relative tumor transduction was observed (Figure 4F, study IV). Even in the presence of neutralizing antiserum, which was incubated with adenovirus alone or adenovirus previously incubated with either lymphocytes or erythrocytes at room temperature before injecting the mice, adenovirus bound to either lymphocytes or erythrocytes showed enhanced transduction of the tumor when compared with the liver (Figure 5A,F study IV). In contrast, we observed more viral DNA in the liver when the virus was administered alone than when it was mixed with antiserum (Figure 5B, Study IV).

As every tumor is different regarding the TME and tumor vasculature, they may respond differently to oncolytic viral therapy (Wojton and Kaur 2010). Therefore, we repeated the experiment described above using a different tumor model (human lung adenocarcinoma A549 tumors). In this experiment, the experimental dose of TILT-123 alone and TILT-123 bound to lymphocytes had a comparable delivery efficacy of virus to tumors (Figure 6A, study IV) and to a lesser extent to the liver (Figure 6B, study IV) and spleen (Supplementary figure 4A, study IV). In the group receiving 10 times more virus (positive control group), we observed more viral DNA in the tumors, spleen, and liver. Viral DNA was not found in the blood serum except for one case from the positive control group (Supplementary figure 4B, study IV). Although the tumor-to-liver ratio of viral DNA was higher when the virus was bound to lymphocytes, we did not find significant differences between the groups (Figure 6C, study IV). We also detected the expression of IL-2 and TNF-α in the tumor samples, as TILT-123 (adenovirus armed with IL-2 and TNF- α) expressed these cytokines when replicating in the tumor. Thus, this further confirmed that the virus is functional when delivered to the tumors (Supplementary figure 5 A-B, study IV).

Upon addition of neutralizing antiserum, the mice that received virus bound to erythrocytes plus antiserum showed higher viral DNA levels in tumors and to a lesser extent in the liver and spleen when compared with other groups (Figure 6D, E, F Supplementary Figure 4C, study IV). In this experiment, antiserum had a greater neutralizing effect on the virus alone, which resulted in more virus in liver. This is because liver uptake (by e.g. Kupffer cells) is not dependent on the interaction with the primary receptor of the virus. We also observed a notable neutralizing effect in case of virus plus lymphocytes and antiserum (Figure 6D-F, Supplementary figure 4C, study IV). We detected the expression of IL-2 and TNF-α in the tumor samples, indicative of the replication of TILT-123 (adenovirus armed with IL-2 and TNF-α) within the tumor, thus confirming virus replication (Supplementary figure 5 C-D, study IV).

In conclusion, we discovered that the Ad5/3 chimeric adenovirus can hitchhike on human lymphocytes and erythrocytes to reach non-injected tumors *in vivo*. It does so by binding to erythrocytes and lymphocytes in a reversible manner. Moreover, this binding does not inhibit viral functionality.

5 Summary and conclusions

This study examined the cancer therapy potential of a novel oncolytic adenovirus Ad3-hTERT-CMV-hCD40L, which is fully serotype 3 and designed to improve DC therapy. Results from this study suggest that Ad3-hTERT-CMV-CD40L transduces tumors upon intravenous administration, and induces antitumor responses. These responses were more pronounced with adoptive transfer of DCs. The antitumor responses were derived both from the virus infection (oncolysis) and the expression of an immunostimulatory transgene (CD40L). Virus-mediated lysis of tumor cells is important for the spread of virus within the tumor and release of tumor epitopes, while transgene expression induces immunological changes. This study revealed the ability of the virus to induce DC maturation, to direct T-cell responses towards Th1, and to promote cytotoxic T cells. Treatment with Ad3-hTERT-CMV-hCD40L did not cause any toxicity. Thus, Ad3-hTERT-CMV-hCD40L is a potent enabler of DC therapy. Our findings provide a rationale for a phase I trial to investigate the safety of Ad3-hTERT-CMV-CD40L together with DC treatment in patients with solid tumors.

The fourth part of this study also demonstrated the ability of a chimeric adenovirus to transduce non-injected tumors despite the presence of neutralizing antibodies. Reversible interaction of virus with blood cells, such as lymphocytes and erythrocytes, was shown to enhance tumor transduction upon intravenous administration.

6 Perspectives

Tumors and their microenvironments are highly heterogeneous due to the ability of tumors to escape immune responses and their constantly evolving nature. Therefore, targeting of multiple pathways is required to ensure active control of evolving tumor cells. Emerging targets for cancer therapy are co-stimulatory or co-inhibitory molecules. These could have additional synergistic effects when combined with oncolytic adenovirus therapies. Oncolytic adenoviruses are safe and effective for cancer treatment. Adenoviruses can be armed with immunostimulatory molecules (transgenes), which allows localized (i.e. within the tumor) expression of these molecules. However, the possible negative influence of these transgenes on the oncolytic capacity of virus should not be neglected.

The contributions of DCs in initiating antitumor immune responses are recognized as targetable. DC therapies as monotherapy have been minimally successful. Lack of favorable responses to current DC-based immunotherapies are potentially due to the immunosuppressive TME, dysfunction of administered DCs, lack of prognostic biomarkers, low tumor mutational burden, and low T-cell infiltration in tumors. Hence, many clinical trials are combining DC therapy with other therapies, such as radiotherapy, chemotherapy or checkpoint inhibitor therapy. Nevertheless, this combination often lacks immunological rationale.

Comparison of immunological responses and clinical efficacy between different studies is challenging due to variations in treatment schedule and dosing. Different clinical trials with DC therapy are also difficult to compare due to variations in use of DC subsets, DC maturation status or process, DC dose per injection, number of injections, or interval between injections. Comparisons may be simplified with appropriate immunomonitoring. The oncolytic adenovirus Ad3-hTERT-CMV-CD40L is a potent enhancer of DC therapy; in the future a combination of DC therapy and this oncolytic adenovirus could provide clinical benefit for patients suffering from currently incurable cancers.

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8 References

Aarntzen, E. H., C. G. Figdor, G. J. Adema, C. J. Punt & I. J. de Vries (2008) Dendritic cell vaccination and immune monitoring. *Cancer Immunol Immunother,* 57**,** 1559-68.

Ahi, Y. S. & S. K. J. F. i. m. Mittal (2016) Components of adenovirus genome packaging. 7**,** 1503.

Ahmed, K. A., L. Wang, M. A. Munegowda, S. J. Mulligan, J. R. Gordon, P. Griebel & J. Xiang (2012) Direct in vivo evidence of CD4+ T cell requirement for CTL response and memory via pMHC-I targeting and CD40L signaling. *J Leukoc Biol,* 92**,** 289-300.

Alfaro, C., J. L. Perez-Gracia, N. Suarez, J. Rodriguez, M. Fernandez de Sanmamed, B. Sangro, S. Martin-Algarra, A. Calvo, M. Redrado, A. Agliano, A. Gonzalez, I. Rodriguez, E. Bolaños, S. Hervás-Stubbs, J. Perez-Calvo, A. Benito, I. Peñuelas, C. Vigil, J. Richter, I. Martinez-Forero & I. Melero (2011) Pilot Clinical Trial of Type 1 Dendritic Cells Loaded with Autologous Tumor Lysates Combined with GM-CSF, Pegylated IFN, and Cyclophosphamide for Metastatic Cancer Patients. 187**,** 6130-6142.

