PeptiENV – PERSONALIZED ONCOLYTIC CANCER VACCINE PLATFORM

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Abstract

Cancer immunotherapies aim to target the immune defence mechanisms of the body specifically and efficiently against the tumour tissue. Cancer vaccines and oncolytic viruses are forms of active immunotherapies, which require patients having a properly functioning immune system. The vaccines are based on the administration of tumour antigens into the body to which the immune system reacts. However, often the response is not robust enough. The oncolytic viruses in turn kill the cancer cells which causes the release of antigens from the tumour tissue. Viruses usually elicit a strong immune response but sometimes it is targeted too much against the virus instead of the tumour.

Oncolytic vaccine is a composition of an oncolytic virus and a cancer vaccine. Tumour antigens can be coded to the genome of the virus therefore, when the virus invades tumour cells they start to produce the antigens. Eventually the cancer cells are also destroyed due to viral replication. The antigens can be tumour-associated that is, they are expressed in healthy tissues too. Their usage is not always efficient which is why an interest towards utilizing tumour-specific antigens has been increased. Considering the expression of antigens, tumour tissue is very heterogenous and distinctive between patients. Hence, utilizing mutated patient unique neoantigens would enable the development of personalized tumour-specific oncolytic vaccines. Genetic modification of viruses is complicated thus, an easier way to insert the neoantigens to the virus has been invented. The developed oncolytic vaccine platform is called PeptiENV, and it is designed to use with enveloped viruses. The idea is to fuse tumour-specific antigens onto the envelope of the virus and eliminate the need of gene insertion.

The aim of this study is to investigate *in vivo* the efficacy of PeptiENV in preventing tumour growth and eliciting a tumour-specific immune response. An object is also to observe survival times of the treated animals. Furthermore, the preservation of infectivity is studied *in vitro*. The research was executed with two potential oncolytic viruses, vaccinia virus (VACV) and herpes simplex virus type 1 (HSV-1). The PeptiENV complex was formed by using an artificial tumour antigen, ovalbumin epitope SIINFEKL, which was attached to the viral envelope with cell penetrating peptide (CPP) or cholesterol anchor. The preservation of infectivity was examined by measuring cell viability of PeptiENV infected cells. Animal experiments instead were performed with a mouse melanoma model created with B16-OVA cells, which express ovalbumin and therefore the antigen epitope SIINFEKL. PeptiENV was compared to control treatments which were virus, SIINFEKL peptide and complexation medium only. Treatments were administered as intratumoural injections. Tumour growth was followed by measuring the size of implanted tumours every other day. With flow cytometry, tumour-specific immune response was assessed by acquiring the relative amount of SIINFEKL-specific CD8+ T cells in the tumour tissue. Euthanizing dates were registered in order to observe the survival of the mice.

According to the *in vitro* results, conjugation of peptides to the virus does not affect infectivity. In addition, the *in vivo* studies show that PeptiENV VACV CPP prevents tumour growth the most. Difference in tumour growth between PeptiENV VACV CPP and control treatments is significant. Mice injected with the same treatment also lived considerably longer than mice injected with virus, peptide or medium only. Also, PeptiENV HSV-1 hinders tumour growth distinctly more than virus only and slightly more than SIINFEKL only, but unfortunately it did not have an evident impact on the survival time. In both experiments, the PeptiENV treatment elicits the largest proportional amount of SIINFEKL-specific CD8+ T cells. In other words, PeptiENV engenders a tumour-specific immune response. In the PeptiENV VACV study the difference to control treatments is clearer than in the PeptiENV HSV-1 study.

At present, the PeptiENV platforms performs better with VACV than HSV-1. With further investigations however, the results can be verified and improved. All in all, the results are encouraging. The PeptiENV platform shows great promise for being a part of personalized cancer immunotherapy developments in the future.

Keywords

cancer, immunotherapy, oncolytic virus, cancer vaccine, oncolytic vaccine, personalized immunotherapy

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Tiivistelmä

Syövän immunoterapian tavoitteena on kohdistaa kehon immuunipuolustusreaktio tehokkaasti ja tarkasti syöpäsoluja vastaan. Syöpärokotteet ja onkolyyttiset virukset ovat niin kutsuttuja aktiivisia immunoterapioita. Jotta potilas hyötyisi näistä hoidoista, immuunijärjestelmän täytyy toimia riittävästi. Syöpärokotteet perustuvat immuunijärjestelmän stimuloimiseen annostelemalla tuumoriantigeenejä potilaaseen. Hoidon aiheuttama immuunivaste ei kuitenkaan ole usein tarpeeksi voimakas. Onkolyyttiset virukset taas tuhoavat syöpäsoluja, minkä seurauksena tuumorikudoksesta vapautuu antigeenejä. Virukset saavat aikaan vahvan puolustusreaktion, mutta usein vaste muodostuu liikaa virusta vastaan syövän sijaan. Onkolyyttinen rokote on onkolyyttisen viruksen ja syöpärokotteen yhdistelmä. Tuumoriantigeenejä voidaan asentaa viruksen genomiin, jolloin viruksen infektoidessa syöpäsolun se alkaa tuottamaan kyseistä antigeeniä. Lopulta syöpäsolut myös tuhoutuvat johtuen viruksen replikoitumisesta. Koodatut antigeenit voivat olla tuumorispesifisiä tarkoittaen, että ne ilmentyvät ainoastaan syöpäsolujen pinnalla. On myös tuumoriantigeenejä, jotka voivat esiintyä samanaikaisesti sekä terveessä että pahanlaatuisessa kudoksessa. Tällaisten antigeenien käyttö ei ole aina tehokasta, minkä vuoksi kiinnostus tuumorispesifisiä antigeenejä kohtaan on lisääntynyt. Tuumorikudos on erittäin heterogeeninen, mikä johtuu syöpäsolujen jatkuvista mutaatioista. Tämän seurauksena syöpäsolujen pinnalla ilmennetään laajasti erilaisia antigeenejä, joiden vaihtelu potilaiden välillä on myös suurta. Mutatoituneiden potilasspesifisten neoantigeenien käyttö mahdollistaisi personoitujen tuumorispesifisten onkolyyttisten rokotteiden kehittämisen. Virusten geneettinen manipulaatio on kuitenkin monimutkaista, minkä vuoksi luotiin käytännöllisempi onkolyyttinen syöpärokotealusta PeptiENV, joka on suunniteltu vaipallisille viruksille. Ideana on kiinnittää tuumoriantigeenit suoraan viruksen vaippaan, jolloin geenien muokkaus ei ole tarpeellista.

Tämän tutkimuksen tavoitteena on selvittää *in vivo* PeptiENV alustan tehokkuus tuumorikudoksen kasvun ehkäisyssä sekä tuumorispesifisen immuunivasteen tuottamisessa. Tarkoituksena on myös havainnoida PeptiENV alustan vaikutusta eläinten elossaoloaikaan. Lisäksi alustan infektiivisyyden säilyminen analysoidaan *in vitro.* Tutkimus toteutettiin käyttämällä kahta onkolyyttistä virusta, vaccinia virusta (VACV) sekä herpes simplex virus tyyppiä 1 (HSV-1). PeptiENV kompleksi muodostettiin kiinnittämällä ovalbumiinista peräisin oleva keinotekoinen antigeeniepitooppi SIINFEKL virusten vaippaan. Epitoopin liittämiseen käytettiin solukalvon läpäisevää peptidiä (cell penetrating peptide, CPP) tai kolesterolia. Infektiivisyyden säilyminen tutkittiin määrittämällä PeptiENV alustalla infektoitujen solujen kuolleisuus. Eläinkokeet taas suoritettiin hiirillä, joihin implantoitiin B16-OVA melanoomasoluja, jotka ilmentävät ovalbumiinia ja siten SIINFEKL-epitooppia. PeptiENV hoitoa verrattiin kontrolleihin, jotka olivat onkolyyttinen virus, peptidit ja kompleksaatioon käytetty liuos. Hoidot injektoitiin intratumoraalisesti. Tuumoreiden kasvua seurattiin mittaamalla tuumorien koko joka toinen päivä. Sen sijaan virtaussytometriaa käytettiin selvittämään SIINFEKL-spesifisten CD8+ T-solujen suhteellinen osuus kaikista T soluista tuumoreissa. Hiirten eutanasiapäivämäärät tallennettiin elossaoloaikojen seurantaa varten.

In vitro kokeen tulosten mukaan PeptiENV kompleksaatio ei vaikuta viruksen infektointikykyyn. *In vivo* tutkimukset puolestaan osoittavat, että PeptiENV VACV CPP estää tuumorien kasvua tehokkaimmin. Ero kasvussa PeptiENV VACV CPP:n ja kontrollihoitojen välillä on merkittävä. Samaa hoitoa saaneet hiiret myös elivät huomattavasti pidempään. Myös PeptiENV HSV-1 hidastaa tuumorien kasvua selvästi enemmän kuin HSV-1 ja hieman enemmän kuin SIINFEKL-peptidi. Valitettavasti PeptiENV HSV-1 ei kuitenkaan kasvattanut hiirten elossaoloaikaa merkittävästi. Molemmissa tutkimuksissa PeptiENV alustalla hoidetuissa tuumoreissa on suurin suhteellinen määrä SIINFEKL-spesifisiä CD8+ T-soluja. Toisin sanoen PeptiENV saa aikaan tuumorispesifisen immuunivasteen. PeptiENV VACV kokeessa ero kontrollihoitoihin on selkeämpi kuin PeptiENV HSV-1 kokeessa.

Tällä hetkellä PeptiENV VACV näyttää toimivan paremmin kuin PeptiENV HSV-1. Jatkotutkimukset ovat joka tapauksessa tarpeellisia, sillä niiden avulla tuloksia pystytään vahvistamaan sekä parantamaan. Kaiken kaikkiaan tulokset ovat kuitenkin rohkaisevia. On mahdollista, että PeptiENV syöpärokotealusta on potentiaalinen osa tulevaisuuden yksilöllisten syöpäimmunoterapioiden kehitystä.

Avainsanat

syöpä, immunoterapia, onkolyyttinen virus, syöpärokote, onkolyyttinen rokote, personoitu immunoterapia

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ABBREVIATIONS

ANOVA	Analysis of variance
APC	Antigen presenting cell
CAR	Chimeric antigen receptor
CD	Cluster of differentiation
CD40L	Cluster of differentiation 40 ligand
chol	Cholesterol
CPP	Cell penetrating peptide
CTLA-4	Cytotoxic T lymphocyte-associated protein 4
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DRR	Durable response rate
FBS	Fetal bovine serum
FDA	U.S. Food and Drug Administration
GM-CSF	Granulocyte-macrophage colony stimulating factor
HSV-1	Herpes simplex virus type 1
ICI	Immune checkpoint inhibitor
IFN	Interferon
IL	Interleukin
irAE	Immune-related adverse event
JX-594	Pexastimogene devacirepvec
mAb	Monoclonal antibody
MAGE-A3	Melanoma-associated antigen 3
MHC	Major histocompatibility complex
MOI	Multiplicity of infection
NK	Natural killer
OVA	Ovalbumin
PBS	Phosphate buffered saline
PD-1	Programmed cell death protein 1

PD-L1	Programmed cell death ligand 1
PeptiCRAd	Peptide-coated oncolytic adenovirus
Pexa-Vec	Pexastimogene devacirepvec
RECIST	Response evaluation criteria in solid tumours
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute medium
TAA	Tumour-associated antigen
TCR	T cell receptor
TSA	Tumour-specific antigen
T-VEC	Talimogene laherparepvec
VACV	Vaccinia virus
VSV	Vesicular stomatitis virus

I LITERATURE REVIEW

1 INTRODUCTION

Cancer is a general term for various diseases that involve abnormal growth of cells which are able to spread into surrounding tissues in the body and generate metastasis (National Cancer Institute 2015). It occurs worldwide and has become one of the leading causes of death (WHO 2017). In 2015, cancer resulted in 8.8 million fatalities globally. Cancer develops when the normal circle of cell renewal is disturbed (Hanahan and Weinberg 2000). Normally, old or damaged cells go through apoptosis and are replaced with new ones, but in cancer the normal cells have gone through genetic changes which causes their harmful proliferation. Often the growing cells form a solid tissue mass that is called a tumour (National Cancer Institute 2015). If the tumour has the ability to spread into other organs, it is cancerous or malignant.

Surgery is a cancer treatment which is frequently used for removing malignant tumours mechanically (National Cancer Institute 2017). It is not always applicable since many times the cell mass is located so that it is impossible to reach, or the cancer has formed several metastases. Other well-known cancer treatments are radiation therapy and chemotherapy. Radiation therapy utilizes ionizing radiation to destroy the DNA in cancer cells (Baskar et al. 2012). Damaged DNA prevents the cells from growing and promotes cell death. The treatment can be used locally or systemically depending on the cancer type (National Cancer Institute 2017). In chemotherapy different drugs are used for killing the cancer cells and hindering tumour growth. It is given systemically and usually applied for cancers that grow rapidly.

Although surgery, radiation therapy and chemotherapy are very common treatments of cancer, they have challenges. In many cases, it is impossible to extirpate the tissue completely with surgery, and sometimes healthy tissue has to be removed also (National Cancer Institute 2017). Radiation and chemotherapy always affect healthy cells too which can cause damage in normal tissue and hence various side effects.

To the side of conventional cancer treatments, other remedies have been developed. One of these is immunotherapy which aims to target the immune defence mechanism of the body towards cancer (Papaioannou et al. 2016). The fact that immune system can recognize and fight tumour growth, has been discovered already in the 19th century, but due to limited clinical efficiency, eagerness for immunotherapy was modest (Yang 2015). For the last few decades, increasing knowledge of anti-cancer immune responses has led to extensive research, and during recent years great progress has been achieved in the development of cancer immunotherapies (Yang 2015; Voena and Chiarle 2016). Several types are available nowadays, for example cancer vaccines and oncolytic viruses (Chiocca and Rabkin 2014; Butterfield 2015).

The main goal and challenge in cancer immunotherapy is to induce a strong enough immune response that is targeting only the cancer tissue (Papaioannou et al. 2016). Guiding the immune system is complicated as such, but tumour tissue also has the ability to evade the immune defence mechanisms which creates an additional difficulty (Voena and Chiarle 2016). Via different mode of actions, the therapies attempt to achieve the best efficacy, tumour-specificity and overcome the immunosuppressive tumour microenvironment. Like the traditional cancer treatments, also various immunotherapies can be used together (Butterfield 2015). By availing treatments that fulfil each other, it is possible to obtain their best advantages.

The objective of this literature review is to present the main points and limitations of cancer immunotherapies and clarify what kind of treatments are currently available. First, the interactions between cancer development and the immune system are illustrated, and later different treatments are scrutinized. The focus will be on cancer vaccines, oncolytic viruses and their combinations. In the end, a novel oncolytic cancer vaccine platform called PeptiENV is presented after which the thesis continues with experimental part.

2 IMMUNOLOGY AND CANCER

2.1 The immune system

The immune system is divided into two parts which are innate and adaptive immunity (Netea et al. 2011; Murphy 2012). People are born with the innate immunity, and it includes mechanic defences such as the skin and mucous, the complement cascade, and also certain white-blood-cells or leucocytes. Macrophages, dendritic cells, neutrophils and natural killer (NK) cells are very important leucocytes in the innate immune response (Murphy 2012). Macrophages and neutrophils participate in inflammation reaction and inhibit, for instance, entrance of bacteria into the body. Dendritic cells, as well as macrophages, are also essential for the adaptive immunity because they present antigens to lymphocytes which is critical for their activity. NK cells kill tumour cells or virus-infected cells without additional activation. They identify invaded host cells by recognizing their altered surface proteins. The innate immunity is also called non-specific immune system since the response is generic and does not change even if the same antigen comes across. Although, the reaction is fast and the first defence mechanism that pathogens have to encounter (Netea et al. 2011).

The response of the adaptive immune system on the contrary is specific and can be either fast or slow, depending on whether the antigen has been fought against before (Netea et al. 2011; Murphy 2012). That is to say, the system can form a memory towards antigens which enables more efficient and faster immune response if the same antigens are confronted again. The adaptive immune system composes of leucocytes called lymphocytes, T cells and B cells. T cells transmit cell-mediated immune response while B cells are in charge of humoral or antibody-mediated immunity (Murphy 2012).

