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USING SFV-BASED VECTORS, MELANOMA-SPECIFIC ANTIBODIES AND IL-2/VLPs IN MELANOMA IMMUNE THERAPY

Master’s Thesis

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# Table of contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abbreviations</td>
<td>4</td>
</tr>
<tr>
<td>Introduction</td>
<td>5</td>
</tr>
<tr>
<td>1. Background</td>
<td>7</td>
</tr>
<tr>
<td>1.1. Genetic basis for pathogenesis of cancer</td>
<td>7</td>
</tr>
<tr>
<td>1.2. Genetic vaccination</td>
<td>8</td>
</tr>
<tr>
<td>1.2.1. Naked DNA vaccination</td>
<td>8</td>
</tr>
<tr>
<td>1.2.2. SFV vector system</td>
<td>9</td>
</tr>
<tr>
<td>1.2.2.1. Semliki forest virus</td>
<td>9</td>
</tr>
<tr>
<td>1.2.2.2. Alphaviruses as self-replicating vectors</td>
<td>11</td>
</tr>
<tr>
<td>1.2.2.3. Less cytopathic vectors</td>
<td>15</td>
</tr>
<tr>
<td>1.2.2.4. SFV vectors in gene therapy</td>
<td>17</td>
</tr>
<tr>
<td>1.3. Tyrosinase-related protein-2 as a tumor antigen</td>
<td>19</td>
</tr>
<tr>
<td>1.3.1. Tyrosinase-related protein-2</td>
<td>19</td>
</tr>
<tr>
<td>1.3.2. Tyrosinase-related protein-2 in antitumor therapy</td>
<td>21</td>
</tr>
<tr>
<td>1.4. Anti-tyrosinase antibodies in cancer therapy</td>
<td>23</td>
</tr>
<tr>
<td>1.5. Interleukin-2 in cancer therapy</td>
<td>25</td>
</tr>
<tr>
<td>2. Experimental part</td>
<td>27</td>
</tr>
<tr>
<td>2.1. Aim of the study</td>
<td>27</td>
</tr>
<tr>
<td>2.2. Materials and methods</td>
<td>28</td>
</tr>
<tr>
<td>2.2.1. Used plasmids</td>
<td>28</td>
</tr>
<tr>
<td>2.2.2. Used cell lines</td>
<td>29</td>
</tr>
<tr>
<td>2.2.3. Western blotting</td>
<td>29</td>
</tr>
<tr>
<td>2.2.4. In vitro transcription</td>
<td>30</td>
</tr>
<tr>
<td>2.2.5. RNA electroporation</td>
<td>31</td>
</tr>
<tr>
<td>2.2.6. Harvesting VLP</td>
<td>32</td>
</tr>
<tr>
<td>2.2.7. Infection of BHK-21 cells with VLPs</td>
<td>32</td>
</tr>
<tr>
<td>2.2.8. Immunofluorescent stainig of cells</td>
<td>32</td>
</tr>
<tr>
<td>2.2.9. Preparation of DNA/gold cartridges</td>
<td>33</td>
</tr>
</tbody>
</table>
2.2.10. Tumor inoculation.................................................................34
2.2.11. DNA injection.................................................................34
2.2.12. Antibody injection............................................................35
2.2.13. IL-2 and SFV VLP injection...............................................35
2.2.14. Enzyme-linked immunosorbent assay..................................35
2.2.15. ELISpot............................................................................36

2.3. Results.........................................................................................38
2.3.1. Western blot........................................................................38
2.3.2. DNA injection......................................................................38
2.3.3. Antibody injection.................................................................41
2.3.4. Vaccination with wild-type and mutated SFV vectors..................44
2.3.5. Vaccination by i.t. administration of VLPs and IL-2..................47
2.3.6. Enzyme-linked immunosorbent assay....................................48
2.3.7. ELISpot.................................................................................50

2.4. Discussion...................................................................................53

Acknowledgements...........................................................................55

Conclusion.........................................................................................56