Almand, B., J. R. Resser, B. Lindman, S. Nadaf, J. I. Clark, E. D. Kwon, D. P. Carbone & D. I. Gabrilovich (2000) Clinical significance of defective dendritic cell differentiation in cancer. *Clin Cancer Res,* 6**,** 1755-66.

Andtbacka, R. H., M. Ross, I. Puzanov, M. Milhem, F. Collichio, K. A. Delman, T. Amatruda, J. S. Zager, L. Cranmer & E. J. A. o. s. o. Hsueh (2016) Patterns of clinical response with talimogene laherparepvec (T-VEC) in patients with melanoma treated in the OPTiM phase III clinical trial. 23**,** 4169-4177.

Andtbacka, R. H. I., F. Collichio, K. J. Harrington, M. R. Middleton, G. Downey, K. Öhrling & H. L. Kaufman (2019) Final analyses of OPTiM: a randomized phase III trial of talimogene laherparepvec versus granulocyte-macrophage colony-stimulating factor in unresectable stage III–IV melanoma. *Journal for ImmunoTherapy of Cancer,* 7**,** 145.

Bajor, D. L., R. Mick, M. J. Riese, A. C. Huang, B. Sullivan, L. P. Richman, D. A. Torigian, S. M. George, E. Stelekati, F. Chen, J. J. Melenhorst, S. F. Lacey, X. Xu, E. J. Wherry, T. C. Gangadhar, R. K. Amaravadi, L. M. Schuchter & R. H. Vonderheide (2018) Long-term outcomes of a phase I study of agonist CD40 antibody and CTLA-4 blockade in patients with metastatic melanoma. *Oncoimmunology,* 7**,** e1468956.

Banchereau, J., F. Briere, C. Caux, J. Davoust, S. Lebecque, Y.-J. Liu, B. Pulendran & K. Palucka (2000) Immunobiology of Dendritic Cells. 18**,** 767-811.

Banchereau, J. & R. M. Steinman (1998) Dendritic cells and the control of immunity. *Nature,* 392**,** 245-52.

Bandola-Simon, J. & P. A. Roche (2019) Dysfunction of antigen processing and presentation by dendritic cells in cancer. *Molecular Immunology,* 113**,** 31-37.

Bartlett, D. L., Z. Liu, M. Sathaiah, R. Ravindranathan, Z. Guo, Y. He & Z. S. J. M. c. Guo (2013) Oncolytic viruses as therapeutic cancer vaccines. 12**,** 103.

Beatty, G. L. & W. L. Gladney (2015) Immune Escape Mechanisms as a Guide for Cancer Immunotherapy. 21**,** 687-692.

Bender, A., M. Sapp, G. Schuler, R. M. Steinman & N. Bhardwaj (1996) Improved methods for the generation of dendritic cells from nonproliferating progenitors in human blood. *Journal of Immunological Methods,* 196**,** 121-135.

Benencia, F., M. C. Courrèges & G. Coukos (2008) Whole tumor antigen vaccination using dendritic cells: Comparison of RNA electroporation and pulsing with UV-irradiated tumor cells. *Journal of Translational Medicine,* 6**,** 21.

Bourgeois, C., B. Rocha & C. Tanchot (2002) A role for CD40 expression on CD8+ T cells in the generation of CD8+ T cell memory. *Science,* 297**,** 2060-3.

Bray, F., J. Ferlay, I. Soerjomataram, R. L. Siegel, L. A. Torre & A. J. C. a. c. j. f. c. Jemal (2018) Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. 68**,** 394-424.

Byrne, K. T. & R. H. Vonderheide (2016) CD40 Stimulation Obviates Innate Sensors and Drives T Cell Immunity in Cancer. *Cell Rep,* 15**,** 2719-32.

Carreno, B. M., V. Magrini, M. Becker-Hapak, S. Kaabinejadian, J. Hundal, A. A. Petti, A. Ly, W.-R. Lie, W. H. Hildebrand, E. R. Mardis & G. P. Linette (2015) A dendritic cell vaccine increases the breadth and diversity of melanoma neoantigen-specific T cells. 348**,** 803-808.

Casamayor-Palleja, M., M. Khan & I. C. MacLennan (1995) A subset of CD4+ memory T cells contains preformed CD40 ligand that is rapidly but transiently expressed on their surface after activation through the T cell receptor complex. *J Exp Med,* 181**,** 1293-301.

Caux, C., C. Massacrier, B. Vanbervliet, B. Dubois, C. Van Kooten, I. Durand & J. Banchereau (1994) Activation of human dendritic cells through CD40 cross-linking. *J Exp Med,* 180**,** 1263-72.

Cella, M., D. Scheidegger, K. Palmer-Lehmann, P. Lane, A. Lanzavecchia & G. Alber (1996) Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T cell stimulatory capacity: T-T help via APC activation. *J Exp Med,* 184**,** 747-52.

Cervera-Carrascon, V., M. Siurala, J. M. Santos, R. Havunen, S. Tähtinen, P. Karell, S. Sorsa, A. Kanerva & A. Hemminki (2018) TNFa and IL-2 armed adenoviruses enable complete responses by anti-PD-1 checkpoint blockade. *OncoImmunology,* 7**,** e1412902.

Cerwenka, A., A. B. Bakker, T. McClanahan, J. Wagner, J. Wu, J. H. Phillips & L. L. J. I. Lanier (2000) Retinoic acid early inducible genes define a ligand family for the activating NKG2D receptor in mice. 12**,** 721-727.

Chang, J., X. Zhao, X. Wu, Y. Guo, H. Guo, J. Cao, Y. Guo, D. Lou, D. Yu & J. Li (2009) A Phase I study of KH901, a conditionally replicating granulocyte-macrophage colony-stimulating factor: armed oncolytic adenovirus for the treatment of head and neck cancers. *Cancer biology & therapy,* 8**,** 676-682.

Chen, D. S. & I. Mellman (2013) Oncology meets immunology: the cancer-immunity cycle. *Immunity,* 39**,** 1-10.

Cheng, P.-H., S. L. Wechman, K. M. McMasters & H. S. Zhou (2015) Oncolytic Replication of E1b-Deleted Adenoviruses. *Viruses,* 7**,** 5767-5779.

Chesney, J., I. Puzanov, F. Collichio, P. Singh, M. M. Milhem, J. Glaspy, O. Hamid, M. Ross, P. Friedlander, C. Garbe, T. F. Logan, A. Hauschild, C. Lebbé, L. Chen, J. J. Kim, J. Gansert, R. H. I. Andtbacka & H. L. Kaufman (2018) Randomized, Open-Label Phase II Study Evaluating the Efficacy and Safety of Talimogene Laherparepvec in Combination With Ipilimumab Versus Ipilimumab Alone in Patients With Advanced, Unresectable Melanoma. 36**,** 1658-1667.

Chiang, C. L.-L., G. Coukos & L. E. J. V. Kandalaft (2015) Whole tumor antigen vaccines: where are we? 3**,** 344-372.

Chiang, C. L.-L., L. E. Kandalaft, J. Tanyi, A. R. Hagemann, G. T. Motz, N. Svoronos, K. Montone, G. M. Mantia-Smaldone, L. Smith, H. L. Nisenbaum, B. L. Levine, M. Kalos, B. J. Czerniecki, D. A. Torigian, D. J. Powell, R. Mick & G. Coukos (2013) A Dendritic Cell Vaccine Pulsed with Autologous Hypochlorous Acid-Oxidized Ovarian Cancer Lysate Primes Effective Broad Antitumor Immunity: From Bench to Bedside. 19**,** 4801-4815.