2.1.1 Cell-mediated immune response

When a naïve T cell encounters with an antigen for the first time it develops into an effector T cell (Yatim and Lakkis 2015). Several types of effector T cells exist but if coarsely categorized, they can be divided into four groups which are helper, cytotoxic

(killer), memory and regulatory T cells (Murphy 2012). Helper T cells are crucial for the whole adaptive immunity since their activation is needed for the function of cytotoxic T cells and B cells. Cytotoxic T cells are important for eliminating intracellular pathogens, especially viruses. Regulatory T cells, on the other hand, suppress the activity of other lymphocytes and assist in controlling the immune response (Yatim and Lakkis 2015). Memory T cells, in turn, maintain an immunity that has evolved after a disease or vaccination. Helper and cytotoxic T cells express cluster of differentiation (CD) 4 and 8 glycoproteins on their surfaces, and therefore they are often referred to as effector CD4+ and CD8+ T cells, respectively (Murphy 2012).

In order that naïve CD4+ and CD8+ T cells are able to become mature, antigens have to be presented to them by other cells (Zhu and Chen 2009). The presentation happens with major histocompatibility complex (MHC) proteins on antigen presenting cells (APC), which are dendritic cells, macrophages and B cells (Zhu and Chen 2009; Murphy 2012; Yatim and Lakkis 2015). First, the antigen is taken into an APC and processed (Murphy 2012). Then a small part of it, an antigen epitope, is complexed with an MHC protein and transported to the cell surface. There are two classes of MHC molecules: MHC class I molecules are found in most cells, whereas MHC class II proteins exist only in APCs. CD4+ T cells have T cell receptors (TCRs) that only recognize antigen epitopes that are presented with MHC class II proteins, while the TCRs on CD8+ T cells react to epitopes complexed with MHC class I molecules.

The antigen presentation is not the only requirement for T cell activation, but additional stimulating signals are needed as well. (Zhu and Chen 2009; Murphy 2012). Along with TCRs, CD4+ and CD8+ T cells have CD28 and cytotoxic T lymphocyte antigen-4 (CTLA-4) receptors on their cell surfaces, which bind to B7 proteins on APCs (Figure 1). The CD28-B7 pairing acts as a costimulating effect and is necessary for the activation of both CD4+ and CD8+ T cells (Zhu and Chen 2009). Instead, the interaction of CTLA-4 and B7 operates as an immune checkpoint which inhibits the activating signals of above mentioned MHC I/II-TCR and CD28-B7 pairs and thus downregulates the immune response (Murphy 2012). CD4+ T cells are activated more easily than CD8+ T cells, which often demand further stimulation. The maturated effector CD4+ T cells provide

the stimulus by secreting cytokines that accelerate the proliferation and differentiation of the CD8+ T cells. Consequently, they gain their full capacity to kill, for example, cancerous and virus-infected cells.

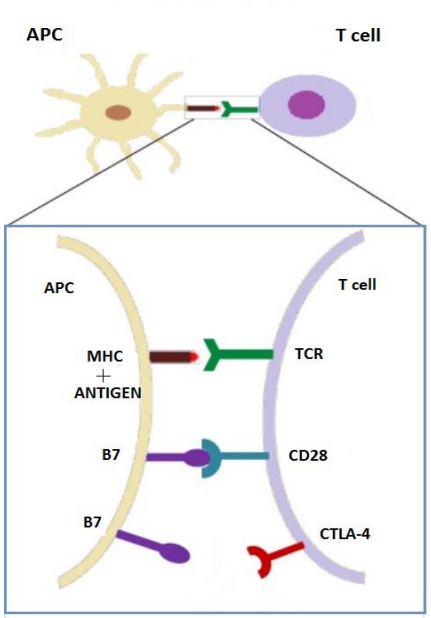

Figure 1. T cell activation. Antigen is presented to T cells on APCs as a complex with MHC proteins class I and II. T cells recognize the complex with specific TCRs and become mature. The binding of CD28 receptor and B7 protein offers a costimulating signal that ensures the complete activation of the T cells. The interaction between CTLA-4 and B7 will impede the activating signals and limit the T cell response. APC = antigen presenting cell; TCR = T cell receptor (Murphy 2012; picture adapted from Papaioannou et al. 2016)

2.1.2 Antibody-mediated immune response

In contrast to T cells, B cells are capable of recognizing antigens directly with their surface receptors (Murphy 2012). After the receptors have recognized and bound to an antigen, the antigen-receptor complex is taken into the cell by endocytosis after which the antigen epitope is presented on the cell surface with MHC class II protein. B cells also need additional stimulation from the CD4+ T cells in order to become completely functional. CD4+ T cells express CD40 ligand (CD40L), which interacts with CD40 receptors on APCs. Binding of the CD40L to CD40 activates the B cells further. Thus, following the epitope presentation, the CD4+ T cells recognize the complex and by subsequent cytokine secretion and binding of the CD40 and CD40L, the B cells transform

into plasma cells that produce antibodies that resemble the surface receptors. Part of the antigen-stimulated B cells develop into memory cells.

2.2 Cancer pathogenesis

The proliferation of cells and programmed cell death is highly regulated by various genes. They affect multiple cell signalling pathways, and when performing correctly they maintain homeostasis of cells and tissue integrity (Hanahan and Weinberg 2000). Alterations in these genes endanger to increased risk of developing cancer. Normal genes that are in charge of cell division are called proto-oncogenes (Torry and Cooper 1991). If mutated, they have potential to become more dangerous oncogenes, which lead to the growth of malignant tumours if activated. The genome of cells also contains tumour suppressor genes and "healing" genes that hinder cell division and repair DNA damages, respectively (Hanahan and Weinberg 2000). Defects in these functions may cause cancer as well.

In order that normal tissue cells can evolve into malignant cancer cells, they have to acquire capabilities that enable the development (Hanahan and Weinberg 2011). The acquisition happens when appropriate oncogenes becomes active and disturb the regulatory circuits within cells. For example, many tumour cells have the ability to produce their own growth signals and hence, stimulate their proliferation independently. Also, tumours are insensitive to anti-growth signals and they are able to evade apoptosis. Capacity to replicate countlessly, produce new blood vessels around the tissue, that is angiogenesis, and generate metastasis are included to the properties of cancer cells as well.

One component that enables cancer cells to obtain above said capabilities is that the genome of tumour cells is highly instable (Hanahan and Weinberg 2011). The instability increases with every cell division, which spawn to the emergence of spontaneous mutations. The advantageous qualities obtained from the mutated genes are then inherited to the next subclone of cells which promotes their outgrowth further. The rate of spontaneous mutations also tends to elevate during tumour progression, which leads to

that more tumour growth supporting properties occur. The hyperproliferation and faster appearing mutations causes that tumours become genetically heterogeneous. That is to say, the physiology and morphology of cancer cells can be remarkably variant, and different subpopulations can even exist within the same tumour (Hanahan and Weinberg 2000; Hanahan and Weinberg 2011). Furthermore, the expression of cell surface proteins varies greatly (Vinay et al. 2015).

Another significant factor in tumour development and obtaining earlier mentioned abilities is inflammation (Hanahan and Weinberg 2011). In general, inflammation is an acute protective local reaction that helps to restore normal cell function in an infected or damaged area (Zamarron and Chen 2011). However, many carcinogens such as tobacco smoke and UV-radiation cause chronic inflammation which has been linked with cancer progression (Aggarwal et al. 2009). Carcinogens can trigger certain inflammatory cell signalling pathways, which affect transcription and proliferation of cells. Various tumour-associated genes are regulated by these pathways and they have been proved to be active in many types of tumours. Inflammation reaction is also essential in the later stages of tumour development since cancer cells can create inflammatory environment by themselves (Mantovani et al. 2008). For instance, they produce mediators that allure inflammatory leucocytes. Overall, the immune cells have several roles in carcinogenesis, and they are discussed in the following chapter (Zamarron and Chen 2011).

2.3 Cancer and immune response

As told, it demands many steps that normal cells can become cancerous, and most of the time the immune system notices abnormal cells and destroys them (Dunn et al. 2004b). Though, tumours possess qualities that make them able to evade the immune defence mechanisms (Vinay et al. 2015). Consequently, even correctly functioning immune system is not always able to prevent tumours to develop.

The overall phenomenon, which includes the success and failure of the immune system protecting from cancer development is called cancer immunoediting (Dunn et al. 2004a). It is divided into three parts that are elimination (also called immunosurveillance),

equilibrium and escape. The elimination part is the one that usually averts tumour progression entirely. If the elimination does not work properly the tumour enters the equilibrium phase where the tumour is maintained or modified. Variant cancer cells may obtain properties that allow the evasion of immune defence which leads to the escape part in which the tumour progresses into a clinically significant cancer.

The elimination part starts when emerging tumour cells disrupt the local tissue and cause the production of pro-inflammatory molecules and chemokines (Dunn et al. 2004b). Leucocytes from the innate immune system such as macrophages, NK cells and dendritic cells becomes alerted by these signals and arrive to the site of the tumour. By recognizing certain tumour-specific ligands and complexes, the leucocytes are activated and begin to secrete a cytokine called interferon-ɣ (IFN-ɣ) which amplifies the effects of the innate immunity and triggers processes that results in partial killing of the tumour cells. The destruction of the cancer cells liberates tumour antigens, which in turn activates the adaptive immunity. Dendritic cells present the antigens to naïve CD4+ T cells in lymph nodes causing their maturation which is followed by the activation of CD8+ T cells.

CD8+ T cells and CD4+ T cells are major anti-tumour lymphocytes, and by recognizing the tumour antigens, they are able to find the cancer cells, and eventually inhibit their growth or destroy them (Dunn et al. 2004b; Zamarron and Chen 2011). The suppression of tumours happens via different cytokines of which IFN-ɣ is the most significant and produced by both effector CD8+ and CD4+ T cells (Zamarron and Chen 2011). Additionally, effector CD8+ T cells secrete cancer cell killing cytotoxins such as granzymes and perforin (Mittal et al. 2014). As mentioned earlier, cancer cells can be genetically very heterogenous which is why new tumour antigens occur constantly and therefore, the immune defence process has to be repeated multiple times so that all cancer cells can be eradicated (Dunn et al. 2004b; Hanahan and Weinberg 2011).

If the elimination part fails and cancer cells are not killed completely, the tumour shifts into equilibrium state (Dunn et al. 2004b). In this state, the immune system constantly destroys cancer cells while new ones arise. The problem is that the new cells have

differently mutated genome and increased ability to resist the immune attack. Also, the tumour may become less immunogenic.

There are many complex mechanisms behind the immune evasion. One significant part is that the tumour microenvironment is immunosuppressive (Vinay et al. 2015). By secreting cytokines, tumour cells are able to draw immunosuppressive lymphocytes into the microenvironment, for instance certain regulatory T cells. Normally, regulatory T cells are critical for the human physiology because they obviate other T cells reacting to self-antigens and thus creating autoimmunity (Zamarron and Chen 2011). However, considering tumour progression their activity is not wanted. Inflammation induced by the tumour also causes the presence of other suppressive immune cells (Vinay et al. 2015). Myeloid derived suppressor cells and modulated dendritic cells can decrease the immunity of T cells, whereas tumour associated macrophages produce tumour growth promoting cytokines such as interleukin 10 (IL-10) and transforming growth factor beta (Vinay et al. 2015; Yang 2015). Cancer cells and also non-cancerous cells in the microenvironment are able to secrete immune suppressive mediators too (Vinay et al. 2015).

Another way that tumours aid the evasion of immune defence is downregulating of antigen presenting pathways such as MHC class I (Vinay et al. 2015). Consequently, antigens are not presented properly on the cancer cell surface, and CD8+ T cells cannot recognize them. Additionally, most tumour cells are not able to express costimulatory molecules which are crucial for enabling the functions of effector CD4+ and CD8+ T cells. Instead, they can express protein ligands for the immune checkpoints that inhibit the activation of T cells. One of these proteins is programmed cell death ligand 1 (PD-L1) which is a member of the B7 protein family (Becker et al. 2013). By binding to the PD-1 receptor of T cells, some key pathways in the T cell activation process are disrupted, and hence their proliferation, survival and cytokine production are impeded.

The equilibrium phase can last even for years before any detectable cancer is developed (Dunn et al. 2004b). The tumour enters escape phase, in other words, begins to grow when

the genetic changes confer enough resistance against the immune defence system, and the evasion mechanisms overcome the elimination processes.

3 IMMUNOTHERAPY OF CANCER

Cancer immunotherapy includes different treatments that utilizes the immune defence mechanisms to battle cancer (Papaioannou et al. 2016). Significant leaps in the research have been made during the decade after 2010, and currently various immunotherapies exist in the market (Papaioannou et al. 2016; Voena and Chiarle 2016). The treatments are divided into passive and active immunotherapies (Papaioannou et al. 2016). Passive treatments are suitable for cancer patients who have insufficient immune responses against the disease. Different immune cells or molecules are stimulated *ex vivo* after which they are injected to the patient. Active treatments, conversely, are based on initiating the immune defence mechanisms inside the body which then requires that patient's immune system works adequately. These therapies consist of various cancer vaccines, oncolytic viruses and immune checkpoint inhibitors, and they are discussed more detailed in the following chapters.

Monoclonal antibodies (mAbs) that are targeted to specific tumour antigens and used to recruit immune cells to kill cancer cells is a form of passive immunotherapy (Papaioannou et al. 2016). Several kinds of these mAbs are available such as rituximab, which is used for treating Non-Hodgkin's lymphoma. Adoptive cell transfer is also a passive immunotherapy in which patient's own T cells are exploited (Voena and Chiarle 2016). Tumour infiltrated lymphocytes are collected from the tumour of which specific anti-tumour T cells are expanded and re-administered unmodified back into the patient. Peripheral T cells can also be used. Before reinfusion, they are genetically armed either with TCRs or chimeric antigen receptors (CARs) that enable the T cells to recognize the tumour antigens specifically. The CAR T cell therapy has been more promising, and in 2017, the first two CAR T cell therapies were approved by the U.S. Food and Drug Administration (FDA) (FDA 2017a; FDA 2017b). One is for treating children with acute lymphoblastic leukaemia, and the other is for adults with advanced B cell lymphomas.

Third form of passive immunotherapy is the use of cytokines, for instance, IL-2 and IFN-α, which are both approved by the FDA (FDA 2012; Papaioannou et al. 2016).

3.1 Immune checkpoint inhibitors

In order that effector T cells gain their full function, the costimulating bindings discussed in chapter 2.1.1 need to happen (Murphy 2012). As said, the T cells also express CTLA-4 receptors, which interact with the B7 molecules too, and thus compete with the activating interaction of CD28 and B7 (Ito et al. 2015). The binding of CTLA-4 to B7 averts naïve T cells from activating and operates as a "brake" for the immune response (Ito et al. 2015; Papaioannou et al. 2016). Another inhibitory surface protein on T cells is PD-1 (Ito et al. 2015). It binds to PD-L1, which is expressed, inter alia, on tumour cells and also belongs to B7 protein ligands. The PD-1-PD-L1 complex works in the later stages of T cell activation and causes their exhaustion. The purpose of these hindering signals is to prevent autoimmunity and excessive tissue damage during infections, but they also induce immunosuppressive tumour microenvironment (Ito et al. 2015; Voena and Chiarle 2016). The anti-tumour T cell response can be boosted by blocking the inhibitory signalling with specifically targeted mAbs that are called immune checkpoint inhibitors (ICIs) (Papaioannou et al. 2016).

The first ICI, ipilimumab, which binds directly to the CTLA-4 receptor and blocks the CTLA-4/B7 interaction, was approved by the FDA in 2011 for treating metastatic melanoma (FDA 2011; Ito et al. 2015). The approval was received after two prominent phase III studies (Hodi et al. 2010; Robert et al. 2011; Ito et al. 2015). Hodi (2010) and Robert (2011) with their research groups showed that ipilimumab, compared to melanoma vaccine and dacarbazine (chemotherapy medication), respectively, significantly prolonged the overall survival of patients with advanced melanoma. Another CTLA-4 blocker in development is tremelimumab (Comin-Anduix et al. 2016). It has been in several clinical trials for treating cancer, but it has not yet been approved by the FDA.

Two other ICIs for treating advanced melanoma, pembrolizumab and nivolumab, were approved by the FDA in 2014 (FDA 2014a; FDA 2014b; Papaioannou et al. 2016). Both

of them target the PD-1 receptor on T cells, but pembrolizumab with higher affinity (Papaioannou et al. 2016). By 2016, they were approved for several other indications too.

Despite the successful outcomes, the inhibitors have limitations too. For instance, they cause immune-related adverse effects (irAEs) (Koster et al. 2015). Mild and moderate irAEs with ipilimumab occur in 60 % of patients, and the incidence for severe toxicities is 10–15 % (Hodi et al. 2010). Anti-PD-1 treatment also produces irAEs but not as much as blocking the CTLA-4 (Koster et al. 2015). Another major issue is that many patients do not or poorly respond to the treatment (Voena and Chiarle 2016; Ribas et al. 2017). Topalian et al. (2012) reported an objective response to anti-PD-1 therapy in 27 % of patients with melanoma, and only 19 % had a durable response longer than six months. As told, the ICI treatment is a form of active immunotherapy, and to function properly a T cell response against the tumour has to exist even at some extent (Obeid et al. 2015; Papaioannou et al. 2016). Additionally, high immunogenicity of the tumour is noted to be advantageous, for example, by Snyder et al. (2014) and Rizvi et al. (2015) who detected that patients with highly mutated cancers responded to ICIs better. According to the studies, this could be derived from the emergence of new mutations, and thus, new tumour antigens to which the immune system reacts.