Résumé............................................................................................58

References........................................................................................60
Abbreviations

ADCC – antibody-dependent cellular cytotoxicity
APC – antigen-presenting cell
BHK-21 – baby hamster kidney cell line
CDC – complement-dependent cytotoxicity
CMV – cytomegalovirus
DC – dendritic cell
FCS – fetal calf serum
HSP – heat-shock protein
IFN-γ – interferon γ
MHC – major histocompatibility complex
NK – natural killer cell
PBS – phosphate buffered saline
PFA – paraformaldehyde
PKR – protein kinase R
VLP – virus-like particles
**Introduction**

Melanoma is less common than the familiar basal and squamous cell tumors of the skin, but much more fatal. In Europe, approximately 26,100 males and 33,300 females are diagnosed each year with melanoma, and around 8,300 males and 7,600 females die of it [1] (Fig. 1). It is the 8th most commonly diagnosed cancer in females and 17th in males. Melanoma has shown some of the fastest rates of secular increase in incidence in white populations, and has a complicated relationship with sun exposure, that is not completely understood. When melanomas are detected at early stages they are curable, but once advanced they are very difficult to treat[2].

There has been a lot of studies performed using vaccination with different antigens of melanoma encoded by different vectors, vaccination with VLPs, antibodies, systemic administration of IL-2.

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**Figure 1.** Time trends of malignant melanoma in males and females in Europe countries. (E. De Vries, EnCR Cancer Fact Sheets, 2003)
In this study, in the background part genetic vaccination against melanoma will be discussed, as well as a short review will be given on the known treatments – like antigens and antibodies, and interleukin-2.

In the practical part some studies that we have performed are described, such as treatment of mice with inoculated tumors with different antigens and antibodies and combined vaccinations and comparing their efficacy.
1. Background

1.1. Genetic basis for pathogenesis of cancer

Understanding and achieving immunity to cancer does not fit nearly into the self/nonself paradigm because cancer is not an exogenous pathogen, but rather arises from normal host cells. In this regard, cancer antigens recognized by the human immune system are self or mutated self molecules.

Protooncogenes and tumor suppressor genes are normal cellular genes that control crucial cell functions, particularly growth and survival. Mutations in these genes lead to the emergence of cancer. In addition, loss of expression of tumor suppressor genes contributes to malignant transformation.

T cells, which are immune cells crucial for rejecting tumors, use their TCRs to recognize short antigenic peptides bound to MHC-I and –II molecules on the surface of host antigen presenting cells (APC). Naive T cells that have never been activated by antigen are initially triggered by TCR recognition of specific peptide/MHC complexes presented by dendritic cells (DC), which are specialized, professional APCs. DCs capture exogenous antigens from pathogens as well as from host cells. After recognition of specific peptide/MHC complexes by naive T cells they provide additional potent costimulatory signals for T cell activation. The ability of DCs to take up and present antigens is the basis for vaccination to induce T cell immunity. Activated T cells can go on to destroy antigen-positive host cells, e.g. cells infected with pathogens or cancer cells [3].
1.2. Genetic vaccination.

1.2.1. Naked DNA vaccination

DNA vaccination has become the fastest growing field in vaccine technology following reports at the beginning of the 90’s that plasmid DNA induces immune response to the plasmid-encoded antigen[4, 5]. This unexpectedly successful new method is considered by some to be one of the most important discoveries in the history of vaccinology[6, 7]. In contrast to vaccines that employ recombinant bacteria and viruses, genetic vaccines consist only of DNA (as plasmids) or RNA (as mRNA), which is taken up by cells and translated into protein. Genetic vaccines can be delivered into the host by several routes and methods - needle injection into the muscle or skin (the most common method), spleen, and variety of mucosal surfaces, nose and gut are also used as targets. In case of gene-gun delivery, plasmid DNA is precipitated on to the inert particles (generally gold beads) and forced into the cells by a helium blast. Transfected cells then express the antigen encoded on the plasmid resulting in an immune response. Like live or attenuated viruses, DNA vaccines effectively engage both MHC-I and MHC-II pathways allowing for the induction of CD8⁺ and CD4⁺ T cells[8].