Chiba, S., M. Baghdadi, H. Akiba, H. Yoshiyama, I. Kinoshita, H. Dosaka-Akita, Y. Fujioka, Y. Ohba, J. V. Gorman, J. D. Colgan, M. Hirashima, T. Uede, A. Takaoka, H. Yagita & M. Jinushi (2012) Tumor-infiltrating DCs suppress nucleic acid–mediated innate immune responses through interactions between the receptor TIM-3 and the alarmin HMGB1. *Nature Immunology,* 13**,** 832-842.

Coley, W. B. J. T. A. J. o. t. M. S. (1893) The treatment of malignant tumors by repeated inoculations of erysipelas: With a report of ten original cases. 1. 105**,** 487.

Conrad, C., J. Gregorio, Y.-H. Wang, T. Ito, S. Meller, S. Hanabuchi, S. Anderson, N. Atkinson, P. T. Ramirez, Y.-J. Liu, R. Freedman & M. Gilliet (2012) Plasmacytoid Dendritic Cells Promote Immunosuppression in Ovarian Cancer via ICOS Costimulation of Foxp3⁺ T-Regulatory Cells. 72**,** 5240-5249.

Courrèges, M. C., F. Benencia, J. R. Conejo-García, L. Zhang & G. Coukos (2006) Preparation of apoptotic tumor cells with replication-incompetent HSV augments the efficacy of dendritic cell vaccines. *Cancer Gene Therapy,* 13**,** 182-193.

Davison, A. J., M. Benko & B. Harrach (2003) Genetic content and evolution of adenoviruses. *J Gen Virol,* 84**,** 2895-2908.

De Graaf, J., L. de Vor, R. Fouchier, B. J. C. Van Den Hoogen & g. f. reviews (2018) Armed oncolytic viruses: A kick-start for anti-tumor immunity. 41**,** 28-39.

de Mingo Pulido, Á., A. Gardner, S. Hiebler, H. Soliman, H. S. Rugo, M. F. Krummel, L. M. Coussens & B. Ruffell (2018) TIM-3 Regulates CD103(+) Dendritic Cell Function and Response to Chemotherapy in Breast Cancer. *Cancer Cell,* 33**,** 60-74.e6.

DeWeese, T. L., H. van der Poel, S. Li, B. Mikhak, R. Drew, M. Goemann, U. Hamper, R. DeJong, N. Detorie & R. J. C. r. Rodriguez (2001) A phase I trial of CV706, a replication-competent, PSA selective oncolytic adenovirus, for the treatment of locally recurrent prostate cancer following radiation therapy. 61**,** 7464-7472.

Dhodapkar, M. V., R. M. Steinman, J. Krasovsky, C. Munz & N. Bhardwaj (2001) Antigen-specific inhibition of effector T cell function in humans after injection of immature dendritic cells. *J Exp Med,* 193**,** 233-8.

Diaconu, I., V. Cerullo, M. L. Hirvinen, S. Escutenaire, M. Ugolini, S. K. Pesonen, S. Bramante, S. Parviainen, A. Kanerva, A. S. Loskog, A. G. Eliopoulos, S. Pesonen & A. Hemminki (2012) Immune response is an important aspect of the antitumor effect produced by a CD40L-encoding oncolytic adenovirus. *Cancer Res,* 72**,** 2327-38.

Dias, J. D., O. Hemminki, I. Diaconu, M. Hirvinen, A. Bonetti, K. Guse, S. Escutenaire, A. Kanerva, S. Pesonen, A. Löskog, V. Cerullo & A. Hemminki (2012) Targeted cancer immunotherapy with oncolytic adenovirus coding for a fully human monoclonal antibody specific for CTLA-4. *Gene Therapy,* 19**,** 988-998.

Dunn, G. P., A. T. Bruce, H. Ikeda, L. J. Old & R. D. J. N. i. Schreiber (2002) Cancer immunoediting: from immunosurveillance to tumor escape. 3**,** 991-998.

Eissa, I. R., I. Bustos-Villalobos, T. Ichinose, S. Matsumura, Y. Naoe, N. Miyajima, D. Morimoto, N. Mukoyama, W. Zhiwen, M. Tanaka, H. Hasegawa, S. Sumigama, B. Aleksic, Y. Kodera & H. Kasuya (2018) The Current Status and Future Prospects of Oncolytic Viruses in Clinical Trials against Melanoma, Glioma, Pancreatic, and Breast Cancers. *Cancers,* 10**,** 356.

Elgueta, R., M. J. Benson, V. C. de Vries, A. Wasiuk, Y. Guo & R. J. Noelle (2009) Molecular mechanism and function of CD40/CD40L engagement in the immune system. *Immunol Rev,* 229**,** 152-72.

Engeland, C. E., C. Grossardt, R. Veinalde, S. Bossow, D. Lutz, J. K. Kaufmann, I. Shevchenko, V. Umansky, D. M. Nettelbeck, W. Weichert, D. Jäger, C. von Kalle & G. Ungerechts (2014) CTLA-4 and PD-L1 Checkpoint Blockade Enhances Oncolytic Measles Virus Therapy. *Molecular Therapy,* 22**,** 1949-1959.

Engell-Noerregaard, L., T. H. Hansen, M. H. Andersen, P. thor Straten & I. M. Svane (2008) Review of clinical studies on dendritic cell-based vaccination of patients with malignant melanoma: assessment of correlation between clinical response and vaccine parameters. *Cancer Immunology, Immunotherapy,* 58**,** 1.

Farkona, S., E. P. Diamandis & I. M. Blasutig (2016) Cancer immunotherapy: the beginning of the end of cancer? *BMC Medicine,* 14**,** 73.

Ferguson, M. S., N. R. Lemoine & Y. Wang (2012) Systemic delivery of oncolytic viruses: hopes and hurdles. *Adv Virol,* 2012**,** 805629.

Flint, J., V. R. Racaniello, G. F. Rall, A. M. Skalka & T. Hatziioannou. 2020. *Principles of Virology, Volume 1: Molecular Biology*. John Wiley & Sons.

Foy, S. P., S. J. Mandl, T. dela Cruz, J. J. Cote, E. J. Gordon, E. Trent, A. Delcayre, J. Breitmeyer, A. Franzusoff & R. B. Rountree (2016) Poxvirus-based active immunotherapy synergizes with CTLA-4 blockade to increase survival in a murine tumor model by improving the magnitude and quality of cytotoxic T cells. *Cancer Immunology, Immunotherapy,* 65**,** 537-549.

Gabitass, R. F., N. E. Annels, D. D. Stocken, H. A. Pandha & G. W. J. C. I. Middleton, Immunotherapy (2011) Elevated myeloid-derived suppressor cells in pancreatic, esophageal and gastric cancer are an independent prognostic factor and are associated with significant elevation of the Th2 cytokine interleukin-13. 60**,** 1419.

Gabrilovich, D. (2004) Mechanisms and functional significance of tumour-induced dendritic-cell defects. *Nature Reviews Immunology,* 4**,** 941-952.