To enhance the responses, CTLA-4 and PD-1 blockers have been also studied as a combination therapy, since they have different affecting mechanisms (Voena and Chiarle 2016). CTLA-4 inhibitors increase tumour infiltrating lymphocytes, while PD-1 blockers help overcome immune evasion of the tumour (Sharma and Allison 2015). That is why the treatments can complement each other. In addition to inadequate responses, another challenge has been the determination of biomarkers that would predict whether a patient will benefit from ICIs or not (Voena and Chiarle 2016). Nonetheless, the discovery of the inhibitory signalling pathways and development of the checkpoint blockades have been one of the most remarkable advances in cancer immunotherapy (Ito et al. 2015; Voena and Chiarle 2016).

3.2 Cancer vaccines

Cancer vaccines as treatments are also considered to be active immunotherapy (Papaioannou et al. 2016). The basic idea is to provoke anti-tumour immunity, especially specific cytotoxic CD8+ T cell response, by administrating tumour antigens into the body (Butterfield 2015). The antigens can be divided roughly into two classes: tumour-associated (TAAs) and tumour-specific antigens (TSAs) (Buonaguro et al. 2011; Obeid et al. 2015). TAAs are expressed mostly in cancerous tissue, but they also occur in normal cells and are usually shared, that is, found in different patients' tumours. TSAs can also be shared antigens, if the mutations are very typical to cancer cells. However, TSAs are often patient specific. In other words, they are conducted from distinctive mutations in the tumour and often referred to as neoantigens. They are expressed solely on cancer cells.

3.2.1 Peptide-based vaccines

Variable cancer vaccine platforms have been invented to operate as vehicles in delivering and presenting the tumour antigens to the immune system (Butterfield 2015; Obeid et al. 2015; Papaioannou 2016). One method is to deliver antigens as peptides. Single or several kinds can be injected either alone or mixed with an adjuvant. The most frequently used approach is administering a certain antigen as a MHC class I restricted peptide, which is derived from TAAs (Butterfield 2015). These short epitopes, usually 8–11 amino acids in length, are either processed and presented by APCs or bound to the MHC I molecules directly on the surface of APCs (Butterfield 2015; Obeid et al. 2015).

Problem is that these epitopes can only be used with patients who share common HLA haplotype of genes that express the MHC proteins (Butterfield 2015). Also, the epitopes do not stimulate the activation of important CD4+ T cells, which has been tried to achieve by making the peptides longer, approximately 20–45 amino acids in length, to involve epitopes for the CD4+ T cells (Butterfield 2015; Obeid et al. 2015). These peptides necessitate processing by APCs, but the antigen presentation is effective, and the epitopes are complexed with MHC class I and II molecules evoking the activation of both CD8+ and CD4+ T cells. For wider spectrum of MHC antigen peptides, full length tumour

antigen proteins have also been investigated. Even though, the use of longer peptides does not yet offer complete solution because it is more likely that also self-antigens are presented to the immune system which causes an increased risk for autoimmunity (Obeid et al. 2015).

The peptide-based cancer vaccines have been studied in several clinical trials, and they have shown great promise (Aranda et al. 2013). Nevertheless, not one peptide vaccine has been approved by the FDA due to inadequate efficacy. One reason for this is most likely the issue that TAAs are expressed also in healthy tissues which may lead to immune tolerance and activation of regulatory T cells that suppress responses against self-antigens (Buonaguro et al. 2011; Zamarron and Chen 2011). Also, immune attack induced by delivering a single TAA might end up being insufficient since the spectrum of target antigens for the T cell response will be very narrow (Buonaguro et al. 2011). Furthermore, the immunosuppressive activities of the tumour cells, discussed in chapter 2.3, creates an additional hindrance.

3.2.2 Whole cell-based vaccines

Another means utilized for antigen delivery is the use of whole cells such as APCs or tumour cells (Butterfield 2015; Obeid et al. 2015; Papaioannou et al. 2016). The cells can be allogeneic or autologous that is, donated or patient's own cells, respectively. The most availed cells in whole cell vaccines are APCs, more precisely dendritic cells, because they have the ability to initiate and enhance immune responses (Butterfield 2015; Papaioannou et al. 2016). Two variant ways to exploit them have been established (Papaioannou et al. 2016). With targeting vectors, antigens can be bound directly to the surface receptors of the dendritic cells *in vivo* which will lead to epitope presentation with MHC I and II proteins (Tacken et al. 2007; Papaioannou et al. 2016). Also, it is possible to stimulate the dendritic cell functions by administering the antigens with adjuvants (Obeid et al. 2015). A more common approach is to load the cells with antigens *ex vivo* before infusion to the patient (Obeid et al. 2015; Papaioannou et al. 2016).

Dendritic cell-based vaccines possess many advantages; they are safe, achievable, and they can be loaded with many kinds of antigens and combined with range of adjuvants (Butterfield 2015; Papaioannou et al. 2016). Manufacturing can be however, arduous and expensive (Papaioannou et al. 2016) Even so, a dendritic cell-based vaccine sipuleucel-T is the first and so far, the only cancer vaccine approved by the FDA (FDA 2010; Melero et al. 2014). The approval was given in 2010 after a phase III clinical trial executed by Kantoff (2010) and his colleagues (FDA 2010). Sipuleucel-T elongated the survival of men with metastatic castration-resistant prostate cancer. The median survival was 4.1 months longer in the treatment group than in the placebo group. The vaccine is prepared from autologous peripheral-blood mononuclear cells that include APCs. The dendritic cells are collected from the cell preparation by gradient centrifugation after which they are pulsed with a fusion protein that comprises of a TAA prostatic acid phosphatase and an immune cell activator (Kantoff et al. 2010; Butterfield 2015; Obeid et al. 2015).

As mentioned, tumour cells have also been tested as cancer vaccine platforms. They offer a large repertoire of antigens which may contain such that are relevant for the immune response but cannot be identified (Obeid et al. 2015). This benefit could enable more efficient T cell response, although the chance of expressing self-antigens and eliciting autoimmunity might become greater.

Both autologous and allogeneic tumour cells have been examined (Butterfield 2015; Obeid et al. 2015; Papaioannou et al. 2016). Autologous cells supply a spectrum of patient unique neoantigens, which could be beneficial in the development of personalized immunotherapy (Obeid et al. 2015; Papaioannou et al. 2016). Nevertheless, their manufacturing is demanding, and it is noticed that the allogeneic cells are more practical. They are a good source of TAAs, have better suitability for production, and variability between batches is small which allows comparing patients' clinical outcomes (Papaioannou et al. 2016). To be more immunogenic, the allogeneic tumour cells are transfected to secrete certain cytokines and express costimulating molecules which is followed by inactivation with radiation to stop their proliferation. One potential application is modulating the cells to produce cytokine called granulocyte-macrophage colony stimulating factor (GM-CSF) (Butterfield 2015; Papaioannou et al. 2016). It

amplifies the actions of APCs and supports the presentation of the tumour antigens (Papaioannou et al. 2016).

In addition to all vaccine platforms viewed, antigens can also be delivered as DNA or RNA that code the tumour antigens (Obeid et al. 2015). The genetic material is administered either as naked DNA or RNA or, for example, with a viral vector. The immune response is completely dependent on the antigen presentation by APCs, and sometimes the genes are edited so that they also contain stimulating factors such as the GM-CSF. Viruses that destroy tumour cells, or oncolytic viruses, may also be considered as cancer vaccines because the demolition of the cells releases tumour antigens which leads to the activation of immune defence mechanisms (Obeid et al. 2015). Oncolytic viruses are discussed more thoroughly in the following section.

3.3 Oncolytic viruses

Virus is a biological organism that needs a host cell to proliferate (Murphy 2012). It invades the host cell and exploits it by forcing the cell to translate the genome of the virus and perform protein synthesis in order to produce new viral material. The genome, DNA or RNA, is also copied for new viruses. After this process called replication, the viruses abandon the cell leaving it damaged and ultimately dead and try to find new host cells in which to proliferate again. As a result, viruses can engender multiple hazardous diseases.

Oncolytic viruses have a unique ability to selectively replicate in cancer cells and exterminate them without injuring healthy cells and being pathogenic (Chiocca and Rabkin 2014). Some viruses kill tumour cells specifically in their native form, but it is also possible to genetically modify them to do so (Kaufman et al. 2015). Furthermore, they can generate a systemic immune response against cancer which is fundamental for their clinical efficacy. Typically, the viruses are injected straight into tumour tissue in order to bypass physical barriers created by the tumour and its surroundings. Along with ICIs and vaccines, oncolytic viruses are a type of active cancer immunotherapy as well (Papaioannou et al. 2016).

The mechanisms behind the lytic activities and eliciting immune attack are not completely understood, yet several probable factors have been discovered (Kaufman et al. 2015). In healthy cells, where different cell signalling pathways are normal, viral eradication systems operate accordingly. On the contrary, as discussed in chapter 2.2, in cancer cells variable oncogenes are active and regulation of cell functions is impaired which enables efficient proliferation of the viruses in distinctive ways (Hanahan and Weinberg 2011; Kaufman et al. 2015). First of all, the division of cancer cells is rapid and consequently, protein synthesis is intensive (Kaufman et al. 2015). This provides a productive machinery for the virus to utilize for replication and building its own viral components. The capacity of tumour cells to avoid apoptosis also aids the proliferation because the cells will stay alive longer thus, the virus has a prolonged period of time available for replication. For the same reason, spreading of the virus is easier as well. Moreover, specific cell signalling pathways that are responsible in healthy cells for annihilating viruses are often malfunctioning in cancer cells. As a consequence, the amount of virus increases with time in the tumour unlike the dose of conventional drugs which makes the pharmacokinetics of oncolytic viruses extraordinary (Chiocca and Rabkin 2014).

The actions mentioned above result eventually in the lysis of cancer cells, which subsequently releases TAAs and TSAs (Kaufman et al. 2015). The adaptive immune system responds to them and begins to reject the tumour. Because of the recognition, the immune cells are able to fight cancer cells that are not only at the viral infection site, but those as well that are located elsewhere and not affected by the virus. In addition to tumour antigens, the destroyed cells free pathogen- and danger-associated molecular pattern signals and cytokines that further the progress of APC maturation and full activation of CD4+ and CD8+ T cells, after which the CD8+ T cells migrate to established tumour growths.

Beside the anti-tumour immunity, oncolytic viruses elicit anti-viral immune response, which is probably one of the most prominent limiting factors for oncolytic immunotherapy (Chiocca and Rabkin 2014). The defence mechanism composes of adaptive and innate immune responses, which both neutralize and clear the virus from the body. Depending on whether the virus has been recognized before by the immune system,

the immunity can be newly-formed or pre-existing, in which case the viral clearance can be very rapid (Kaufman et al. 2015). Regardless, if the anti-viral defence mechanism functions too dominantly, it can inhibit the tumour rejecting activities by eliminating the virus before it replicates fully and induces cytotoxic effects against the cancer cells (Chiocca and Rabkin 2014). Choosing to deliver the virus intratumourally may have a slight protective impact and is often more rational since intravenous dosing makes the virus even more exposed to the immune attack. Dosing directly into tumour is not unfortunately always possible, and intravenous injection is more pragmatic (Chiocca and Rabkin 2014; Breitbach et al. 2016).

Determining the level of how much the anti-viral immunity hinders the anti-tumour effects is complicated but it is most likely influenced by the tumour microenvironment and the qualities of the virus (Kaufman et al. 2015). For instance, some viruses are capable of modifying the immunosuppressive tumour microenvironment to a direction that promotes cancer eradication. The central point is that the balance between the anti-tumour and anti-viral immunities may govern therapeutic outcomes which is why strengthening the anti-tumour immunity is vital (Chiocca and Rabkin 2014).

Several oncolytic viruses have been discovered and their use as prospective cancer treatments has been researched in clinical trials (Kaufman et al. 2015). Currently, the most significant viruses being tested among others are, for example, vaccinia virus and herpes simplex virus. Vaccinia virus has been proceeded until phase II and III studies while herpes simplex virus has already been approved by the FDA for the treatment of melanoma (FDA 2015; Kaufman et al. 2015; ClinicalTrials.gov 2018a).

3.3.1 Vaccinia virus

Vaccinia virus (VACV) belongs to the poxvirus family and was originally used for vaccination against smallpox (Kaufman et al. 2015). It is a large membrane covered virus that has a DNA genome consisting of circa 190 kilobase pairs (kbs) (Smith et al. 2002; Kaufman et al. 2015). Additionally, it has capacity for foreign DNA of 25 kbs which enables versatile editing of gene expression (Smith et al. 2013). The replication of the

virus happens in the cell cytosol reducing concerns about insertional mutagenesis (Kaufman et al. 2015).

VACV also has natural tropism to cancer cells but still, some genetic modifications have been made in order to emphasize its tumour specificity (Kaufman et al. 2015). The virus has been for example, attenuated by deleting genes that are necessary for the viral replication in normal cells but not in cancer cells which hinders the pathogenic effects. Since being naturally immunosuppressive too, meaning that it has the ability to evade immune responses, its immune effects have been enhanced (Smith et al. 2013; Kaufman et al. 2015). For instance, costimulating B7 molecules and immune stimulating factors have been applied to the genome to magnify anti-tumour T cell responses (Kaufman et al. 2015). VACV has shown great promise and it has been examined in multiple clinical trials.

Kaufman (2005) with his colleagues, executed a phase I clinical study for the first time with a recombinant VACV that expresses B7.1 molecule, or CD80, which binds to the CD28 causing an activating signal for the immune system. The aim was to evaluate immune effects, such as tumour regression and T cell responses. Twelve patients with metastatic melanoma participated in the trial, and once a month they were given intralesional injections of the substance for three months. Six patients were selected into the T cell analysis which showed that all six developed a response against specific melanoma antigens. The tumour regression was defined by using standard response evaluation criteria in solid tumours (RECIST), which tells the size progression of tumour lesions. One person withdrew from the study after the first injections, therefore eleven were able to be included to this evaluation. Two out of eleven showed indications of regression, and one patient experienced complete disappearance of several tumours.

In a later study, performed by Heo et al. (2013), a preparation referred to as pexastimogene devacirepvec (JX-594 or Pexa-Vec) was used. It is a VACV genetically engineered with the stimulating cytokine GM-CSF that was brought up in section 3.2. Cancer selectivity is also improved by disrupting the functions of a viral thymidine kinase gene. The object of this clinical trial was to compare results, such as overall survival,

achieved with two different virus doses. Thirty subjects with advanced hepatocellular carcinoma were enrolled to the study and divided into two dose groups, high and low. Treatments were injected intratumourally on days 1, 15 and 29. Size progression was also used in this study to observe responses, and the results did not differ much between the treatments. Instead, the survival times varied considerably; median overall survivals in the high and low dose groups were 14.1 and 6.7 months, respectively. Because of the potential, JX-594 proceeded to larger clinical trials, and currently recruiting to a phase III study is ongoing (ClinicalTrials.gov 2018a).

3.3.2 Herpes simplex virus type 1

Herpes simplex virus type 1 (HSV-1), like VACV, is an enveloped virus with double stranded DNA (Kaufman et al. 2015). The size of the genome although is smaller, only 152 kbp, and the replication happens in the nucleus instead of the cytosol. HSV-1 is a member of alphaherpesvirus family and a serious pathogen. Infection can lead to skin lesions such as blisters and ulcers (Välimaa et al. 2013). The lesions eventually heal but the virus will stay in the body as latent in the ganglia of peripheral neurons and cause new outbreaks when activated. If the virus spreads to central nervous system, the infection can be lethal. As VACV, HSV-1 have been genetically modified to decrease the pathogenic effects but also to improve its oncolytic properties (Kaufman et al. 2015).

The most successful HSV-1-derived product is talimogene laherparepvec (T-VEC) which is the first oncolytic virus approved by the FDA (FDA 2015; Hamid et al. 2017). Two viral gene deletions have been made in the T-VEC (Liu et al. 2003; Andtbacka et al. 2015). Removal of neurovirulence factor gene (ICP34.5) attenuates the pathogenicity of HSV-1 efficiently since it is mostly responsible for the toxicity of the virus (Andtbacka et al. 2015). That is to say, deleting the gene ensures that the virus cannot grow within neurons or mediate latency (Kaufman et al. 2015). Simultaneously, tumour specificity is strengthened (Liu et al. 2003; Andtbacka et al. 2015). Another gene referred to as ICP47 prevents infected cells to present antigens to CD8+ T cells hence, it has been removed to intensify the stimulation of the immune system (Liu et al. 2003). Furthermore, the gene in charge of the GM-CSF production has been inserted.