In contrast to muscle, skin has important immunological functions as it represents the „first line of defence“ of the immune system. When delivered by gene-gun, the plasmid solubilizes when the plasmid-coated gold bullet penetrates the cell in the skin. Thus, plasmid is directly deposited into cells transfecting up to 20% of the cells in the target-area[9]. Tissue stress resulting from the blast may contribute to the activation of DC. In addition to cellular immunity, gene-gun immunization induces also either IgG1 or mixed IgG1/IgG2-responses[10].
1.2.2. SFV vector system

However, DNA vaccination in many cases is hampered by poor efficacy. "Self-replicating" genetic vaccines are designed to overcome the poor efficacy of some current DNA-based and RNA-based genetic vaccines. The idea and the elements for this new generation of vaccines come from members of the Alphavirus genus (family Togaviridae), which includes Semliki forest virus (SFV), Sindbis virus (SIN) and Venezuelan equine encephalitis (VEE) virus.

1.2.2.1. Semliki Forest Virus

SFV is a positive-stranded RNA virus of the genus Alphavirus. It was first isolated from mosquitoes in Uganda in 1944 [11]. It is a relatively simple virus, encoding only nine functional proteins of unique sequence. The four nonstructural proteins are concerned with viral RNA synthesis, whereas the structural proteins form the capsid (the C protein) and the envelope (the E1, E2 and E3 proteins). A small 6 kDa protein encoded by the structural region of the genome is not incorporated into virions. The structural proteins are encoded by a subgenomic RNA species, usually labeled 26S, whereas the nonstructural proteins are translated from the genomic 42S RNA. The structural and nonstructural proteins are formed from precursors by separate post-translational cleavage pathways [12].

The original isolate of SFV is designated L10 and is neurovirulent for mice, causing encephalitis by infection of the central nervous system (CNS). Subsequently, an avirulent strain designated A7 was isolated from mosquitoes in Mosambique [13]. Most SFV strains used for laboratory studies are derived from these two isolates. The original infectious clone of SFV, constructed from the prototype strain, is designated pSP6-SFV4; the virus produced by transcription of this infectious clone is labelled SFV4 [14].
The SFV virion contains a single copy of RNA encapsidated by a protein/lipid envelope. The viral RNA encodes its own RNA replicase, an autoproteolytic polyprotein that cleaves itself into four nonstructural protein components (nsP1-nsP4) [8]. Replication takes place in the cytoplasm of the host cell and, therefore, is independent of the host’s replication system. Upon infecting a cell, the viral RNA first translates the replicase complex, which in turn drives its own RNA replication. The replicase complex then synthesizes a genomic negative-strand (anti-sense RNA), which is used as a template for the synthesis of genomic positive-strand RNA as well as a subgenomic RNA encoding the structural viral proteins[8] (Fig. 2).
Theoretically up to 200,000 copies of RNA can be produced in a single cell within 4 hours and expression of the encoded antigen can be as much as 25% of total cell protein [8].

The alphavirus replicase functions in a broad range of host cells – mammalian, avian, reptilian, amphibian and insect cells [8].

All the above features, i.e. broad host range, cytoplasmic replication and also high level of expression, are useful features in genetic vaccine development.

1.2.2.2. Alphaviruses as self-replicating vectors

In 1989, the original creation of an alphaviral self-replicating and packaging-deficient expression vector was the starting point of the rapid development of various alphaviral vectors [15].

From at least three different alphaviruses, replicon expression vectors have been engineered from virus strains. In those vectors, the genome for the viral structural proteins has been replaced by a multiple cloning site. They retain the entire nonstructural region as well as the natural subgenomic promoter. Packaged alphavirus-like particles are produced by cotransfection of in vitro-transcribed replicon RNA and helper RNA (or split helper vectors) [16] (Fig. 3).
Productive replication and high level expression of foreign genes can be initiated either by transfection of the genomic RNA into the cytoplasm of the cell or by its infection with packaged alphavirus-like particles. The system is self-limiting because helper RNAs, which lack the packaging sequence are not encapsidated. Thus, replicons are single-cycle vectors incapable of spreading from infected cells [17].