Gabrilovich, D. I., S. Ostrand-Rosenberg & V. Bronte (2012a) Coordinated regulation of myeloid cells by tumours. *Nature Reviews Immunology,* 12**,** 253-268.
Gabrilovich, D. I., S. Ostrand-Rosenberg & V. J. N. R. I. Bronte (2012b) Coordinated regulation of myeloid cells by tumours. 12**,** 253-268.
Gao, Y., P. Whitaker-Dowling, J. A. Griffin, M. A. Barmada & I. Bergman (2009) Recombinant vesicular stomatitis virus targeted to Her2/neu combined with anti-CTLA4 antibody eliminates implanted mammary tumors. *Cancer Gene Therapy,* 16**,** 44-52.
Garber, K. 2006, "China approves world's first oncolytic virus therapy for cancer treatment", Journal-National Cancer Institute, 98(5), 298-300.
Garg, A. D., M. Vara Perez, M. Schaaf, P. Agostinis, L. Zitvogel, G. Kroemer & L. Galluzzi (2017) Trial watch: Dendritic cell-based anticancer immunotherapy. *OncoImmunology,* 6**,** e1328341.
Giberson, A. N., A. R. Davidson & R. J. J. N. a. r. Parks (2012) Chromatin structure of adenovirus DNA throughout infection. 40**,** 2369-2376.
Girardi, M., D. E. Oppenheim, C. R. Steele, J. M. Lewis, E. Glusac, R. Filler, P. Hobby, B. Sutton, R. E. Tigelaar & A. C. J. S. Hayday (2001) Regulation of cutaneous malignancy by γδ T cells. 294**,** 605-609.
Granot, T., T. Senda, D. J. Carpenter, N. Matsuoka, J. Weiner, C. L. Gordon, M. Miron, B. V. Kumar, A. Griesemer, S. H. Ho, H. Lerner, J. J. C. Thome, T. Connors, B. Reizis & D. L. Farber (2017) Dendritic Cells Display Subset and Tissue-Specific Maturation Dynamics over Human Life. *Immunity,* 46**,** 504-515.
Greig, S. L. (2016) Talimogene Laherparepvec: First Global Approval. *Drugs,* 76**,** 147-54.
Guo, Z. S., Z. Liu & D. L. J. F. i. o. Bartlett (2014) Oncolytic immunotherapy: dying the right way is a key to eliciting potent antitumor immunity. 4**,** 74.
Gutschner, T. & S. Diederichs (2012) The hallmarks of cancer: a long non-coding RNA point of view. *RNA biology,* 9**,** 703-719.
Handy, C. E. & E. S. Antonarakis (2018) Sipuleucel-T for the treatment of prostate cancer: novel insights and future directions. 14**,** 907-917.
Hardcastle, J., L. Mills, C. S. Malo, F. Jin, C. Kurokawa, H. Geekiyanage, M. Schroeder, J. Sarkaria, A. J. Johnson & E. J. N.-o. Galanis (2017) Immunovirotherapy with measles virus strains in combination with anti–PD-1 antibody blockade enhances antitumor activity in glioblastoma treatment. 19**,** 493-502.
Havunen, R., M. Siurala, S. Sorsa, S. Grönberg-Vähä-Koskela, M. Behr, S. Tähtinen, J. M. Santos, P. Karell, J. Rusanen, D. M. Nettelbeck, A. Ehrhardt, A. Kanerva & A. Hemminki (2017) Oncolytic Adenoviruses Armed with Tumor Necrosis Factor Alpha and Interleukin-2 Enable Successful Adoptive Cell Therapy. *Molecular Therapy - Oncolytics,* 4**,** 77-86.
Hecht, J. R., R. Bedford, J. L. Abbruzzese, S. Lahoti, T. R. Reid, R. M. Soetikno, D. H. Kirn & S. M. Freeman (2003) A phase I/II trial of intratumoral endoscopic ultrasound injection of ONYX-015 with intravenous gemcitabine in unresectable pancreatic carcinoma. *Clin Cancer Res,* 9**,** 555-61.
Heise, C., T. Hermiston, L. Johnson, G. Brooks, A. Sampson-Johannes, A. Williams, L. Hawkins & D. J. N. m. Kirn (2000) An adenovirus E1A mutant that demonstrates potent and selective systemic anti-tumoral efficacy. 6**,** 1134-1139.
Heise, C., A. Sampson-Johannes, A. Williams, F. Mccormick, D. D. Von Hoff & D. H. J. N. m. Kirn (1997) ONYX-015, an E1B gene-attenuated adenovirus, causes tumor-specific cytolysis and antitumoral efficacy that can be augmented by standard chemotherapeutic agents. 3**,** 639-645.
Hemminki, O., M. Oksanen, K. Taipale, I. Liikanen, A. Koski, T. Joensuu, A. Kanerva & A. J. M. T.-O. Hemminki (2018) Oncograms Visualize Factors Influencing Long-Term Survival of Cancer Patients Treated with Adenoviral Oncolytic Immunotherapy. 9**,** 41-50.
Hoeben, R. C. & T. G. Uil (2013) Adenovirus DNA replication. *Cold Spring Harb Perspect Biol,* 5**,** a013003.
Hsu, F. J., C. Benike, F. Fagnoni, T. M. Liles, D. Czerwinski, B. Taidi, E. G. Engleman & R. Levy (1996a) Vaccination of patients with B-cell lymphoma using autologous antigen-pulsed dendritic cells. *Nat Med,* 2**,** 52-8.

Hsu, F. J., C. Benike, F. Fagnoni, T. M. Liles, D. Czerwinski, B. Taidi, E. G. Engleman & R. Levy (1996b) Vaccination of patients with B–cell lymphoma using autologous antigen–pulsed dendritic cells. *Nature Medicine,* 2**,** 52-58.

Hu, J. C., R. S. Coffin, C. J. Davis, N. J. Graham, N. Groves, P. J. Guest, K. J. Harrington, N. D. James, C. A. Love, I. McNeish, L. C. Medley, A. Michael, C. M. Nutting, H. S. Pandha, C. A. Shorrock, J. Simpson, J. Steiner, N. M. Steven, D. Wright & R. C. Coombes (2006) A phase I study of OncoVEXGM-CSF, a second-generation oncolytic herpes simplex virus expressing granulocyte macrophage colony-stimulating factor. *Clin Cancer Res,* 12**,** 6737-47.

Ilett, E., T. Kottke, J. Thompson, K. Rajani, S. Zaidi, L. Evgin, M. Coffey, C. Ralph, R. Diaz, H. Pandha, K. Harrington, P. Selby, R. Bram, A. Melcher & R. Vile (2017) Prime-boost using separate oncolytic viruses in combination with checkpoint blockade improves anti-tumortherapy. *Gene Therapy,* 24**,** 21-30.

Irenaeus, S. M. M., D. Nielsen, P. Ellmark, J. Yachnin, A. Deronic, A. Nilsson, P. Norlén, N. Veitonmäki, C. S. Wennersten & G. J. Ullenhag (2019) First-in-human study with intratumoral administration of a CD40 agonistic antibody, ADC-1013, in advanced solid malignancies. *Int J Cancer,* 145**,** 1189-1199.

Jiang, H., Y. Rivera-Molina, C. Gomez-Manzano, K. Clise-Dwyer, L. Bover, L. M. Vence, Y. Yuan, F. F. Lang, C. Toniatti & M. B. J. C. r. Hossain (2017) Oncolytic adenovirus and tumor-targeting immune modulatory therapy improve autologous cancer vaccination. 77**,** 3894-3907.

Jiang, H., D. H. Shin, T. T. Nguyen, J. Fueyo, X. Fan, V. Henry, C. C. Carrillo, Y. Yi, M. M. Alonso & T. L. J. C. C. R. Collier (2019) Localized treatment with oncolytic adenovirus delta-24-RGDOX induces systemic immunity against disseminated subcutaneous and intracranial melanomas. 25**,** 6801-6814.