T-VEC was approved in 2015 with the official indication of “the local treatment of unresectable cutaneous, subcutaneous, and nodal lesions in patients with melanoma recurrent after initial surgery” (FDA 2015). The approval was preceded a major, randomized, open-label phase III study that compared T-VEC treatment to GM-CSF only (Andtbacka et al. 2015). From screened patients, 436 were randomized to treatment groups in a ratio of two-to-one thus, 295 would receive T-VEC and 141 GM-CSF only. T-VEC was injected intratumourally, whereas GM-CSF was given subcutaneously. Primary endpoint was durable response rate (DRR), which was defined so that response should be detected within twelve months after starting the treatment and last continuously at least six months or longer. The DRR in the T-VEC group was 16.3 % which is significantly higher than in the GM-CSF group in which it was only 2.1 %. Response was observed not only in the injected, but also in the un-injected lesions which points that to some extent a systemic immune response was achieved too.

After the approval, T-VEC has been examined in many clinical trials alone and in combination with different therapies (ClinicalTrials.gov 2018e). The ICIs, ipilimumab and pembrolizumab, are proven to enhance T cell responses and assist overcoming the immunosuppressive tumour microenvironment (Voena and Chiarle 2016). Hence, they have been studied together with T-VEC with expectations that combination therapy would be more efficient than monotherapy (Papaioannou et al. 2016; Puzanov et al. 2016; Ribas et al. 2017).

In two different phase Ib, open-label, multicentre, non-randomized clinical trials, the safety and efficacy of T-VEC/ipilimumab and T-VEC/pembrolizumab combination therapies were investigated (Puzanov et al. 2016; Ribas et al. 2017). 19 and 21 patients with advanced melanoma enrolled to the studies, respectively. In the T-VEC/ipilimumab research, only 18 participated to the efficacy studies (Puzanov et al. 2016). In both trials, the patients received a dose of T-VEC and ICI once a week (Puzanov et al. 2016; Ribas et al. 2017). The treatments were started with T-VEC, and the ICI was combined to the therapy after 6 weeks from the first T-VEC dosing. The T-VEC was administered as intratumoural injections every other week, except the first two doses, which were administered in four-week intervals. Instead, the ICIs were infused systemically either

every two or three weeks. Treatments were continued until beforehand defined endpoints. Regarding safety, dose-limiting toxicities were not observed in either studies.

The efficiency was assessed with immune-related response criteria (Puzanov et al. 2016; Ribas et al. 2017). The overall response rate in the T-VEC/ipilimumab research was 50 % (9 patients) of which four (22 %) experienced complete response that is, total tumour disappearance, and five (28 %) had a partial response or tumour regression (Puzanov et al. 2016). From these subjects eight had a durable response lasting at least six months. Out of the 21 patients in the T-VEC/pembrolizumab study, 62 % (13 individuals) responded to the treatment and seven of them (33 %) had a complete response (Ribas et al. 2017). Significant perception was that responders had an increased amount of tumour infiltrated CD8+ T cells after the T-VEC injections compared to baseline which was most likely very beneficial for the subsequent pembrolizumab treatment.

The outcomes of the study by Puzanov et al. (2016) were compared to earlier studies performed with T-VEC and ipilimumab alone, and the overall response rate achieved in their trial was higher than in the earlier studies. This indicates that the combination therapy seems to be more potent than monotherapy. Although, the number of patients was much smaller in their research than in the previous trials which is why the conclusion is only directional and has to be verified with results from larger ongoing clinical trials (ClinicalTrials.gov 2018b). The results by Ribas et al. (2017) also imply that using T-VEC with ICI therapy is advantageous. To confirm the outcome of that study, a randomized phase III clinical trial is ongoing as well (ClinicalTrials.gov 2018d). Research of using T-VEC for treating other cancers than melanoma such as pancreatic and breast cancer has also been executed (ClinicalTrials.gov 2018e).

3.4 Oncolytic vaccines

All previously discussed immunotherapies consist of individual successes and potential treatments that are being intensively studied. As more information about the therapies have been found, their characteristic issues have emerged too. All of the main limitations are related to the functions of the immune system; the immune response is not strong

enough, or targeting the attack is problematic (Chiocca and Rabkin 2014; Obeid et al. 2015; Papaioannou et al. 2016). In addition, the tumour microenvironment creates a remarkable challenge because of the immunosuppressive properties (Vinay et al. 2015). However, the treatments also have benefits and combining them is one method to overcome these issues. Different options and modifications have already been developed as presented earlier in this review. This section will focus on the concept of joining together the principles of cancer vaccines and oncolytic viruses.

Their combination is often referred to as an oncolytic vaccine, in which the virus operates as a carrier of tumour antigens meanwhile it replicates in the cancer cells and exterminates them (Lichty et al. 2014). Typically, a TAA is coded to the genome of the oncolytic virus (Elsedawy and Russel 2013). When the virus infects tumour cells, the antigen will be expressed through protein translation. Via destruction, the produced antigens, and other TAAs and TSAs from the tumour cells are introduced to the immune system which will engage APCs, especially dendritic cells that will present the antigens to the adaptive immunity. The virus itself also aids the maturation of the dendritic cells. Furthermore, the tumour antigen expression from the viral genome helps the overall presentation to be prevalent over viral antigens. Consequently, a more extensive and specific T cell response towards the cancer may be inflicted.

It has been noted that the advantage explained in the theory can be accomplished better by using the oncolytic vaccines in prime-boost compositions in which the immune system is first primed with a virus encoding a TAA and later boosted with a different virus expressing the same TAA (Breitbach et al. 2016). Encouraging results have been elucidated. By using murine models, Bridle (2010) and Pol (2014) with their research teams tested replicating vesicular stomatitis virus (VSV) and rhabdovirus Maraba, respectively, which were engineered with a melanoma associated antigen, dopachrome tautomerase. Both studies obtained similar results: the recombinant viruses alone did not prolong the survival of the mice or engender a specific anti-tumour effect. Moreover, in the study by Bridle et al. (2010), the immune response targeting the virus was very high. But, when the mice were first primed with a replication-deficient adenovirus encoding the same melanoma antigen and later boosted with the replicating viruses, the relative

amount of IFN-ɣ secreting tumour-specific CD8+ T cells was significantly increased in the tumour and blood (Bridle et al. 2010; Pol et al. 2014). These results suggest that using replicating viruses as priming or mono treatments is not very sufficient. According to Pol et al. (2014), this might derive from a strong anti-viral immune response against the extremely immunogenic viral antigens that need to be expressed for the replication of the virus.

Maraba virus that encodes the human melanoma associated antigen MAGE-A3 has moved forward to a phase I/II clinical study in which recruiting patients is ongoing (ClinicalTrials.gov 2018c). The purpose is to determine a maximum feasible dose of the oncolytic vaccine when given alone or as a boosting treatment. As a priming vaccine an adenovirus expressing the same MAGE-A3 is used. Efficacy of utilizing the two vaccines as a prime-boost treatment is also assessed by evaluating objective tumour response rate with the RECIST criteria.

The limitation with this approach is that the viruses are expressing only one tumour antigen, when the fact is that tumours present a large repertoire of both TAAs and TSAs (Hanahan and Weinberg 2011; Woller et al. 2014). A method, which has been exploited in trying to circumvent this problem, is to code an entire antigen library to the viral genome (Woller et al. 2014). The idea is to take xenogenic DNA from a healthy tissue, which is corresponding to the treatable tumour tissue, and insert it into the genome of an oncolytic virus (Kottke et al. 2011; Woller 2014). Hence, the virus will express variable TAAs and also self-antigens and generate an immune response against them. But, because the “self-antigens” are from a foreign source they will not hinder the attack and also do not cause strong autoimmunity.

3.4.1 Personalized oncolytic vaccines

Nearly all immunotherapies presented in this review are exploiting TAAs in various manners. TAAs have advantages since they can occur in number of patients permitting broad applicability (Buonaguro et al. 2011; Obeid et al. 2015). Nevertheless, challenging is that TAAs do not necessarily elicit a robust and a specific anti-tumour immunity

because they are types of self-antigens against which the immune system does not attack so vigorously (Gubin et al. 2015; Patel et al. 2017). TSAs instead, are not as prone to immunological tolerance and they are more visible to the immune system. Therefore, exploiting them could enable developing treatments that are more sufficiently targeting only tumours (Woller et al. 2014). Especially utilizing neoantigens of individual patients would be beneficial since cancer cells mutate constantly and thus, expressed tumour antigens can be very variant between different persons (Hanahan and Weinberg 2011). In addition, it has been noted that genetic changes that render missense mutations have the largest probability to be expressed and recognized by the immune system (Zhang et al. 2017). Targeting neoantigens also has lower risk for auto-immunity.

Accordingly, developing of personalized oncolytic vaccines requires the identification of patient's mutated TSAs, and methods which accomplished that were earlier cumbersome and hard (Gubin et al. 2015). Today, however, technologies have been improved which is why an interest towards neoantigen-based immunotherapies has been augmented. The antigens could be engineered to the DNA of an oncolytic virus as done with the TAAs. Genetic modifications, on the other hand, are complex and do not suit for all viruses (Elsedawy and Russel 2013). A large genome allows gene insertions but in turn, those viruses also have very immunogenic epitopes which can lead to viral antigen epitope dominance. Second, performing various genetic manipulations is time-consuming, and every new alteration demands a re-evaluation by the authorities FDA and European Medicines Agency (Capasso et al. 2016).

An effort to bring together the benefits of oncolytic vaccines and TSAs, and to eliminate the complexity of genetic modifications has been made by Capasso (2016) and his colleagues. They created an oncolytic vaccine platform called PeptiCRAd in which MHC class I restricted TSA epitopes are attached with electrostatic interactions directly onto the capsid of an adenovirus while leaving out unnecessary manipulations of the genome. The ability of the platform to hinder tumour growth and elicit a tumour specific immune response was studied by performing an animal experiment with a mouse melanoma model. The mice were implanted with melanoma cells (B16) that express an artificial TSA epitope referred to as SIINFEKL, which is derived from chicken ovalbumin. Hence,

the tested treatments were PeptiCRAd (formed by adsorbing the SIINFEKL peptide to the adenovirus), adenovirus mixed with SIINFEKL, virus only, peptide epitope only and injection medium (mock). The mice were treated three times on days 0, 2 and 7 by intratumoural injections. The results showed that PeptiCRAd significantly slows tumour growth, and in the end of the experiment the volume of the tumours in the PeptiCRAd treatment group were clearly smaller than in the control groups. Since adenovirus does not replicate in murine cells, the efficacy of PeptiCRAd engendered mainly via specific anti-tumour immune response. The relative amount of SIINFEKL targeted CD8+ T cells in the tumour, spleen and draining lymph nodes was significantly larger compared to the control treatments.

3.4.2 PeptiENV platform

The interest towards personalized immunotherapies and the promising results presented above encouraged to research whether the same idea would work with enveloped oncolytic viruses such as the VACV and the HSV-1, which have been pointed to be highly potential as cancer immunotherapies. Thus, a novel cancer vaccine platform called PeptiENV was developed (Figure 2).

The principle, as in PeptiCRAd, is to attach MHC class I restricted tumour antigens onto the virus and therefore, stimulate a specific anti-tumour immune response especially through activating CD8+ T cells to target cancer cells. At first, how the tumour peptide epitopes to be attached would bind to the envelope was examined. The peptide cannot be joined to the membrane as such, but instead, requires an additional link that operates as an anchor between the peptide and the envelope.

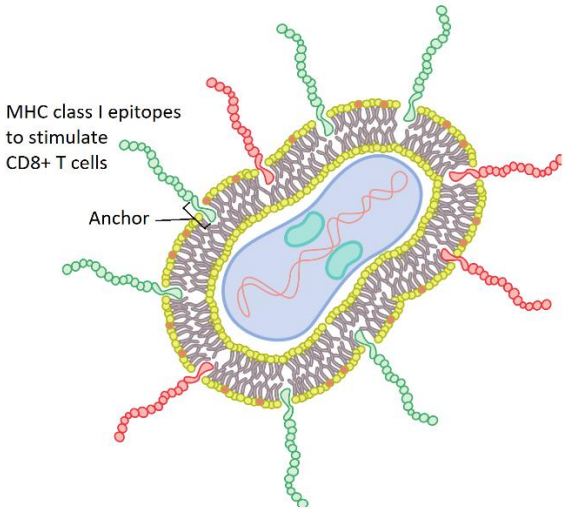

Figure 2. The structure of PeptiENV platform. The antigen epitope is linked to an anchor that attaches the epitope onto the envelope of the oncolytic virus (Ylösmäki et al. submitted).

Several options were screened, and the outcomes pointed that the most secure way is to fix the peptide to either cell penetrating peptide (CPP) or to cholesterol (chol) (Ylösmäki et al. submitted). The anchors were fused to the same chicken derived TSA as in the study by Capasso et al. (2016), or SIINFEKL, and attached to the membranes of the VACV and HSV-1. The interactions between the CPP- and chol-SIINFEKL peptides and the envelopes of both viruses were studied, and they have been proved to be stable (Ylösmäki et al. submitted). If the platform would demonstrate to be efficient in later experiments, it could be utilized as a personalized cancer immunotherapy by attaching neoantigens of individual patients onto the viral envelope.

II EXPERIMENTAL PART

4 AIM OF THE STUDY

The aim of this study is to learn if PeptiENV treatment *in vivo* prevents tumour growth and generates a tumour antigen-specific T cell response more effectively than control treatments, especially an oncolytic virus only. Furthermore, the elongation of survival and the preservation of infectivity of the PeptiENV platform are investigated *in vivo* and *in vitro*, respectively. The research is executed with replicating VACV and HSV-1 thus, an objective is also to detect possible differences between the viruses and discuss them.

The VACV is studied with SIINFEKL epitopes that are conjugated onto the viral envelope either with the CPP or the cholesterol anchor. Instead, the HSV-1 is fused with SIINFEKL by using only CPP. The infectivity of the PeptiENV platform is tested with cell viability assay. Tumour growth instead is examined with laboratory mice by using a murine skin cancer model created with mouse melanoma cells (B16) expressing the artificial tumour antigen SIINFEKL derived from chicken ovalbumin (OVA) that is, B16-OVA cell line. Tumour-specific immune response is assessed by measuring the relative amount of SIINFEKL-specific CD8+ T cells in the tumour tissue with flow cytometry.

5 MATERIALS AND METHODS

5.1 Viruses and peptides

The VACV used in the experiments (VACVdd-tdTomato-mDAI) is a genetically engineered Western Reserve strain of vaccinia virus provided by the ImmunoViroTherapyLab. The virus has double deletion (dd) in order to enhance cancer specific replication: one in the thymidine kinase gene and one in the vaccinia growth factor gene (Hirvinen et al. 2016). Also, it is armed with a murine DNA-dependent activator of IFN-regulatory factors (mDAI) which is a sensor of cytosolic double-stranded

DNA. When stimulated, it activates the innate immune responses hence, it improves the immunogenicity of VACV. Furthermore, a red fluorescent protein (tdTomato) has been inserted.

The HSV-1 used in the experiments (HSV-1 (17^+) Lox-Luc) was a kind gift from Prof. Veijo Hukkanen (University of Turku, Finland). The virus is derived from the laboratory strain HSV-1(17+) and is also genetically modified: the firefly luciferase gene has been inserted (Nygårdas et al. 2013). Suppliers of peptides and full amino acid sequences are found in Table 1.

Table 1. Amino acid sequences and suppliers of the peptides used in the experiments. CPP = cell penetrating peptide

Peptide	Amino acid sequence	Supplier
CPP-SIINFEKL	GRKKRRQRRRPQRWEKI-SIINFEKL	Ontores (China)
chol-SIINFEKL	cholesterol-C-SIINFEKL	Pepscan (The Netherlands)

5.2 Cell lines

Murine melanoma cell line B16-OVA was cultured in Roswell Park Memorial Institute (RPMI) 1640 low glucose medium completed with 10 % fetal bovine serum (FBS), 1 % penicillin-streptomycin and 1 % L-glutamin (GlutaMAX™). Geneticin™ was added to B16-OVA culture flasks with concentration of 5 %. Murine triple negative breast cancer cell line 4T1 was cultured in RPMI 1640 high glucose medium supplemented with 10 % FBS and 1 % penicillin-streptomycin. Human lung cancer cell line A549 and human triple negative breast cancer cell line MDMBA436 were cultured in Dulbecco's modified Eagle medium (DMEM) completed with 10 % FBS and 1 % penicillin/streptomycin. All cell lines were grown in an incubator at temperature 37°C and 5 % of CO_2. When subcultured, cells were washed with phosphate buffered saline (PBS) and detached with TrypLE™ enzyme solution. B16-OVA cell line was provided by Prof. Richard Vile (Mayo Clinic, Rochester, MN, USA). Other cell lines were acquired from ATCC (USA). All cell culture materials were ordered from Gibco® Life Technologies except DMEM, which was purchased from Lonza®.