A subsequent modification of this vector system is the layered DNA/RNA vector. Here the SFV vector is cloned as cDNA under the control of a cytomegalovirus (CMV) immediate early promoter. When such DNA is transfected into cells, the CMV promoter stimulates the transcription of the SFV sequence along with any cloned gene, and high-level transient expression occurs [18]. Both types of SFV vectors induce apoptosis late in infection, so persistence of the virus infection is unlikely to occur [19] (Fig. 4,5).

Figure 4. Self-replicating genetic vaccines. The first product of the self-replicating RNA is a four-subunit-replicase which uses the (+) strand RNA as a template to make (-) strand RNA and more copies of full length (+) strand 'genomic' RNA and (+) strand 'subgenomic' mRNA for the encoded antigen. Due to the high number of RNA-copies, the main product of the transfected cells becomes the encoded antigen. The host cell eventually undergoes apoptosis. (Wolfgang W. Leitner et al., Vaccine 18 (2000) 765-777)
Replicase–based DNA vaccines may be significantly more immunogenic and efficacious than conventional DNA-plasmid vaccines when low doses of the vaccine are given. An analysis performed showed no over-production of antigen in the replicase-based DNA vector compared to conventional DNA vector [20]. Transfection of host cells with replicase-based genetic vaccines triggers a series of „danger signals“ [21] (Fig. 6). dsRNA molecules induce type I interferons and heat shock proteins, thus helping to initiate immune response to a viral infection. Furthermore, replicase-based DNA and RNA constructs cause apoptosis of transfected cells, most likely a consequence of activation of the RNAsL and PKR pathways. Apoptosis has been also shown to stimulate the immune system [22]. Dendritic cells can recognise and engulf apoptotic cells and subsequently become activated. In one study, immunization with pCMV-
Figure 6. Potential factors contributing to the high immunogenicity of self-replicating genetic vaccines. (Starting in the upper centre and moving clockwise): Accumulation of antigen in the transfected cell can result in highly efficient MHC-I-loading. A number of `danger signals' may be generated such as interferon production and interferon release from infected cells resulting from the presence of dsRNA. Interferon may also be produced by bystander cells in response to dsRNA released from dead and lysed transfected cells. Heat shock proteins (HSP) have also been shown to be produced in response to the presence of dsRNA in the cells. Ingestion of antigen-loaded apoptotic cells by APCs can also result in the elicitation of powerful immune responses. Finally, the local release of large amounts of antigen at the site of injection by transfected cells may be fed into resident APC. (Wolfgang W. Leitner et al., Vaccine 18 (2000) 765-777)

mTRP-1 (conventional plasmid) only marginally increased the titer of antibody against mouse TRP-1 above background levels. In contrast, the pSIN-mTRP-1 plasmid induced high antibody titers [20]. In other study, a replicase-based DNA plasmid encoding a non-mutated, tumor-associated self-antigen TRP-1 broke tolerance and protected against tumor, whereas the same antigen encoded by a conventional DNA plasmid was ineffective [20].
1.2.2.3. Less cytopathic vectors

A major drawback of alphaviral vectors has been their substantial inhibition of host cell protein synthesis within hours upon infection, resulting in a rapid decrease of endogenous gene expression, induction of apoptosis, and cell death within 72 hours of infection[12].

The alphaviral nonstructural proteins 1-4 (nsP1-nsP4) form the cytoplasmatic RNA replicase complex and play essential roles for virus function: nsP1 is required for the initiation of minus-strand RNA synthesis and the capping of viral RNAs, nsP2 contains protease and helicase activity, and nsP4 is the catalytic subunit of the viral RNA polymerase[12]. NsP3 is a phosphoprotein involved in alphaviral replication, but its precise function remains unknown [23]. Spontaneous mutations discovered in the nonstructural genes, particularly in nsP2 and nsP4, have considerable effects on the viral pathogenicity.