Jogler, C., D. Hoffmann, D. Theegarten, T. Grunwald, K. Überla & O. J. J. o. v. Wildner (2006) Replication properties of human adenovirus in vivo and in cultures of primary cells from different animal species. 80**,** 3549-3558.

Kantoff, P. W., C. S. Higano, N. D. Shore, E. R. Berger, E. J. Small, D. F. Penson, C. H. Redfern, A. C. Ferrari, R. Dreicer, R. B. Sims, Y. Xu, M. W. Frohlich & P. F. Schellhammer (2010) Sipuleucel-T Immunotherapy for Castration-Resistant Prostate Cancer. 363**,** 411-422.

Karyampudi, L., P. Lamichhane, J. Krempski, K. R. Kalli, M. D. Behrens, D. M. Vargas, L. C. Hartmann, J. M. T. Janco, H. Dong, K. E. Hedin, A. B. Dietz, E. L. Goode & K. L. Knutson (2016) PD-1 Blunts the Function of Ovarian Tumor–Infiltrating Dendritic Cells by Inactivating NF-κB. 76**,** 239-250.

Kasuya, H., Y. Kodera, A. Nakao, K. Yamamura, T. Gewen, W. Zhiwen, Y. Hotta, S. Yamada, T. Fujii, S. Fukuda, N. Tsurumaru, T. Kuwahara, T. Kikumori, Y. Koide, Y. Fujimoto, T. Nakashima, Y. Hirooka, H. Shiku, M. Tanaka, K. Takesako, T. Kondo, B. Aleksic, H. Kawashima, H. Goto & Y. Nishiyama (2014) Phase I Dose-escalation Clinical Trial of HF10 Oncolytic Herpes Virus in 17 Japanese Patients with Advanced Cancer. *Hepatogastroenterology,* 61**,** 599-605.

Kelderman, S., T. N. M. Schumacher & J. B. A. G. Haanen (2014) Acquired and intrinsic resistance in cancer immunotherapy. *Molecular Oncology,* 8**,** 1132-1139.

Khalil, D. N., E. L. Smith, R. J. Brentjens & J. D. Wolchok (2016) The future of cancer treatment: immunomodulation, CARs and combination immunotherapy. *Nature Reviews Clinical Oncology,* 13**,** 273-290.

Kim, R. 2007. Cancer immunoediting: from immune surveillance to immune escape. In *Cancer Immunotherapy*, 9-27. Elsevier.

Kleinpeter, P., L. Fend, C. Thioudellet, M. Geist, N. Sfrontato, V. Koerper, C. Fahrner, D. Schmitt, M. Gantzer, C. Remy-Ziller, R. Brandely, D. Villeval, K. Rittner, N. Silvestre, P. Erbs, L. Zitvogel, E. Quéméneur, X. Préville & J.-B. Marchand (2016) Vectorization in an oncolytic vaccinia virus of an antibody, a Fab and a scFv against programmed cell death -1 (PD-1) allows their intratumoral delivery and an improved tumor-growth inhibition. *OncoImmunology,* 5**,** e1220467.

Komorowski, M., J. Tisonczyk, A. Kolakowska, R. Drozdz & D. J. V. Kozbor (2018) Modulation of the tumor microenvironment by CXCR4 antagonist-armed viral oncotherapy enhances the antitumor efficacy of dendritic cell vaccines against neuroblastoma in syngeneic mice. 10**,** 455.

Koske, I., A. Rössler, L. Pipperger, M. Petersson, I. Barnstorf, J. Kimpel, C. H. Tripp, P. Stoitzner, Z. Bánki & D. von Laer (2019) Oncolytic virotherapy enhances the efficacy of a cancer vaccine by modulating the tumor microenvironment. *Int J Cancer,* 145**,** 1958-1969.

Krempski, J., L. Karyampudi, M. D. Behrens, C. L. Erskine, L. Hartmann, H. Dong, E. L. Goode, K. R. Kalli & K. L. Knutson (2011) Tumor-Infiltrating Programmed Death Receptor-1⁺ Dendritic Cells Mediate Immune Suppression in Ovarian Cancer. 186**,** 6905-6913.

Kuang, D.-M., Q. Zhao, C. Peng, J. Xu, J.-P. Zhang, C. Wu & L. J. J. o. E. M. Zheng (2009) Activated monocytes in peritumoral stroma of hepatocellular carcinoma foster immune privilege and disease progression through PD-L1. 206**,** 1327-1337.

Kumar, A., M. Commane, T. W. Flickinger, C. M. Horvath & G. R. J. S. Stark (1997) Defective TNF-α-induced apoptosis in STAT1-null cells due to low constitutive levels of caspases. 278**,** 1630-1632.

Lamm, D. L., B. A. Blumenstein, E. D. Crawford, J. E. Montie, P. Scardino, H. B. Grossman, T. H. Stanisic, J. A. Smith Jr, J. Sullivan & M. F. J. N. E. J. o. M. Sarosdy (1991) A randomized trial of intravesical doxorubicin and immunotherapy with bacille Calmette–Guerin for transitional-cell carcinoma of the bladder. 325**,** 1205-1209.

Lang, F. F., C. Conrad, C. Gomez-Manzano, F. Tufaro, W. Yung, R. Sawaya, J. Weinberg, S. Prabhu, G. Fuller, K. Aldape & J. Fueyo (2014) FIRST-IN-HUMAN PHASE I CLINICAL TRIAL OF ONCOLYTIC DELTA-24-RGD (DNX-2401) WITH BIOLOGICAL ENDPOINTS: IMPLICATIONS FOR VIRO- IMMUNOTHERAPY. *Neuro-Oncology,* 16**,** iii39-iii39.

Lang, F. F., C. Conrad, C. Gomez-Manzano, W. K. A. Yung, R. Sawaya, J. S. Weinberg, S. S. Prabhu, G. Rao, G. N. Fuller, K. D. Aldape, J. Gumin, L. M. Vence, I. Wistuba, J. Rodriguez-Canales, P. A. Villalobos, C. M. F. Dirven, S. Tejada, R. D. Valle, M. M. Alonso, B. Ewald, J. J. Peterkin, F. Tufaro & J. Fueyo (2018) Phase I Study of DNX-2401 (Delta-24-RGD) Oncolytic Adenovirus: Replication and Immunotherapeutic Effects in Recurrent Malignant Glioma. *J Clin Oncol,* 36**,** 1419-1427.

Laurie, S. A., J. C. Bell, H. L. Atkins, J. Roach, M. K. Bamat, J. D. O'Neil, M. S. Roberts, W. S. Groene & R. M. Lorence (2006) A phase 1 clinical study of intravenous administration of PV701, an oncolytic virus, using two-step desensitization. *Clin Cancer Res,* 12**,** 2555-62.