5.3 Production of PeptiENV

The PeptiENV complex was always produced right before experiments. It is formed by mixing a virus and a peptide in a certain volume of medium. In this study, the medium used was plain RPMI 1640. First, the medium is placed into a microcentrifuge tube. Next, the peptide is added to the tube and finally the virus. Then, the mix is kept at 37°C for 20-30 minutes after which it is put in ice. All different complexes are formed individually in separate microcentrifuge tubes.

Viruses and peptides are always mixed in a known ratio; in experiments performed in this study, 10^6 pfu (plaque forming units) of viruses were combined with 3.1 nmol of peptides. Although, the number of viruses and peptides varied between experiments, the ratio was constantly kept the same.

5.4 Cell viability assay

The preservation of infectivity of the PeptiENV platform was tested to prove that complexation of virus and peptide does not affect the infectivity of the virus. The experiment was performed with cell viability assay in which the used PeptiENV complex was formed with vaccinia virus and CPP-SIINFEKL. Cell viability after infecting with PeptiENV, was compared to infections with naked VACV, CPP-SIINFEKL only and complex formation medium (RPMI 1640). Complexation of PeptiENV was done as described earlier.

Four different cell lines, B16-OVA, A549, 4T1 and MDMBA436, were utilized in the experiment. All cells were plated one day before infection in 96-well plates with density of 50 000 cells/well. The next day, the cells were infected by using four different concentrations of PeptiENV and each control treatment. Concentrations were determined as multiplicity of infection (MOI) which tells the relative amount of virus compared to number of cells. Strongest concentration used was MOI 1, that is, 50 000 viruses was used for infecting 50 000 cells. Lower concentrations were MOI 0.1, MOI 0.01 and MOI

0.001. Four repetitions of each concentration were made with every treatment to calculate means from the raw data. Demonstrative plate layout is shown in Figure 3.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	1	1	1	1	1	1	1	1	1	1	1
B	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
C	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
D	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
E	mock	mock	mock	mock								
F	mock	mock	mock	mock								
G	mock	mock	mock	mock								
H	mock	mock	mock	mock								

Figure 3. Scheme of a 96-well plate in the cell viability assay. Cells were infected with different treatments and MOI concentrations (numbers). The same layout was used with all cell lines. Blue = PeptiENV; pink = vaccinia virus only; green = CPP-SIINFEKL peptide only; orange (mock) = medium only

After infection, cells were kept in an incubator at temperature 37°C and 5 % of CO_2 for three days, and cytopathic effects were checked daily. Cell viability was assessed by using CellTiter-Fluor™ Cell Viability Assay kit from Promega, which exploits protease activity within live cells (Promega.com). Live cells produce a fluorescent signal which was used to indicate the relative number of viable cells. Fluorescence of the cells was measured with Varioskan Lux (Thermo Scientific).

5.5 Animal experiments

Animal experiments were carried out by using laboratory mice. The strain of the mice was C57BL/6JOlaHsd and gender female. Mice were ordered from a commercial breeder Envigo (The Netherlands) at the age of 4–6 weeks. Animals were kept in individually ventilated cages in groups of 3–5 mice. Food and water was provided all times, and cages were changed into clean ones once a week. Light time was 12 hours, from 6:00 to 18:00. All animal experiments were performed according to the Finnish Act (497/2013) and Government Decree (564/2013) on the protection of animals used for scientific or

educational purposes, and Directive (2010/63/EU) of the European Parliament and of the Council on the protection of animals used for scientific purposes. License for animal experiments has been authorized by the national Animal Experiment Board (ELLA, Eläinkoelautakunta).

5.5.1 Tumour implantation

The animal experiments were started with tumour implantation. B16-OVA cells were detached from culture flasks by using TrypLE™ Express (Gibco® Life Technologies) and collected into a sterile falcon tube. In the tube, cells were washed two times with PBS. Centrifugation speed was 400 x g and temperature 23°C (Allegra® X15R Centrifuge, Beckman Coulter). After second wash, the cell pellet was resuspended into plain RPMI 1640 medium. The aim was to implant 300 000 tumour cells into every mouse hence, the cells were counted, and concentration was adjusted to $6x10^6$ cells/ml. Each mouse was injected with 50 µl of the cell suspension subcutaneously into the right flank. Mice were held under inhalational anaesthesia with 3 % isoflurane during injections. Following the implantation, mice were checked for growing tumours every other day and treatments were started when the tumour diameter reached approximately 4–5 mm (or volume 50 mm^3). The mice that had not developed sufficient tumours were excluded from experiments. Animals that were included were randomized into different treatment groups.

5.5.2 PeptiENV with vaccinia virus

The first PeptiENV experiment was executed with VACV, CPP and cholesterol conjugated SIINFEKL peptides. Treatment and control groups are presented in Table 2. Tumours were grown enough after 11 days post implantation thus the treatments were started on that day. The treatments were prepared right before they were injected. The PeptiENV complexes were produced as described in chapter 5.3, and the amount of VACV used for one mouse was 10^6 pfu and therefore peptide amount was 3.1 nmol. The control treatments were also formed according to the PeptiENV production protocol but only virus or peptide were added to the medium containing tubes. The same amount of

virus and peptides as in PeptiENV were used in the VACV only group, and the CPP- and chol-SIINFEKL only groups. Substances were injected intratumourally in volume of 50 µl, and inhalational anaesthesia with 3 % isoflurane was used during all injections. Over all, the mice were treated three times of which the first two were given in the beginning on days 11 and 13. The third injection was administered later as boosting treatment on day 19.

Table 2. Treatment and control groups used in the PeptiENV VACV experiment. PeptiENV VACV CPP (vaccinia virus conjugated with CPP-SIINFEKL) and PeptiENV VACV chol (vaccinia virus conjugated with chol-SIINFEKL) were the studied treatments. VACV (vaccinia virus only) CPP-SIINFEKL (peptide only), chol-SIINFEKL (peptide only) and mock (injection/complexation medium only) were used as controls.

Group	Number of mice
PeptiENV VACV CPP	7
PeptiENV VACV chol	7
VACV	7
CPP-SIINFEKL	6
chol-SIINFEKL	6
Mock	7

Tumour growth was followed by measuring the diameter of the tumours with a calliper every other day. Because the tumours are not completely symmetrical, the diameters were measured from two points, the narrowest and the widest part. Measurements were started the same day as the treatments, and they were always performed before injections. As well as the treatments, measurements were also executed while the mice were under anaesthesia. Inhalational anaesthesia with 3 % isoflurane was used. To reduce stress and pain in the animals, the endpoint to the experiment was defined so that maximum tumour diameter allowed was 17 mm. When that diameter was reached or if the mice seemed unwell they were euthanized. Euthanizing dates were marked in order that survival times could be observed.

5.5.3 PeptiENV with herpes simplex virus type 1

The basic principle of the second animal experiment was similar to the first one except, HSV-1 was used. Also, the results from the first experiment indicated that virus conjugated with chol-SIINFEKL does not have as effective outcome as the complex with CPP-SIINFEKL which is why HSV-1 was fused only with the CPP-SIINFEKL (Figure 5). These results are presented later. The treatment and control groups are shown in Table 3.

Table 3. Treatment and control groups used in the PeptiENV HSV-1 experiment. PeptiENV HSV-1 CPP (herpes simplex virus type 1 conjugated with CPP-SIINFEKL peptide) was the studied treatment. HSV-1 (virus only), CPP-SIINFEKL (peptide only) and mock (injection/complexation medium) were used as controls.

Group	Number of mice
PeptiENV HSV-1 CPP	8
HSV-1	8
CPP-SIINFEKL	5
Mock	6

Virus amount was increased from 10^6 pfu to 5 x 10^6 pfu per mouse and thus, peptide amount from 3.1 nmol to 15.5 nmol. Substances were injected intratumourally in volume of 50 µl. Injections and measurements were started on day 10 when the tumours were large enough. In this experiment mice were treated also three times. The second treatments were injected on day 12 and third injections were administered on day 20 as boosting treatments. Measurements were continued every other day until previously described endpoint.

5.5.4 Tissue collecting

In both animal experiments, after euthanizing the mice, certain tissues were collected in order to perform immunological analysis of them later. The collected tissues were spleen, tumour draining lymph node and the tumour. Tissues were put in complete RPMI 1640 medium and in ice after which they were immediately transferred for processing. By using

cell strainers, all organs were individually isolated into single-cell suspensions. Dimethyl sulfoxide (DMSO), obtained from Sigma-Aldrich, was added to the suspensions so that DMSO concentration was 10 %. Then, the suspensions were aliquoted into cryogenic vials and stored in – 80 °C.

5.6 Immunological analysis

Extracellular staining and flow cytometry were used to determine the relative amounts of SIINFEKL-specific CD8+ T cells in the tumours collected from the PeptiENV VACV and PeptiENV HSV-1 animal experiments. Also, the relative amount of VACV-specific CD8+ T cells was obtained from PeptiENV VACV study. Samples were taken from three different mice from each treatment and control group. Due to weaker results, PeptiENV VACV chol and thus chol-SIINFEKL only treatment groups were excluded from the analysis (Figure 5). Mice included to the analysis were chosen so that euthanizing dates were as close to each other as possible to reduce variability in the results.

As soon as the samples were melted, they were placed into a 96-well plate and washed once with PBS. Fc receptors, which occur in the surface of many immune cells and bind several kinds of antibodies, were blocked by using TruStain fcX^{TM} to eliminate false positive results. After, the samples were protected from light throughout the protocol. Samples were also pooled together according to treatment groups. Incubation time with the Fc block was 15 minutes and temperature 4°C. Following the incubation, fluorophore-conjugated pentamers were put to the pooled samples. APC labelled SIINFEKL was used in both PeptiENV VACV and HSV-1 analysis to detect SIINFEKL-specific CD8+ T cells. Instead, PE labelled VACV pentamer was used only in the PeptiENV VACV study to find VACV-specific CD8+ T cells. Pentamers were incubated 30 minutes at 23°C. Next, the samples were washed two times with PBS.

In order to exclude B cells from the results and detect all T cells, fluorophore-conjugated antibodies were placed to the samples. The antibody used to find B cells was anti-CD19-PeCy7, whereas anti-CD3-PerCpCy5.5 and anti-CD8-FITC were used to discover all T cells and CD8+ T cells, respectively. The antibodies were added as a mixture, and then

the samples were incubated for 30 minutes at 4°C. After the staining, cells were washed with PBS and fixed with 4 % Formalin (Sigma-Aldrich) followed by one additional wash and resuspension with PBS. Finally, the samples were transferred through cell strainer caps to tubes that are applicable for flow cytometry. Samples were analysed with Gallios Flow cytometer (Beckman Coulter).

SIINFEKL pentamer (APC labelled Pro5 MHC I Pentamer, H-2Kb SIINFEKL), VACV pentamer (R-PE labelled Pro5 Pentamer, H-2Kb TSYKFESV) and CD8-FITC (rat isotype) antibody were purchased from Proimmune®. Instead, Fc block (TruStain fcXTM, anti-mouse CD16/32) and antibodies CD19-PeCy7 (rat isotype) and CD3-PerCpCy5.5 (hamster isotype) were supplied by BioLegend®.

5.7 Data processing

Raw data from the experiments was processed with different software programs. In animal experiments Microsoft Excel (2016) was used for calculating relative tumour volumes for each mouse from the measured diameters. Tumour volume growth percentages were obtained by comparing absolute volumes to the original volumes measured on the first treatment day. Averages for these values, standard deviations and growth curves were formed with GraphPad Prism 7 (GraphPad Software, Inc. La Jolla, CA, USA). Microsoft Excel and GraphPad Prism were also used in data analysis in the cell viability assay. Means and standard deviations were calculated. Data from flow cytometry was analysed with FlowJo Software (Tree Star, Ashland, OR, USA). Results were transferred to GraphPad Prism 7 which was used for creating graphs and calculating averages and standard deviations. Additionally, statistics of the PeptiENV VACV immunological analysis were tested with one-way analysis of variance (one-way ANOVA).

6 RESULTS

6.1 Cell viability assay

The outcomes of the *in vitro* cell viability assay, which was executed to study the preservation of infectivity of PeptiENV, are presented in Figures 4A–4D. They show that the ability of infecting cells does not decrease when VACV is conjugated with CPP-SIINFEKL. The viability of cells, when infected with PeptiENV or VACV only, does not differ significantly from each other. In addition, the Figures 4A–4D point that treating the cells with CPP-SIINFEKL only, does not affect viability more than treating with medium only.

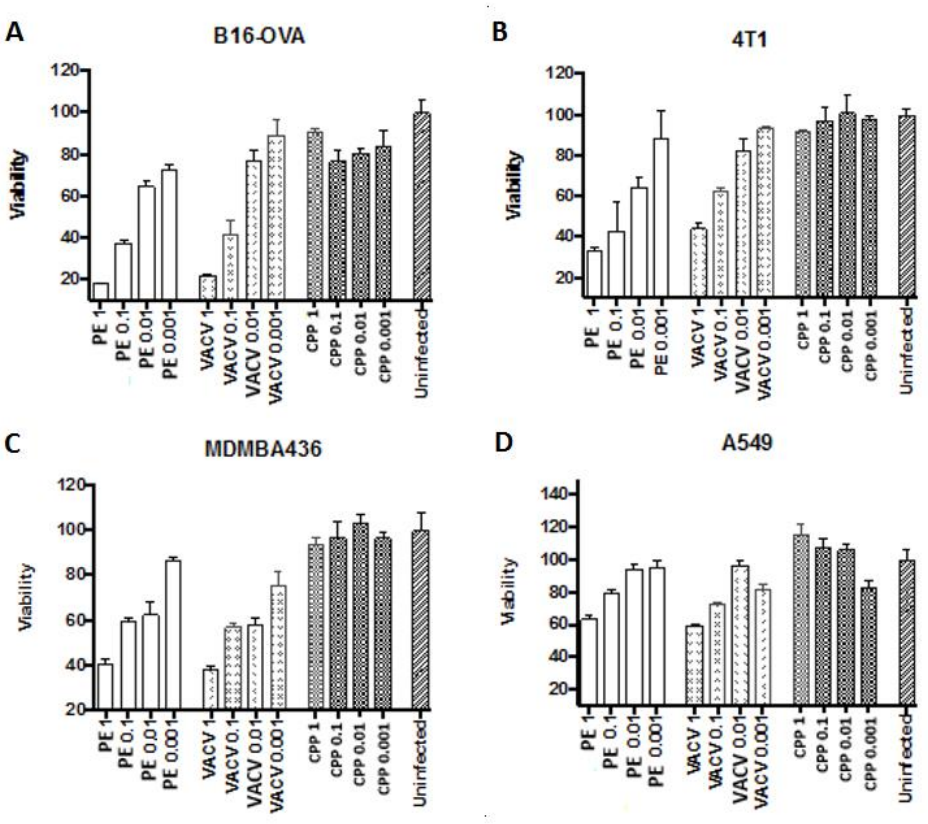

Figure 4. The viability of different cells (A–D), when they are treated with variable concentrations of PeptiENV, VACV only, CPP-SIINFEKL peptide only and medium only. Viability is presented as percentage, and in each graph cell viability is compared to uninfected cells (treated with medium) in which the viability is set to be 100 %. On the x-axis are shown different MOI (multiplicity of infection) concentrations. The values of the columns are averages and shown with standard deviations. PE = PeptiENV; VACV = vaccinia virus only; CPP = CPP-SIINFEKL peptide only; uninfected = medium only

6.2 Animal experiments: tumour growth and survival

Tumour volume data was obtained to investigate tumour growth in PeptiENV VACV and HSV-1 animal experiments. Also, survival time was researched by collecting the euthanizing dates of the treated mice from both studies.

6.2.1 PeptiENV with vaccinia virus

Tumour growth curves from the PeptiENV VACV study are presented in Figure 5. They show that tumours treated with PeptiENV VACV CPP grow considerably slower than tumours that are injected with control substances.