Liau, L. M., K. Ashkan, D. D. Tran, J. L. Campian, J. E. Trusheim, C. S. Cobbs, J. A. Heth, M. Salacz, S. Taylor, S. D. D'Andre, F. M. Iwamoto, E. J. Dropcho, Y. A. Moshel, K. A. Walter, C. P. Pillainayagam, R. Aiken, R. Chaudhary, S. A. Goldlust, D. A. Bota, P. Duic, J. Grewal, H. Elinzano, S. A. Toms, K. O. Lillehei, T. Mikkelsen, T. Walbert, S. R. Abram, A. J. Brenner, S. Brem, M. G. Ewend, S. Khagi, J. Portnow, L. J. Kim, W. G. Loudon, R. C. Thompson, D. E. Avigan, K. L. Fink, F. J. Geoffroy, S. Lindhorst, J. Lutzky, A. E. Sloan, G. Schackert, D. Krex, H.-J. Meisel, J. Wu, R. P. Davis, C. Duma, A. B. Etame, D. Mathieu, S. Kesari, D. Piccioni, M. Westphal, D. S. Baskin, P. Z. New, M. Lacroix, S.-A. May, T. J. Pluard, V. Tse, R. M. Green, J. L. Villano, M. Pearlman, K. Petrecca, M. Schulder, L. P. Taylor, A. E. Maida, R. M. Prins, T. F. Cloughesy, P. Mulholland & M. L. Bosch (2018) First results on survival from a large Phase 3 clinical trial of an autologous dendritic cell vaccine in newly diagnosed glioblastoma. *Journal of Translational Medicine,* 16**,** 142.

Lizée, G., W. W. Overwijk, L. Radvanyi, J. Gao, P. Sharma & P. Hwu (2013) Harnessing the Power of the Immune System to Target Cancer. 64**,** 71-90.

Loskog, A., A. Maleka, S. Mangsbo, E. Svensson, C. Lundberg, A. Nilsson, J. Krause, M. Agnarsdóttir, A. Sundin & H. J. B. j. o. c. Ahlström (2016) Immunostimulatory AdCD40L gene therapy combined with low-dose cyclophosphamide in metastatic melanoma patients. 114**,** 872-880.

Malmström, P.-U., A. S. Loskog, C. A. Lindqvist, S. M. Mangsbo, M. Fransson, A. Wanders, T. Gårdmark & T. H. J. C. C. R. Tötterman (2010) AdCD40L immunogene therapy for bladder carcinoma—the first phase I/IIa trial. 16**,** 3279-3287.

Mastelic-Gavillet, B., K. Balint, C. Boudousquie, P. O. Gannon & L. E. Kandalaft (2019) Personalized Dendritic Cell Vaccines-Recent Breakthroughs and Encouraging Clinical Results. *Front Immunol,* 10**,** 766.

Matzinger, P. J. A. r. o. i. (1994) Tolerance, danger, and the extended family. 12**,** 991-1045.

Meier, O. & U. F. Greber (2004) Adenovirus endocytosis. 6**,** S152-S163.

Mohme, M., S. Riethdorf & K. Pantel (2017) Circulating and disseminated tumorcells — mechanisms of immune surveillance and escape. *Nature Reviews Clinical Oncology,* 14**,** 155-167.

Moon, E. K., L.-C. S. Wang, K. Bekdache, R. C. Lynn, A. Lo, S. H. Thorne & S. M. Albelda (2018) Intra-tumoral delivery of CXCL11 via a vaccinia virus, but not by modified T cells, enhances the efficacy of adoptive T cell therapy and vaccines. *OncoImmunology,* 7**,** e1395997.

Motz, G. T. & G. J. I. Coukos (2013) Deciphering and reversing tumor immune suppression. 39**,** 61-73.

Motz, G. T., S. P. Santoro, L.-P. Wang, T. Garrabrant, R. R. Lastra, I. S. Hagemann, P. Lal, M. D. Feldman, F. Benencia & G. J. N. m. Coukos (2014) Tumor endothelium FasL establishes a selective immune barrier promoting tolerance in tumors. 20**,** 607-615.

Mulvihill, S., R. Warren, A. Venook, A. Adler, B. Randlev, C. Heise & D. Kirn (2001) Safety and feasibility of injection with an E1B-55 kDa gene-deleted, replication-selective adenovirus (ONYX-015) into primary carcinomas of the pancreas: a phase I trial. *Gene Ther,* 8**,** 308-15.

Nakao, A., H. Kasuya, T. T. Sahin, N. Nomura, A. Kanzaki, M. Misawa, T. Shirota, S. Yamada, T. Fujii, H. Sugimoto, T. Shikano, S. Nomoto, S. Takeda, Y. Kodera & Y. Nishiyama (2011) A phase I dose-escalation clinical trial of intraoperative direct intratumoral injection of HF10 oncolytic virus in non-resectable patients with advanced pancreatic cancer. *Cancer Gene Ther,* 18**,** 167-75.

Nemerow, G. R., L. Pache, V. Reddy & P. L. Stewart (2009) Insights into adenovirus host cell interactions from structural studies. *Virology,* 384**,** 380-8.

Nestle, F. O., S. Alijagic, M. Gilliet, Y. Sun, S. Grabbe, R. Dummer, G. Burg & D. Schadendorf (1998) Vaccination of melanoma patients with peptide- or tumorlysate-pulsed dendritic cells. *Nature Medicine,* 4**,** 328-332.

Nishio, N., I. Diaconu, H. Liu, V. Cerullo, I. Caruana, V. Hoyos, L. Bouchier-Hayes, B. Savoldo & G. Dotti (2014) Armed Oncolytic Virus Enhances Immune Functions of Chimeric Antigen Receptor–Modified T Cells in Solid Tumors. 74**,** 5195-5205.

Norian, L. A., P. C. Rodriguez, L. A. O'Mara, J. Zabaleta, A. C. Ochoa, M. Cella & P. M. Allen (2009) Tumor-Infiltrating Regulatory Dendritic Cells Inhibit CD8⁺ T Cell Function via l-Arginine Metabolism. 69**,** 3086-3094.

O'Rourke, M. G. E., M. Johnson, C. Lanagan, J. See, J. Yang, J. R. Bell, G. J. Slater, B. M. Kerr, B. Crowe, D. M. Purdie, S. L. Elliott, K. A. O. Ellem & C. W. Schmidt (2003) Durable complete clinical responses in a phase I/II trial using an autologous melanoma cell/dendritic cell vaccine. *Cancer Immunology, Immunotherapy,* 52**,** 387-395.

O'Rourke, M. G. E., M. K. Johnson, C. M. Lanagan, J. L. See, L. E. O'Connor, G. J. Slater, D. Thomas, J. A. Lopez, N. R. Martinez, K. A. O. Ellem & C. W. Schmidt (2007) Dendritic cell immunotherapy for stage IV melanoma. *Melanoma Research,* 17.

O'Sullivan, B. & R. Thomas (2003) Recent advances on the role of CD40 and dendritic cells in immunity and tolerance. *Curr Opin Hematol,* 10**,** 272-8.

Ott, P. A., E. Elez, S. Hiret, D.-W. Kim, A. Morosky, S. Saraf, B. Piperdi & J. M. Mehnert (2017) Pembrolizumab in Patients With Extensive-Stage Small-Cell Lung Cancer: Results From the Phase Ib KEYNOTE-028 Study. 35**,** 3823-3829.

Palmer, D. H., R. S. Midgley, N. Mirza, E. E. Torr, F. Ahmed, J. C. Steele, N. M. Steven, D. J. Kerr, L. S. Young & D. H. J. H. Adams (2009) A phase II study of adoptive immunotherapy using dendritic cells pulsed with tumor lysate in patients with hepatocellular carcinoma. 49**,** 124-132.

Palucka, K. & J. Banchereau (2012) Cancer immunotherapy via dendritic cells. *Nature Reviews Cancer,* 12**,** 265-277.

Patel, D. M., P. M. Foreman, L. B. Nabors, K. O. Riley, G. Y. Gillespie & J. M. Markert (2016) Design of a Phase I Clinical Trial to Evaluate M032, a Genetically Engineered HSV-1 Expressing IL-12, in Patients with Recurrent/Progressive Glioblastoma Multiforme, Anaplastic Astrocytoma, or Gliosarcoma. *Hum Gene Ther Clin Dev,* 27**,** 69-78.

Paulie, S., A. Rosén, B. Ehlin-Henriksson, S. Braesch-Andersen, E. Jakobson, H. Koho & P. Perlmann (1989) The human B lymphocyte and carcinoma antigen, CDw40, is a phosphoprotein involved in growth signal transduction. *J Immunol,* 142**,** 590-5.

Pesonen, S., I. Diaconu, L. Kangasniemi, T. Ranki, A. Kanerva, S. K. Pesonen, U. Gerdemann, A. M. Leen, K. Kairemo & M. J. C. r. Oksanen (2012) Oncolytic immunotherapy of advanced solid tumors with a

CD40L-expressing replicating adenovirus: assessment of safety and immunologic responses in patients. 72**,** 1621-1631.
Pinzon-Charry, A., T. Maxwell & J. A. López (2005) Dendritic cell dysfunction in cancer: A mechanism for immunosuppression. 83**,** 451-461.
Pol, J. G., S. A. Acuna, B. Yadollahi, N. Tang, K. B. Stephenson, M. J. Atherton, D. Hanwell, A. El-Warrak, A. Goldstein & B. J. O. Moloo (2019) Preclinical evaluation of a MAGE-A3 vaccination utilizing the oncolytic Maraba virus currently in first-in-human trials. 8**,** e1512329.
Postow, M. A., M. K. Callahan & J. D. J. J. o. c. o. Wolchok (2015) Immune checkpoint blockade in cancer therapy. 33**,** 1974.
Puzanov, I., M. M. Milhem, D. Minor, O. Hamid, A. Li, L. Chen, M. Chastain, K. S. Gorski, A. Anderson, J. Chou, H. L. Kaufman & R. H. Andtbacka (2016) Talimogene Laherparepvec in Combination With Ipilimumab in Previously Untreated, Unresectable Stage IIIB-IV Melanoma. *J Clin Oncol,* 34**,** 2619-26.
Qiao, J., Z. Liu & Y.-X. Fu (2016) Adapting conventional cancer treatment for immunotherapy. *Journal of Molecular Medicine,* 94**,** 489-495.
Radko, S., R. Jung, O. Olanubi & P. J. P. o. Pelka (2015) Effects of adenovirus type 5 E1A isoforms on viral replication in arrested human cells. 10**,** e0140124.
Rajani, K., C. Parrish, T. Kottke, J. Thompson, S. Zaidi, L. Ilett, K. G. Shim, R.-M. Diaz, H. Pandha & K. J. M. T. Harrington (2016) Combination therapy with reovirus and anti-PD-1 blockade controls tumor growth through innate and adaptive immune responses. 24**,** 166-174.
Ribas, A., R. Dummer, I. Puzanov, A. VanderWalde, R. H. I. Andtbacka, O. Michielin, A. J. Olszanski, J. Malvehy, J. Cebon, E. Fernandez, J. M. Kirkwood, T. F. Gajewski, L. Chen, K. S. Gorski, A. A. Anderson, S. J. Diede, M. E. Lassman, J. Gansert, F. S. Hodi & G. V. Long (2018) Oncolytic Virotherapy Promotes Intratumoral T Cell Infiltration and Improves Anti-PD-1 Immunotherapy. *Cell,* 174**,** 1031-1032.
Ridgway, D. (2003) The First 1000 Dendritic Cell Vaccinees. *Cancer Investigation,* 21**,** 873-886.
Riley, R. S., C. H. June, R. Langer & M. J. Mitchell (2019) Delivery technologies for cancer immunotherapy. *Nature Reviews Drug Discovery,* 18**,** 175-196.
Rojas, J., M. Cascallo, S. Guedan, A. Gros, J. Martinez-Quintanilla, A. Hemminki & R. J. G. t. Alemany (2009) A modified E2F-1 promoter improves the efficacy to toxicity ratio of oncolytic adenoviruses. 16**,** 1441-1451.
Rojas, J. J., P. Sampath, W. Hou & S. H. Thorne (2015) Defining Effective Combinations of Immune Checkpoint Blockade and Oncolytic Virotherapy. 21**,** 5543-5551.
Rojas, L. A., R. Moreno, H. Calderon & R. Alemany (2016a) Adenovirus coxsackie adenovirus receptor-mediated binding to human erythrocytes does not preclude systemic transduction. *Cancer Gene Ther,* 23**,** 411-414.
Rojas, L. A., R. Moreno, H. Calderón & R. Alemany (2016b) Adenovirus coxsackie adenovirus receptor-mediated binding to human erythrocytes does not preclude systemic transduction. *Cancer Gene Therapy,* 23**,** 411.
Rosenberg, S. A., J. C. Yang & N. P. Restifo (2004) Cancer immunotherapy: moving beyond current vaccines. *Nature medicine,* 10**,** 909-915.
Rowe, W. P., R. J. Huebner, L. K. Gilmore, R. H. Parrott & T. G. Ward (1953) Isolation of a cytopathogenic agent from human adenoids undergoing spontaneous degeneration in tissue culture. *Proc Soc Exp Biol Med,* 84**,** 570-3.
Rüter, J., S. J. Antonia, H. A. Burris, R. D. Huhn & R. H. Vonderheide (2010) Immune modulation with weekly dosing of an agonist CD40 antibody in a phase I study of patients with advanced solid tumors. *Cancer Biol Ther,* 10**,** 983-93.
Salmon, H., J. Idoyaga, A. Rahman, M. Leboeuf, R. Remark, S. Jordan, M. Casanova-Acebes, M. Khudoynazarova, J. Agudo, N. Tung, S. Chakarov, C. Rivera, B. Hogstad, M. Bosenberg, D. Hashimoto, S. Gnjatic, N. Bhardwaj, A. K. Palucka, B. D. Brown, J. Brody, F. Ginhoux & M. Merad (2016) Expansion and Activation of CD103(+) Dendritic Cell Progenitors at the Tumor Site Enhances Tumor Responses to Therapeutic PD-L1 and BRAF Inhibition. *Immunity,* 44**,** 924-38.

Santos, J. M., V. Cervera-Carrascon, R. Havunen, S. Zafar, M. Siurala, S. Sorsa, M. Anttila, A. Kanerva & A. Hemminki (2018) Adenovirus Coding for Interleukin-2 and Tumor Necrosis Factor Alpha Replaces Lymphodepleting Chemotherapy in Adoptive T Cell Therapy. *Molecular Therapy,* 26**,** 2243-2254.

Särkioja, M., S. Pesonen, M. Raki, T. Hakkarainen, J. Salo, M. T. Ahonen, A. Kanerva & A. Hemminki (2008) Changing the adenovirus fiber for retaining gene delivery efficacy in the presence of neutralizing antibodies. *Gene Therapy,* 15**,** 921-929.

Saxena, M., S. Balan, V. Roudko & N. Bhardwaj (2018) Towards superior dendritic-cell vaccines for cancer therapy. *Nature Biomedical Engineering,* 2**,** 341-346.

Scarlett, U. K., M. R. Rutkowski, A. M. Rauwerdink, J. Fields, X. Escovar-Fadul, J. Baird, J. R. Cubillos-Ruiz, A. C. Jacobs, J. L. Gonzalez, J. Weaver, S. Fiering & J. R. Conejo-Garcia (2012) Ovarian cancer progression is controlled by phenotypic changes in dendritic cells. *Journal of Experimental Medicine,* 209**,** 495-506.