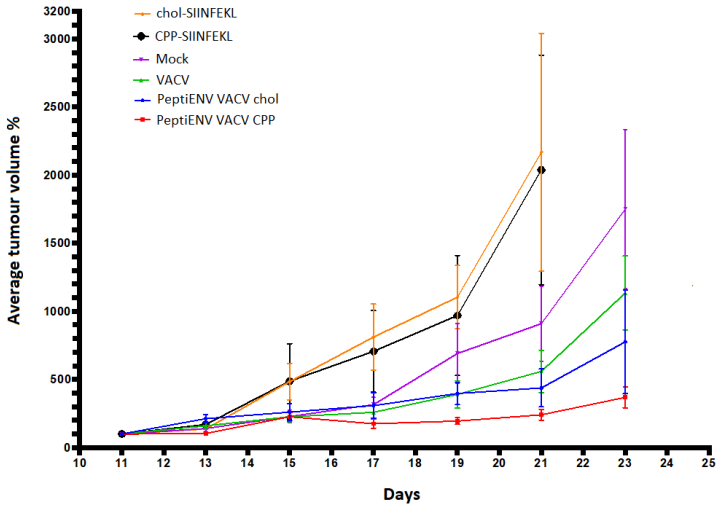

Figure 5. Tumour growth in different treatment groups in the PeptiENV VACV animal experiment. Sizes of the tumours were measured every other day and they are presented as average relative volumes (%) with standard deviations.

With PeptiENV VACV CPP tumours expanded on average only three-fold until day 23. The second-best treatments after PeptiENV VACV CPP is PeptiENV VACV chol and VACV only. Those treatments delay tumour growth to some extent, but still, on day 23 the mean volumes are 7 and 9 times larger than on day 11, respectively. Tumours treated with injection medium, CPP-SIINFEKL and chol-SIINFEKL only grow the fastest. On day 21, mean tumour volumes in the peptide only groups are 21 times larger than in the

beginning (day 11) while tumours injected with medium are on average 17 times bigger on day 23 compared to the start.

Also, the survival curves in Figure 6 and 7 indicate that PeptiENV VACV CPP is the best treatment because majority of the mice in that treatment group stayed alive the longest, until day 45. Mice injected with PeptiENV VACV chol were mostly sacrificed until day 35. Animals treated with chol- and CPP-SIINFEKL only were euthanized already on days 27 and 33, respectively. There is no difference in survival times between the virus only treatment and mock. In both groups all mice were sacrificed until day 35.

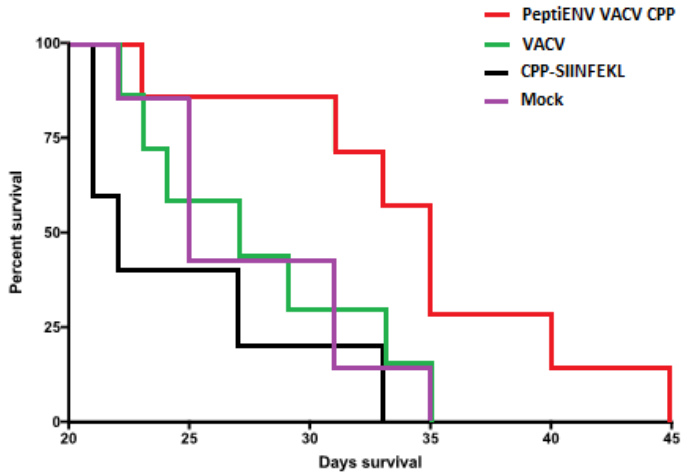

Figure 6. Survival percentages of different treatment groups in PeptiENV VACV experiment presented as Kaplan-Meier curve.

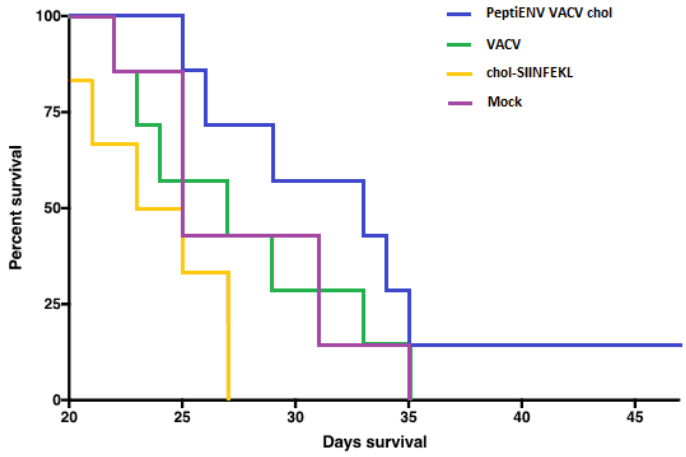

Figure 7. Survival percentages of different treatment groups in PeptiENV VACV experiment presented as Kaplan-Meier curve.

6.2.2 PeptiENV with herpes simplex virus type 1

According to tumour growth curves displayed in Figure 8, PeptiENV HSV-1 appears to inhibit tumour enlargement slightly more than CPP-SIINFEKL and HSV-1 only treatments. Injections were started on day 10, and on day 26 the average tumour volumes of PeptiENV and peptide only are 11 and 16 times larger than in the beginning, respectively. The difference is not unfortunately very significant. Instead, PeptiENV hinders tumour growth distinctly more than HSV-1 only. On day 22, mice treated with virus only has tumour sizes expanded on average to 12-fold while tumours injected with PeptiENV are approximately 6-fold on the same day.

CPP-SIINFEKL peptide slows the growth slightly more than HSV-1 but the difference is not quite clear. Tumours injected with the peptide only reaches the same mean volume as the tumours in the HSV-1 group but slightly later, approximately between days 22–24. Tumours in the mock group grow clearly the fastest. On day 20, the mean tumour volume is 24 times larger than on day 10.

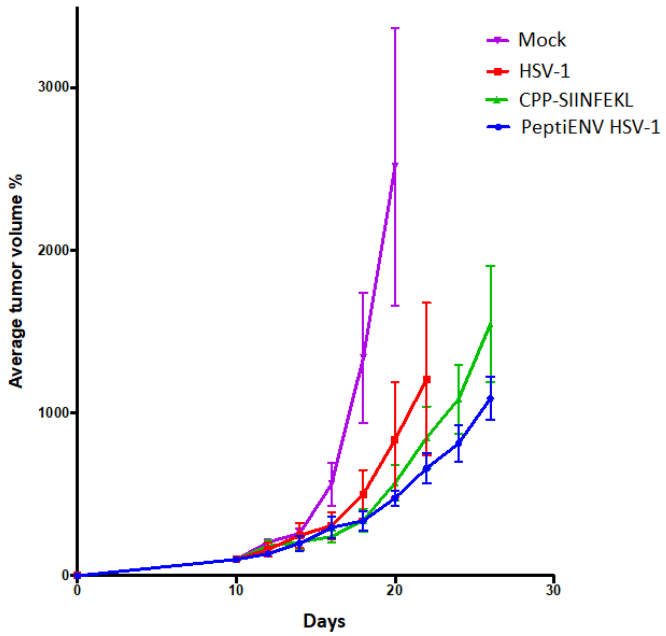

Figure 8. Tumour growth in the PeptiENV HSV-1 animal experiment. Size of the tumours were measured every other day and they are presented as average relative volumes (%) with standard deviations.

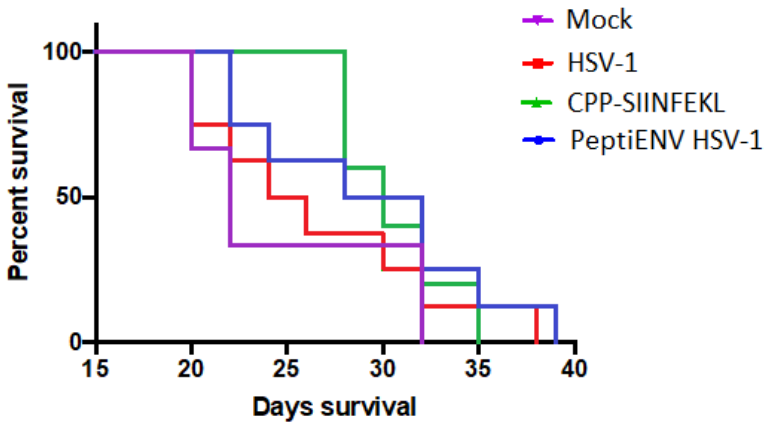

Figure 9. Survival percentages of different treatment groups in PeptiENV HSV-1 experiment presented as Kaplan-Meier curve.

Survival times of the mice seems to be partly in line with the growth rates; small differences are observed between PeptiENV, HSV-1 and CPP-SIINFEKL only treatments (Figure 8; Figure 9). Mice injected with PeptiENV stayed alive the longest, until day 38 and 39, respectively, whereas all mice treated with mock had to be sacrificed until day 32. As can be seen, the differences are not very significant.

6.3 Immunological analysis of tumours

The aim in these experiments was to determine the relative amount of tumour antigen SIINFEKL-specific CD8+ T cells in tumours collected from the animal experiments. The used method was extracellular staining of cell markers and flow cytometry.

6.3.1 PeptiENV with vaccinia virus

The proportional amounts of VACV- and tumour-specific CD8+ T cells are presented in Figure 10. Graph 10A shows that PeptiENV has the highest percentage of SIINFEKL-specific CD8+ T cells in the analysed tumour tissue compared to the control treatments.

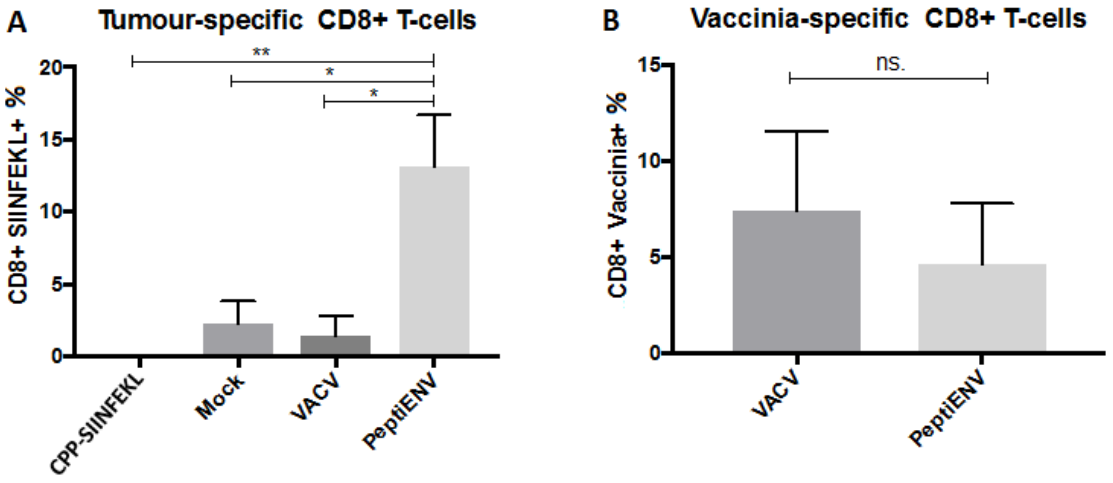

Figure 10. The relative amounts of tumour-specific (A) and VACV-specific (B) CD8+ T cells from total CD8+ population in tumours in the PeptiENV VACV experiment. The values of the columns are averages and displayed with standard deviations. Statistical analysis was performed with one-way ANOVA. $P < 0.05$*; $P < 0.01$**; ns. = not significant

The difference is statistically significant (Figure 10A). VACV only and mock groups also generates few tumour-specific CD8+ T cells, but in CPP-SIINFEKL only treatment group specific CD8+ T cells were not detected. According to the Figure 10B, the quantities of VACV-specific CD8+ T cells do not differ significantly between PeptiENV and VACV only groups.

6.3.2 PeptiENV with herpes simplex type 1

The results in Figure 11. suggest that tumours treated with PeptiENV HSV-1 have the biggest relative amount of SIINFEKL-specific CD8+ T cells compared to others. In turn, tumours injected with HSV-1 only does not seem induce tumour infiltration of SIINFEKL-specific CD8+ T cells at all. Mock and CPP-SIINFEKL only seem to elicit approximately the same amount of tumour-specific T cell-infiltration into the treated tumours.

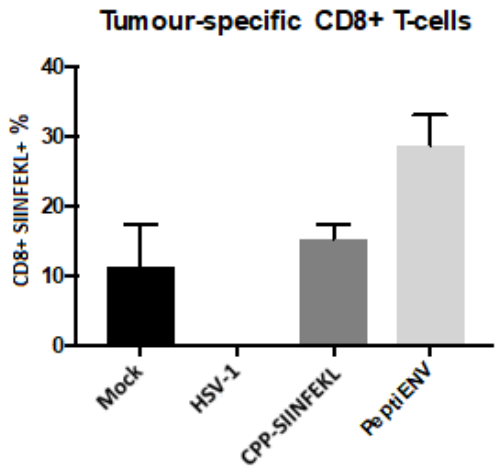

Figure 11. The relative amounts of SIINFEKL-specific CD8+ T cells from total CD8+ T cell population in tumours in the PeptiENV HSV-1 experiment. The values of the columns are calculated averages shown with standard deviations.

7 DISCUSSION

The PeptiENV platform was studied with two different viruses, VACV and HSV-1, which were coated with MHC class I antigen epitope, SIINFEKL. Tumour growth was investigated *in vivo* by using a murine melanoma model, and treatments were given as intratumoural injections. Beside PeptiENV, control therapies used were virus only, peptide only and mock (complexation/injection medium only). The survival time of the mice was also observed. Subsequently, the tumour tissues of the treated mice were analysed with flow cytometry to obtain the relative amount of SIINFEKL-specific CD8+ T cells.

7.1 Tumour growth and survival

According to Figure 5. the PeptiENV VACV CPP hinders tumour growth most efficiently, and the difference between PeptiENV VACV CPP and VACV only is significant. This result is quite similar than in the study with PeptiCRAd by Capasso et al. (2016) which suggests that conjugating an oncolytic virus with tumour antigens has an advantage compared to treating with virus only. The PeptiENV VACV chol on the other hand, does not differ from VACV only considerably. Probably one reason for this

is that chol-SIINFEKL is not presented by APCs as strongly as CPP-SIINFEKL (Ylösmäki et al. submitted). As told in chapter 2.1.1, antigen presentation with MHC molecules is vital in activating the adaptive immune system and effector CD8+ and CD4+ T cells (Murphy 2012). In other words, the PeptiENV VACV chol might affect mainly through the oncolytic functions of the virus.

Another interesting matter is that the growth rate of tumours treated with CPP- and chol-SIINFEKL only is the highest, even greater than with medium (Figure 5). The peptides however, are tumour antigens thus, they could have vaccine-like effects that might have slowed the growth speed at least more than mock. Although, usually peptide-vaccines are administered with adjuvants that activates and matures dendritic cells (Butterfield 2015). Hence, delivering tumour peptides as such could cause immunological tolerance which might be a possible reason for CPP- and chol-SIINFEKL not showing any effect.

The survivals of the mice are mostly in line with the tumour growth rates (Figure 5; Figure 6; Figure 7). Mice treated with PeptiENV VACV CPP lived the longest compared to control treatments. Virus, peptides and mock in turn did not prolong the lifetime of the mice distinctively when compared to each other. As in preventing tumour growth, PeptiENV VACV chol did not increase survival time much from VACV only (Figure 5; Figure 7). Also, animals treated with chol- and CPP-SIINFEKL peptides only had the most rapid tumour growth and they had to be euthanized the soonest too (Figure 5; Figure 6; Figure 7). The difference in these results compared to the growth curves is that VACV only does not seem to have any benefit compared to mock while it prevents tumour growth visibly more. The survival curves with these treatments are practically identical. May be that VACV hinders tumour enlargement only via oncolysis and is not able to induce a sufficient anti-tumour immunity, which is important for the clinical efficacy of oncolytic viruses (Kaufman et al. 2015). Hence, the survival might remain short even if tumour growth is prevented to some extent. In addition to the above said, the fact that PeptiENV VACV CPP prevents tumour enlargement and elongates survival the most indicates that PeptiENV therapy is beneficial over oncolytic treatment only. This observation is highly inciting.

The outcomes in the PeptiENV HSV-1 experiment resembles the results of the PeptiENV VACV study. The tumour growth rate is delayed by PeptiENV the most, and the difference compared to HSV-1 only is significant (Figure 8). The biggest variant is that CPP-SIINFEKL peptide averts the growth speed more than virus only, while in the PeptiENV VACV study the tumours expanded the most with peptide only treatments (Figure 5; Figure 8). Also, in this study, the difference in tumour growth rates between PeptiENV and peptide only is very small, whereas in the PeptiENV VACV experiment the PeptiENV VACV CPP clearly decreased tumour growth more than all control treatments. Furthermore, the survival of the mice in all treatment groups did not differ significantly from each other (Figure 9). Even though the tumour growth rate was obviously slower with PeptiENV than HSV-1 only, the survival times were virtually the same (Figure 8; Figure 9). Mice treated with CPP-SIINFEKL only had to be euthanized only slightly sooner than animals injected with PeptiENV and virus only (Figure 9).