Schiza, A., J. Wenthe, S. Mangsbo, E. Eriksson, A. Nilsson, T. Tötterman, A. Loskog & G. J. J. o. t. m. Ullenhag (2017) Adenovirus-mediated CD40L gene transfer increases Teffector/Tregulatory cell ratio and upregulates death receptors in metastatic melanoma patients. 15**,** 1-11.

Schoenberger, S. P., R. E. Toes, E. I. van der Voort, R. Offringa & C. J. Melief (1998) T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. *Nature,* 393**,** 480-3.

Senzer, N. N., H. L. Kaufman, T. Amatruda, M. Nemunaitis, T. Reid, G. Daniels, R. Gonzalez, J. Glaspy, E. Whitman, K. Harrington, H. Goldsweig, T. Marshall, C. Love, R. Coffin & J. J. Nemunaitis (2009) Phase II clinical trial of a granulocyte-macrophage colony-stimulating factor-encoding, second-generation oncolytic herpesvirus in patients with unresectable metastatic melanoma. *J Clin Oncol,* 27**,** 5763-71.

Sharma, P. & J. P. J. C. Allison (2015) Immune checkpoint targeting in cancer therapy: toward combination strategies with curative potential. 161**,** 205-214.

Shen, W., M. M. Patnaik, A. Ruiz, S. J. Russell & K.-W. Peng (2016) Immunovirotherapy with vesicular stomatitis virus and PD-L1 blockade enhances therapeutic outcome in murine acute myeloid leukemia. *Blood,* 127**,** 1449-1458.

Shields, J. D., I. C. Kourtis, A. A. Tomei, J. M. Roberts & M. A. J. S. Swartz (2010) Induction of lymphoidlike stroma and immune escape by tumors that express the chemokine CCL21. 328**,** 749-752.

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

Smyth, M. J., D. I. Godfrey & J. A. J. N. i. Trapani (2001) A fresh look at tumor immunosurveillance and immunotherapy. 2**,** 293-299.

Sun, L., P. Funchain, J. M. Song, P. Rayman, C. Tannenbaum, J. Ko, M. McNamara, C. Marcela Diaz-Montero & B. Gastman (2018) Talimogene Laherparepvec combined with anti-PD-1 based immunotherapy for unresectable stage III-IV melanoma: a case series. *Journal for ImmunoTherapy of Cancer,* 6**,** 36.

Sun, Y., D. Peng, J. Lecanda, V. Schmitz, M. Barajas, C. Qian & J. Prieto (2000) In vivo gene transfer of CD40 ligand into colon cancer cells induces local production of cytokines and chemokines, tumor eradication and protective antitumor immunity. *Gene Therapy,* 7**,** 1467-1476.

Tanyi, J. L., S. Bobisse, E. Ophir, S. Tuyaerts, A. Roberti, R. Genolet, P. Baumgartner, B. J. Stevenson, C. Iseli, D. Dangaj, B. Czerniecki, A. Semilietof, J. Racle, A. Michel, I. Xenarios, C. Chiang, D. S. Monos, D. A. Torigian, H. L. Nisenbaum, O. Michielin, C. H. June, B. L. Levine, D. J. Powell, D. Gfeller, R. Mick, U. Dafni, V. Zoete, A. Harari, G. Coukos & L. E. Kandalaft (2018) Personalized cancer vaccine effectively mobilizes antitumor T cell immunity in ovarian cancer. 10**,** eaao5931.

Topalian, S. L., G. J. Weiner & D. M. J. J. o. C. O. Pardoll (2011) Cancer immunotherapy comes of age. 29**,** 4828.

van Kooten, C. & J. Banchereau (1997) Functions of CD40 on B cells, dendritic cells and other cells. *Curr Opin Immunol,* 9**,** 330-7.

Vonderheide, R. H., J. P. Dutcher, J. E. Anderson, S. G. Eckhardt, K. F. Stephans, B. Razvillas, S. Garl, M. D. Butine, V. P. Perry, R. J. Armitage, R. Ghalie, D. A. Caron & J. G. Gribben (2001) Phase I study of recombinant human CD40 ligand in cancer patients. *J Clin Oncol,* 19**,** 3280-7.

Vonderheide, R. H., K. T. Flaherty, M. Khalil, M. S. Stumacher, D. L. Bajor, N. A. Hutnick, P. Sullivan, J. J. Mahany, M. Gallagher & A. J. J. o. C. O. Kramer (2007) Clinical activity and immune modulation in cancer patients treated with CP-870,893, a novel CD40 agonist monoclonal antibody. 25**,** 876-883.

Wang, D. & R. N. J. C. DuBois (2015) Immunosuppression associated with chronic inflammation in the tumor microenvironment. 36**,** 1085-1093.

Webb, E. S., P. Liu, R. Baleeiro, N. R. Lemoine, M. Yuan & Y. J. J. o. b. r. Wang (2018) Immune checkpoint inhibitors in cancer therapy. 32**,** 317.

Wojton, J. & B. Kaur (2010) Impact of tumor microenvironment on oncolytic viral therapy. *Cytokine Growth Factor Rev,* 21**,** 127-34.

Yokoyama, W. M. J. N. i. (2000) Now you see it, now you don't! 1**,** 95-97.

Zafar, S., S. Parviainen, M. Siurala, O. Hemminki, R. Havunen, S. Tähtinen, S. Bramante, L. Vassilev, H. Wang, A. Lieber, S. Hemmi, T. de Gruijl, A. Kanerva & A. Hemminki (2017) Intravenously usable fully serotype 3 oncolytic adenovirus coding for CD40L as an enabler of dendritic cell therapy. *OncoImmunology,* 6**,** e1265717.

Zafar, S., S. Sorsa, M. Siurala, O. Hemminki, R. Havunen, V. Cervera-Carrascon, J. M. Santos, H. Wang, A. Lieber, T. De Gruijl, A. Kanerva & A. Hemminki (2018) CD40L coding oncolytic adenovirus allows long-term survival of humanized mice receiving dendritic cell therapy. *OncoImmunology,* 7**,** e1490856.

Zamarin, D., R. B. Holmgaard, S. K. Subudhi, J. S. Park, M. Mansour, P. Palese, T. Merghoub, J. D. Wolchok & J. P. J. S. t. m. Allison (2014) Localized oncolytic virotherapy overcomes systemic tumor resistance to immune checkpoint blockade immunotherapy. 6**,** 226ra32-226ra32.

Zeh, H. J., S. Downs-Canner, J. A. McCart, Z. S. Guo, U. N. Rao, L. Ramalingam, S. H. Thorne, H. L. Jones, P. Kalinski, E. Wieckowski, M. E. O'Malley, M. Daneshmand, K. Hu, J. C. Bell, T. H. Hwang, A. Moon, C. J. Breitbach, D. H. Kirn & D. L. Bartlett (2015) First-in-man study of western reserve strain oncolytic vaccinia virus: safety, systemic spread, and antitumor activity. *Mol Ther,* 23**,** 202-14.

Zitvogel, L., J. I. Mayordomo, T. Tjandrawan, A. B. DeLeo, M. R. Clarke, M. T. Lotze & W. J. Storkus (1996) Therapy of murine tumors with tumor peptide-pulsed dendritic cells: dependence on T cells, B7 costimulation, and T helper cell 1-associated cytokines. *J Exp Med,* 183**,** 87-97.