The Kaplan-Meier curves and the perception that HSV-1 alone hinders tumour enlargement more weakly than CPP-SIINFEKL are obscure (Figure 8; Figure 9). As seen in the PeptiENV VACV study, the peptide only treatments did not have any improving effect on tumour growth or survival (Figures 5; Figure 6; Figure 7). The used CPP-SIINFEKL was exactly the same in both experiments therefore, the outcome in the PeptiENV HSV-1 study was expected to be similar with the PeptiENV VACV study. However, the tumour growth prevention with both HSV-1 only and PeptiENV compared to peptide only did not differ much, and survival is not prolonged in either groups (Figure 8; Figure 9).

The lack of efficiency of both virus only and PeptiENV treatments may be explained with the observation that HSV-1 does not replicate and cause oncolysis in the B16-OVA cells (Miller et al. 2001). An oncolytic virus destroys cancer cells and elicits anti-tumour immunity while single tumour peptides induce a very narrow immune response (Buonaguro et al. 2011; Kaufman et al. 2015). Therefore, in animal models in which the virus can replicate, an oncolytic virus would work better than single antigen epitope only, as seen in the PeptiENV VACV experiment. For this reason, the PeptiENV HSV-1 should

be investigated with more suitable model than B16-OVA in order to see bigger differences in the results.

Improving the properties of the HSV-1 might also affect the outcome. The FDA approved T-VEC has several genetic modifications to enhance cancer specificity and immunogenicity, when the HSV-1 that was used in this research had only the firefly luciferase gene inserted (Nygårdas et al. 2013; Andtbacka et al. 2015). It would be fascinating to see if different genetic changes of the HSV-1 would better the results. Presumably, the peptide conjugation should not affect the infectivity of the virus and thus, the efficiency of PeptiENV, according to the cell viability assay shown in section 6.1 (Figure 4). Although, that option cannot be entirely ruled out based on this study only. Nonetheless, by altering the qualities of HSV-1 and using a more convenient animal model, upgrade of the results is achievable. In the end and despite the small differences, PeptiENV seems to prevent tumour growth most efficiently.

7.2 Immunological analysis

Considering Figure 10 in the PeptiENV VACV study, it is clear that tumours injected with PeptiENV has the largest relative amount of SIINFEKL-specific CD8+ T cells which points that PeptiENV induces a major anti-tumour immune response. VACV also generates a few tumour-specific T cells, probably via lysis of the tumour cells, but the amount is insignificant since medium only has almost the same value. Tumours treated with peptide only have so few SIINFEKL-specific T cells that they were not able to be detected which suggests that the peptide alone does not cause very strong immune response.

When scrutinizing both Figures 10A and 10B, it can be seen that VACV only does elicit an immune response, but it is mainly against the virus. In the analysed tumours injected with virus only, the proportion of VACV-specific CD8+ T cells is higher than SIINFEKL-specific T cells. This supports the presented possible argument, discussed in section 7.1, why the virus only did not improve the survival compared to other control treatments even though it hinders tumour growth more. Important discovery is that the number of

virus-specific CD8+ T cells does not differ much between PeptiENV and VACV only treatments which points that fusing VACV with SIINFEKL peptides does not diminish the immune response against the virus (Figure 10A; Figure 10B). That is to say, the immune attack against VACV is similar whether using naked virus or PeptiENV but when using PeptiENV, the magnitude of tumour-specific T cell response increases. Consequently, the balance between anti-viral and anti-tumour immunity shifts towards the latter which is the aim in using oncolytic viruses and cancer vaccines (Butterfield 2015; Kaufman et al. 2015). This observation most likely explains a big part of the efficacy of PeptiENV VACV CPP in both preventing tumour growth and elongating the survival of the treated mice (Figure 5; Figure 6). In other words, induced anti-tumour immunity is a dominant mechanism of action with PeptiENV.

The relative amounts of SIINFEKL-specific CD8+ T cells in the PeptiENV HSV-1 experiment are also positive. Tumours injected with PeptiENV seem to have distinctly more SIINFEKL-specific T cells than other tumours treated with control substances (Figure 11). Previously in chapter 7.1, the properties of the HSV-1 were discussed. The immunological analysis indicates that the virus is at least immunogenic since the PeptiENV elicits higher anti-tumour immune response than peptide only, which has the same amount of SIINFEKL-specific CD8+ T cells than mock (Figure 11). Although, the used B16-OVA murine model is not ideal, the missing of HSV-1 oncolysis and the displayed results point that the efficacy of PeptiENV HSV-1 is primarily derived from the induced anti-tumour immunity.

Nevertheless, the absolute values are interesting. In the PeptiENV treated tumours, the amount of SIINFEKL-specific CD8+ T cells is approximately 30 % and thus extremely high (Figure 11). The numbers of control treatments are also quite large being between 10–15 % except HSV-1 in which no tumour-specific CD8+ T cells are detected. The values do not seem realistic since in the PeptiENV VACV experiment the PeptiENV treatment has absolute percentage of SIINFEKL-specific T cells circa 12.5 %, which is the highest value in that study (Figure 10). In addition, the PeptiENV treated mice lived significantly longer which is not the case in the PeptiENV HSV-1 research (Figure 6; Figure 9). If the PeptiENV elicits this high immune response against the tumour, it could

be presumed that it would be seen as more efficient tumour growth prevention and elongated survival of the mice.

On the other hand, the relative difference between the absolute values is not large (Figure 11). Tumours treated with PeptiENV HSV-1 has two- to three-fold the amount of tumour-specific CD8+ T cells compared to peptide only and mock whereas in the PeptiENV VACV experiment the difference between PeptiENV and other treatments is approximately 5-fold (Figure 10; Figure 11). Hence, the small differences are in line with the narrow variations in the tumour growth and the survival curves (Figure 8; Figure 9; Figure 11). It is possible that the zero-result of HSV-1 and the large absolute percentages derive from insufficient number of detectable events in the flow cytometry which leads to singular events having a big impact on the final values. Because of this, the results from the PeptiENV HSV-1 immunological analysis are mainly directional but still hopeful.

To produce more accurate results in the measurements of the tumour-specific CD8+ T cells, the mice should be euthanized on a predestined day. The obtaining of the Kaplan-Meier curves however, demands that the animals are sacrificed when a defined endpoint is reached. Studying the survival is important, but the disadvantage is that the euthanizing dates of the mice are distinctive. This can have an impact on the outcome since the animals may be in different stages considering the development of the immune response. The progression of the response is demonstrated in the study by Capasso et al. (2016) in which the relative amounts of SIINFEKL-specific CD8+ T cells varied depending on whether the mice were sacrificed early or later in the experiment. Because observing the survival is also crucial, an option would be executing an experiment with large enough number of mice to acquire both analyses. Of course, the ethical rules of animal experiments should also be noticed.

Altogether, the results from both analyses are positive. PeptiENV clearly increases the amount of tumour-specific CD8+ T cells in the malignant tissue. This is extremely relevant since anti-tumour immunity is the basic objective in cancer immunotherapy, and

it has been discovered that in many cancers large number of tumour infiltrating CD8+ T cells is connected to favourable prognosis (Pagès et al. 2010; Papaioannou et al. 2016).

7.3 General assessment

As presented, the results in the PeptiENV VACV study are better than in the HSV-1 experiment. One already discussed reason might be the insufficiency of the B16-OVA model for HSV-1. Instead, the model suits well for VACV because the virus is able to lyse the cells efficiently as seen in the cell viability assay shown in section 6.1 (Figure 4). This can explain the superiority of the PeptiENV VACV compared to PeptiENV HSV-1. Furthermore, as told in chapter 5.1, the HSV-1 used had very minimal genetic modifications, whereas the VACV had insertions for improving tumour cell specific replication and immunogenicity. These qualities are very important for oncolytic viruses, as discussed in chapter 3.3. By developing the properties of the HSV-1 further and choosing a better model, obtaining more significant results is feasible and thus, repetition of the animal study in the future is rational. Still, thorough *in vitro* investigations in advance are necessary to ensure the proper functioning of the virus.

Although the outcomes in both experiments are quite satisfactory, especially in the PeptiENV VACV study, it has to be noted that the murine melanoma model (B16-OVA) used is artificial and does not represent an authentic situation. Utilizing preclinical models that are as close as possible to human physiology aids the prediction of how developed treatments will conduct in patients. Therefore, to verify the performance of the platform it would be reasonable to repeat the experiments with more realistic cancer models. For PeptiENV VACV study murine melanoma model B16-F10 would be appropriate since the cells express true melanoma antigens instead of artificial ones (Capasso et al. 2016).

Hence the HSV-1 replicates poorly in the B16 cells, a completely different model could be tested. Workenhe et al. (2014) studied an oncolytic HSV-1 with murine HER-2/neu breast cancer cell line (TUBO) and demonstrated that the virus lyses the cells and replicates in them *in vitro*. The used HSV-1 shows also therapeutic efficacy *in vivo* by inhibiting the growth of implanted tumours in mice. In addition, it induces HER-2-

specific anti-tumour immune response. The TUBO cell line might be a good model for PeptiENV HSV-1 experiment too. However, using an HSV-1 pentamer in the immunological analysis would be important because it would demonstrate the magnitude of the immune response against the virus, as in the PeptiENV VACV study, and verify the mode of action. Even more genuine approach for both experiments would be using humanized mice bearing human derived tumours. Moreover, because the tumour-antigen spectrum is very heterogenous, conjugating several kinds of TSAs onto the viral envelope might enhance the immune attack against the tumour tissue and improve results.

It would also be interesting to integrate the prime-boost concept discussed in chapter 3.4 with PeptiENV. In other words, different viruses would be utilized as the base of the platform, but the attached TSAs would remain the same. As told, Bridle (2010) and Pol (2014) with their teams applied the concept to their studies and managed to considerably increase the amount of tumour-specific CD8+ T cells in the tumour and blood, respectively. Additionally, the survival time of the prime-boost treated mice prolonged significantly. In the PeptiENV VACV animal experiment the same treatments were given as boosting treatments too thus, changing the viral backbone in the first or later treatments could improve the outcome even further. Because the results of the PeptiENV HSV-1 experiment are relatively good, the HSV-1 may be suitable to be joined together with VACV in the future. Albeit, it was noted that using replicating viruses as primers might not work therefore, non-replicating version of PeptiCRAd could also be a valid option for priming (Bridle et al 2010; Pol et al. 2014). As discussed in section 3.4.1, the ability of it to induce a strong anti-tumour immune response has been addressed.

Cancer immunotherapies are constantly used as compositions which is why examining the efficiency of PeptiENV with other treatments is subsequently essential (Butterfield 2015; Papaioannou et al. 2016). For example, a very potential combination would be using PeptiENV with ICIs. The main purpose of PeptiENV is to induce a sufficient and specific anti-tumour immune response however, the platform does not have initial tools for overcoming the immunosuppressive tumour microenvironment. In turn, the ICIs are designed to block the T cell function impeding signals, but in order to work adequately they require a pre-existing anti-tumour T cell response (Obeid et al. 2015; Papaioannou

et al. 2016). The PeptiENV can provide that and thus, the two treatments could reinforce each other well. Furthermore, it is possible that PeptiENV would broaden the number of patients responding to ICI therapy.

In addition to be an efficient and specific cancer treatment, the PeptiENV has been developed to function as a personalized immunotherapy platform. In other words, identified neoantigens of a patient would be attached to the envelope of the oncolytic virus. A major benefit is that the conjugation of peptides to the envelope is easier than modifying the viral genome which alleviates the use of variable antigens. The attachment of the SIINFEKL peptide has been successful, and the complexation process is quite simple as described in section 5.3 (Ylösmäki et al. submitted). A forthcoming challenge can rather perhaps be the production of the neoantigen epitopes. At present, it requires several steps before appropriate antigens are identified and can be transferred to a vaccine platform (Zhang et al. 2017). Individual tumour might contain thousands of mutations and all of them are not expressed. Hence, the neoantigen expression needs to be confirmed and also their binding to relevant MHC molecules. After, the epitopes must be further validated with *in vitro* studies. On the other hand, the technology has been dramatically improved and earlier this kind of neoantigen identification was extremely hard. Nevertheless, and considering the future, the manufacturing should be flexible and not too time-consuming or expensive.

When viewing both animal experiments, tumour growths and immunological analyses, it is inciteful to see that the outcomes are quite similar despite the small differences presented. Especially PeptiENV VACV study showed that the combination of an oncolytic virus and TSAs is more efficient treatment than virus only. The HSV-1 experiment did not produce as good results as the VACV, but the outcome is developable. Above all, the platform requires additional investigations for verifying the results displayed in this thesis and for further improvements.

8 CONCLUSION

The PeptiENV platform was studied with VACV and HSV-1. Tumour-specific antigen epitope SIINFEKL was coated onto the envelopes of the viruses by using CPP or cholesterol anchor. By using VACV and CPP-SIINFEKL, it was shown that conjugation of the peptide does not decrease the infectivity of the virus. Additionally, in the first animal experiment, the PeptiENV VACV CPP is the best treatment since it prevents tumour growth remarkably more than virus, peptides or injection medium only. PeptiENV VACV CPP elicits significantly stronger tumour-specific immune response than control treatments as well. Conjugation of peptides does not reduce the immune response but in turn shifts it towards the tumour instead of the virus. In addition, PeptiENV VACV CPP clearly prolongs the survival of tumour bearing mice.

In the second animal study, PeptiENV HSV-1 hinders tumour enlargement slightly more than virus and peptide only, and the difference is distinct when compared to HSV-1 only. However, there was no considerable variations in the survival times of the treated mice. PeptiENV HSV-1 also engenders a tumour-specific immune response, but the result is mainly directional. Currently, the PeptiENV platform seems to function with VACV more efficiently than with HSV-1. Nevertheless, further investigations are needed regarding both viruses. Overall, the results from both animal experiments are very encouraging and hence, the PeptiENV platform has remarkable potential in future cancer immunotherapy developments.

REFERENCES

Aggarwal BB, Vijayalekshmi RV, Sung B: Targeting inflammatory pathways for prevention and therapy of cancer: short-term friend, long-term foe. Clin Cancer Res 15(2): 425–430, 2009

Andtbacka RHI, Kaufman HL, Collichio F, Amatruda T, Senzer N, Chesney J, Delman KA, Spitler LE, Puzanov I, Agarwala SS, Milhem M, Cranmer L, Curti B, Lewis K, Ross M, Guthrie T, Linette GP, Daniels GA, Harrington K, Middleton MR, Miller J, Wilson H, Zager JS, Ye Y, Yao B, Li A, Doleman S, VanderWalde A, Gansert J, Coffin RS: Talimogene laherparepvec improves durable response rate in patients with advanced melanoma. J Clin Oncol 33(25): 2780–2788, 2015

Aranda F, Vacchelli E, Eggermont A, Galon J, Sautès-Fridman C, Tartour E, Zitvogel L, Kroemer G, Galluzzi L: Trial watch. OncoImmunology 2(12): e26621, 2013

Baskar R, Lee KA, Yeo R, Yeoh K. Cancer and radiation therapy: current advances and future directions. Int J Med Sci 9(3): 193–199, 2012

Becker J, Andersen M, Schrama D, thor Straten P: Immune-suppressive properties of the tumor microenvironment. Cancer Immunol Immunother 62(7): 1137–1148, 2013

Breitbach CJ, Lichty BD, Bell JC: Oncolytic Viruses: therapeutics with an identity crisis. EBioMedicine 9: 31–36, 2016

Bridle BW, Stephenson KB, Boudreau JE, Koshy S, Kazdhan N, Pullenayegum E, Brunellière J, Bramson JL, Lichty BD, Wan Y: Potentiating cancer immunotherapy using an oncolytic virus. Mol Ther 18(8): 1430–1439, 2010

Buonaguro L, Petrizzo A, Tornesello ML, Buonaguro FM: Translating tumor antigens into cancer vaccines. Clin Vaccine Immunol 18(1): 23–34, 2011

Butterfield L: Cancer vaccines. BMJ 350: h988, 2015

Capasso C, Hirvinen M, Garofalo M, Romaniuk D, Kuryk L, Sarvela T, Vitale A, Antopolsky M, Magarkar A, Viitala T, Suutari T, Bunker A, Yliperttula M, Urtti A, Cerullo V: Oncolytic adenoviruses coated with MHC-I tumor epitopes increase the antitumor immunity and efficacy against melanoma. Oncoimmunology 5(4): e1105429, 2016

Chiocca EA, Rabkin SD: Oncolytic viruses and their application to cancer immunotherapy. Cancer Immunol Res 2(4): 295–300, 2014

ClinicalTrials.gov (a): A phase 3 randomized, open-label study comparing Pexa-Vec (vaccinia GM-CSF/thymidine kinase-deactivated virus) followed by sorafenib versus sorafenib in patients with advanced hepatocellular carcinoma (HCC) without prior systemic therapy, NCT02562755. (cited 6.2.2018) Available on the Internet: www.clinicaltrials.gov

ClinicalTrials.gov (b): Ipilimumab with or without talimogene laherparepvec in unresected melanoma, NCT01740297. (cited 16.2.2018) Available on the Internet: www.clinicaltrials.gov

ClinicalTrials.gov (c): MG1 Maraba/MAGE-A3, with and without adenovirus vaccine, with transgenic MAGE-A3 insertion in patients with incurable MAGE-A3-expressing solid tumours (I214), NCT02285816. (cited 13.2.2018) Available on the Internet: www.clinicaltrials.gov

ClinicalTrials.gov (d): Pembrolizumab With or Without Talimogene Laherparepvec or Talimogene Laherparepvec Placebo in Unresected Melanoma (KEYNOTE-034), NCT02263508. (cited 16.4.2018) Available on the Internet: www.clinicaltrials.gov

ClinicalTrials.gov (e): 11 Studies found for: Active, not recruiting, Completed studies, Interventional studies, Cancer, T-VEC. Searched 6.2.2018. Available on the Internet: www.clinicaltrials.gov

Comin-Anduix B, Escuin-Ordinas H, Ibarrondo FJ: Tremelimumab: research and clinical development. Onco Targets Ther 9: 1767–1776, 2016

Dunn GP, Old LJ, Schreiber RD: The immunobiology of cancer immunosurveillance and immunoediting. Immunity 21(2): 137–148, 2004(a)

Dunn GP, Old LJ, Schreiber RD. The three Es of cancer immunoediting. Annu Rev Immunol 22(1): 329–360, 2004(b)

Elsedawy NB, Russell SJ: Oncolytic vaccines. Expert Rev Vaccines 12(10): 1155–1172, 2013

Gubin MM, Artyomov MN, Mardis ER, Schreiber RD: Tumor neoantigens: building a framework for personalized cancer immunotherapy. J Clin Invest 125(9): 3413–3421, 2015

Hamid O, Hoffner B, Gasal E, Hong J, Carvajal R: Oncolytic immunotherapy: unlocking the potential of viruses to help target cancer. Cancer Immunol Immunother 66(10): 1249–1264, 2017

Hanahan D, Weinberg R: Hallmarks of cancer. Cell 100(1): 57–70, 2000

Hanahan D, Weinberg R: Hallmarks of cancer: the next generation. Cell 144(5): 646–674, 2011

Hirvinen M, Capasso C, Guse K, Garofalo M, Vitale A, Ahonen M, Kuryk L, Vaehae-Koskela M, Hemminki A, Fortino V, Greco D, Cerullo V: Expression of DAI by an oncolytic vaccinia virus boosts the immunogenicity of the virus and enhances antitumor immunity. Mol Ther Oncolytics 3: 16002, 2016

Hodi FS, O'Day SJ, McDermott DF, Weber RW, Weber JS, Sosman JA, Haanen JB, Gonzalez R, Robert C, Schadendorf D, Hassel JC, Akerley W, van den Eertwegh, Alfons JM, Lutzky J, Lorigan P, Vaubel JM, Linette GP, Hogg D, Ottensmeier CH, Lebbé C, Peschel C, Quirt I, Clark JI, Wolchok JD, Tian J, Yellin MJ, Nichol GM, Hoos A, Urba WJ: Improved survival with ipilimumab in patients with metastatic melanoma. N Engl J Med 363(8): 711–723, 2010

Heo J, Reid T, Ruo L, Breitbach CJ, Rose S, Bloomston M, Cho M, Lim HY, Chung HC, Kim CW, Burke J, Lencioni R, Hickman T, Moon A, Lee YS, Kim MK, Daneshmand M, Dubois K, Longpre L, Ngo M, Rooney C, Bell JC, Rhee B, Patt R, Hwang T, Kirn DH: Randomized dose-finding clinical trial of oncolytic immunotherapeutic vaccinia JX-594 in liver cancer. Nat Med 19(3): 329–336, 2013

Ito A, Kondo S, Tada K, Kitano S: Clinical development of immune checkpoint inhibitors. Biomed Res Int 2015: 605478, 2015

Kantoff PW, Higano CS, Shore ND, Berger ER, Small EJ, Penson DF, Redfern CH, Ferrari AC, Dreicer R, Sims RB, Xu Y, Frohlich MW, Schellhammer PF: Sipuleucel-T immunotherapy for castration-resistant prostate cancer. N Engl J Med 363(5): 411–422, 2010

Kaufman HL, Deraffele G, Mitcham J, Moroziewicz D, Cohen SM, Hurst-Wicker KS, Cheung K, Lee DS, Divito J, Voulo M, Donovan J, Dolan K, Manson K, Panicali D, Wang E, Hörig H, Marincola FM: Targeting the local tumor microenvironment with vaccinia virus expressing B7.1 for the treatment of melanoma. J Clin Invest 115(7): 1903–1912, 2005

Kaufman HL, Kohlhapp FJ, Zloza A: Oncolytic viruses: a new class of immunotherapy drugs. Nat Rev Drug Discov 14(9): 642–662, 2015

Koster BD, de Gruijl TD, van den Eertwegh AJM: Recent developments and future challenges in immune checkpoint inhibitory cancer treatment. Curr Opin Oncol 27(6): 482–488, 2015

Kottke T, Errington F, Pulido J, Galivo F, Thompson J, Wongthida P, Diaz RM, Chong H, Ilett E, Chester J, Pandha H, Harrington K, Selby P, Melcher A, Vile R: Broad antigenic coverage induced by vaccination with virus-based cDNA libraries cures established tumors. Nat Med 17(7): 854–859, 2011

Lichty BD, Breitbach CJ, Stojdl DF, Bell JC: Going viral with cancer immunotherapy. Nat Rev Cancer 14(8): 559–567, 2014

Liu BL, Robinson M, Han ZQ, Branston RH, English C, Reay P, McGrath Y, Thomas SK, Thornton M, Bullock P, Love CA, Coffin RS: ICP34.5 deleted herpes simplex virus with enhanced oncolytic, immune stimulating, and anti-tumour properties. Gene Ther 10(4): 292–303, 2003

Mantovani A, Allavena P, Sica A, Balkwill F: Cancer-related inflammation. Nature 454: 436–444, 2008

Melero I, Gaudernack G, Gerritsen WR, Huber C, Parmiani G, Scholl S, Thatcher N, Wagstaff J, Zielinski C, Faulkner I, Mellstedt H: Therapeutic vaccines for cancer: an overview of clinical trials. Nat Rev Clin Oncol 11(9): 509–524, 2014

Miller CG, Krummenacher C, Eisenberg RJ, Cohen GH, Fraser NW: Development of a syngenic murine B16 cell line-derived melanoma susceptible to destruction by neuroattenuated HSV-1. Mol Ther 3(2): 160–168, 2001

Mittal D, Gubin MM, Schreiber RD, Smyth MJ: New insights into cancer immunoediting and its three component phases--elimination, equilibrium and escape. Curr Opin Immunol 27: 16–25, 2014

Murphy K: Janeway's immunobiology. 8th edition. Garland Science, Taylor & Francis Group, LLC, New York and Abingdon 2012

National Cancer Institute: What is Cancer? February 2015 (cited 30.10.2017). Available on the Internet: www.cancer.gov

National Cancer Institute: Types of Cancer Treatment. April 2017 (cited 30.10.2017). Available on the Internet: www.cancer.gov

Netea MG, Quintin J, van der Meer JWM: Trained immunity: A memory for innate host defense. Cell Host Microbe 9(5): 355–361, 2011

Nygårdas M, Paavilainen H, Müther N, Nagel CH, Röyttä M, Sodeik B, Hukkanen V. A herpes simplex virus-derived replicative vector expressing LIF limits experimental demyelinating disease and modulates autoimmunity. PLoS One 8(5): e64200, 2013

Obeid JM, Hu Y, Slingluff Jr. CL: Vaccines, adjuvants and dendritic cell activators – current status and future challenge. Semin Oncol 42: 549–561, 2015

Papaioannou NE, Beniata OV, Vitsos P, Tsitsilonis O, Samara P: Harnessing the immune system to improve cancer therapy. Anna Transl Med 4(14): 261, 2016

Pagès F, Galon J, Dieu-Nosjean MC, Tartour E, Sautès-Fridman C, Fridman WH: Immune infiltration in human tumors: a prognostic factor that should not be ignored. Oncogene 29(8): 1093–1102, 2010

Patel A, Kaufman HL, Disis ML: Next generation approaches for tumor vaccination. Chin Clin Oncol 6(2): 19, 2017

Pol JG, Zhang L, Bridle BW, Stephenson KB, Resseguier J, Hanson S, Chen L, Kazdhan N, Bramson JL, Stojdl DF, Wan Y, Lichty BD: Maraba virus as a potent oncolytic vaccine vector. Mol Ther 22(2): 420–429, 2014

Puzanov I, Milhem MM, Minor D, Hamid O, Li A, Chen L, Chastain M, Gorski KS, Anderson A, Chou J, Kaufman HL, Andtbacka RHI: Talimogene laherparepvec in combination with ipilimumab in previously untreated, unresectable stage IIIB-IV melanoma. J Clin Oncol 34(22): 2619–2626, 2016

Ribas A, Dummer R, Puzanov I, VanderWalde A, Andtbacka RHI, Michielin O, Olszanski AJ, Malvehy J, Cebon J, Fernandez E, Kirkwood JM, Gajewski TF, Chen L, Gorski KS, Anderson AA, Diede SJ, Lassman ME, Gansert J, Hodi FS, Long GV: Oncolytic virotherapy promotes intratumoral T cell infiltration and improves anti-PD-1 immunotherapy. Cell 170(6): 1109–1119, 2017

Rizvi NA, Hellmann MD, Snyder A, Kvistborg P, Makarov V, Havel JJ, Lee W, Yuan J, Wong P, Ho TS, Miller ML, Rekhtman N, Moreira AL, Ibrahim F, Bruggeman C, Gasmi B, Zappasodi R, Maeda Y, Sander C, Garon EB, Merghoub T, Wolchok JD, Schumacher TN, Chan TA: Cancer immunology. Mutational landscape determines sensitivity to PD-1 blockade in non-small cell lung cancer. Science 348(6230): 124–128, 2015

Robert C, Thomas L, Bondarenko I, O'Day S, M.D JW, Garbe C, Lebbe C, Baurain J, Testori A, Grob J, Davidson N, Richards J, Maio M, Hauschild A, Miller WH, Gascon P, Lotem M, Harmankaya K, Ibrahim R, Francis S, Chen T, Humphrey R, Hoos A, Wolchok JD: Ipilimumab plus dacarbazine for previously untreated metastatic melanoma. N Engl J Med 364(26): 2517–2526, 2011

Sharma P, Allison JP: The future of immune checkpoint therapy. Science 348: 56–61, 2015

Smith GL, Benfield CT, Maluquer de Motes C, Mazzon M, Ember SW, Ferguson BJ, Sumner RP: Vaccinia virus immune evasion: mechanisms, virulence and immunogenicity. J Gen Virol 94(Pt11): 2367–2392, 2013

Smith GL, Vanderplasschen A, Law M: The formation and function of extracellular enveloped vaccinia virus. J Gen Virol 83: 2915–2931, 2002

Snyder A, Makarov V, Merghoub T, Yuan J, Zaretsky JM, Desrichard A, Walsh LA, Postow MA, Wong P, Ho TS, Hollmann TJ, Bruggeman C, Kannan K, Li Y, Elipenahli C, Liu C, Harbison CT, Wang L, Ribas A, Wolchok JD, Chan TA. Genetic basis for clinical response to CTLA-4 blockade in melanoma. N Engl J Med 371(23): 2189–2199, 2014

Tacken PJ, de Vries IJM, Torensma R, Figdor CG: Dendritic-cell immunotherapy: from ex vivo loading to in vivo targeting. Nat Rev Immunol 7: 790–802, 2007

Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, Powderly JD, Carvajal RD, Sosman JA, Atkins MB, Leming PD, Spigel DR, Antonia SJ, Horn L, Drake CG, Pardoll DM, Chen L, Sharfman WH, Anders RA, Taube JM, McMiller TL, Xu H, Korman AJ, Jure-Kunkel M, Agrawal S, McDonald D, Kollia GD, Gupta A, Wigginton JM, Sznol M: Safety, activity, and immune correlates of anti–PD-1 antibody in cancer. N Engl J Med 366(26): 2443–2454, 2012

Torry DS, Cooper GM. Proto-oncogenes in development and cancer. Am J Reprod Immunol 25(3): 129–132, 1991

U.S. Food and Drug Administration (FDA): Approval Letter –Imlygic® (talimogene laherparepvec). Approved Cellular and Gene Therapy Products. October 2015 (cited 26.1.2018). Available on the Internet: www.fda.gov

U.S. Food and Drug Administration (FDA): Approval Letter – Provenge® (sipuleucel-T). Approved Cellular and Gene Therapy Products. April 2010 (cited 16.1.2018). Available on the Internet: www.fda.gov

U.S. Food and Drug Administration (FDA) (a): Center for Drug Evaluation and Research: Approval package for Keytruda® (pembrolizumab). Drugs@FDA: FDA Approved Drug Products (database). September 2014 (cited 8.1.2018). Available on the Internet: www.fda.gov

U.S. Food and Drug Administration (FDA) (b): Center for Drug Evaluation and Research: Approval package for Opdivo® (nivolumab). Drugs@FDA: FDA Approved Drug Products (database). December 2014 (cited 8.1.2018). Available on the Internet: www.fda.gov

U.S. Food and Drug Administration (FDA): Center for Drug Evaluation and Research: Approval package for Yervoy® (ipilimumab). Drugs@FDA: FDA Approved Drug Products (database). March 2011 (cited 5.1.2018). Available on the Internet: www.fda.gov

U.S. Food and Drug administration (FDA) (a): FDA approval brings first gene therapy to the United States. August 2017 (cited 5.1.2018). Available on the Internet: www.fda.gov

U.S. Food and Drug Administration (FDA) (b): FDA approves CAR-T cell therapy to treat adults with certain types of large B-cell lymphoma. October 2017 (cited 5.1.2018). Available on the Internet: www.fda.gov

U.S. Food and Drug Administration (FDA): Proleukin® (aldesleukin) injection label. Drugs@FDA: FDA Approved Drug Products (database). July 2012 (cited 5.1.2018). Available on the Internet: www.fda.gov

Vinay DS, Ryan EP, Pawelec G, Talib WH, Stagg J, Elkord E, Lichtor T, Decker WK, Whelan RL, , Shantha Kumara HMC, Signori E, Honoki K, Georgakilas AG, Amin A, Helferich WG, Boosani CS, Guha G, Ciriolo MR, Chen S, Mohammed SI, Azmi AS, Keith WN, Bilsland A, Bhakta D, Halicka D, Fujii H, Aquilano K, Ashraf SS, Nowsheen S, Yang X, Choi BK, Kwon BS: Immune evasion in cancer: mechanistic basis and therapeutic strategies. Semin Cancer Biol 35: 185–198, 2015

Voena C, Chiarle R: Advances in cancer immunology and cancer immunotherapy. Discov Med 21(114): 125–133, 2016

Välimaa H, Seppänen M, Hukkanen V: Herpes simplex -virusinfektioiden nykykuva ja hoito. Duodecim 129: 31–40, 2013

Woller N, Gürlevik E, Ureche CI, Schumacher A, Kühnel F: Oncolytic viruses as anticancer vaccines. Front Oncol 4(188): 1–13, 2014

Workenhe S, Simmons G, Pol JG, Lichty BD, Halford WP, Mossman KL: Immunogenic HSV-mediated oncolysis shapes the antitumor immune response and contributes to therapeutic efficacy. Mol Ther 22(1): 123–131, 2014

World Health Organization (WHO): Cancer. Fact Sheet. February 2017 (cited 25.10.2017). Available on the Internet: www.who.int

Yang Y: Cancer immunotherapy: harnessing the immune system to battle cancer. J Clin Invest 125(9): 3335–3337, 2015

Yatim KM, Lakkis FG: A brief journey through the immune system. Clin J Am Soc Nephrol 10(7): 1274–1281, 2015

Zamarron BF, Chen W: Dual roles of immune cells and their factors in cancer development and progression. Int J Biol Sci 7(5): 651–658, 2011

Zhang X, Sharma PK, Goedegebuure SP, Gillanders WE: Personalized cancer vaccines: targeting the cancer mutanome. Vaccine 35(7): 1094–1100, 2017

Zhu Y, Chen L: Turning the tide of lymphocyte costimulation. J Immunol 182(5): 2557–2558, 2009