The SFV nsP2 protein has a nuclear targeting signal and in infected cells about half of the nsP2 synthesized is translocated to the nucleus [24]. NsP2 is involved at a number of stages of viral RNA replication; it has single-stranded-RNA-stimulated ATPase and GTPase activities, RNA triphosphatase activity, and RNA helicase activity; it regulates synthesis of the 26S subgenomic RNA; it is involved in the cessation of negative-strand synthesis; and it contains a papain-like proteinase domain responsible for processing the nonstructural polyprotein [24]. The mechanism by which SFV virus triggers the apoptotic response of the infected cells remain unclear. As some researchers suggest - events in the nucleus would be one possibility [24].

The partial transport of nsP2 into the nucleus of SFV-infected cells has been proposed as one possible mechanism determining the cytopathogenicity of SFV infection [25]. Based on this assumption, NLS mutations that prevent nsP2 movement to the nucleus of infected cells should also reduce SFV cytopathogenicity [26].
In Kristi Tamm’s study, mutations designated DDD, RDD, DDR, RDR, RRD, ∆∆∆, ∆∆R and ∆RR were introduced into the NLS of nsP2 (648RRR659) by PCR-based mutagenesis, in order to screen the NLS sequence for changes with potential impact on SFV cytotoxicity. In results, only slightly slower shutdown of host protein synthesis was detected in cells transfected with ∆RR, RDR, DDR and ∆∆R EGFP constructs. Most replicons (RRD, RDR, DDR, ∆RR, ∆∆R) were also packaged into VLPs as efficiently as the wild-type SFV. It was found that the RRD mutation in the NLS did not prevent the nsP2 transport to the nucleus, whereas the localization of nsP2 with an RDR, DDR, ∆∆R or RDD mutation was mostly cytoplasmic. It was found also that there is no strict correlation between nuclear localization on nsP2 and cytotoxicity. Synthesis of viral RNA is one factor that may contribute to virus-induced cytotoxicity. For RRD and ∆RR replicons the ability to replicate and transcribe RNA was reduced to 4-6-fold [26], thus making them less cytotoxic.

The replicons with one mutation (RRD, RDR, ∆RR) were very similar to wild-type replicon – they have very high levels of marker protein and virus-specific protein production, as well as RNA production, they inhibit the host cell protein synthesis resulting in cell apoptosis [26]. The only difference is that they are a little less cytotoxic because all these processes are very slow compared to wild type replicon. The replicons with more than one mutation are even less cytotoxic than replicon with one mutation, but the rates of protein synthesis are also lower.

1.2.2.4. SFV vectors in cancer gene therapy

The first step towards gene therapy application was the demonstration of high transduction rates of various human prostate tumor cell lines with SFV vectors [27]. This study showed not only high infection rates 70-95% in tumor cell lines, but also efficient β-galacosidase reporter gene expression in ex vivo transduced biopsies from prostate cancer patients. SFV infected cells showed a high degree of apoptosis. In another report, human cancer cell lines were infected with relatively low concentrations
of SFV-GFP and SFV-GFP/TK viruses to mimic an in vivo situation with reduced access to tumor cells [28]. Clearly, the infection rate was modest, although a bystander effect was observed for the therapeutic TK (thymidine kinase) after ganciclovir treatment.

Another application of alphaviruses for cancer therapy has been vaccination with tumor antigens [29]. Application of alphavirus vectors as RNA molecules, DNA plasmids and recombinant particles have all generated antibody responses in rodent and primate models. For instance, immunization with SFV-LacZ RNA led to tumor regression in mice [30]. Antitumor activity and immune protection against melanomas was observed in mice after administration of a SIN plasmid DNA construct carrying the tyrosinase-related protein 1 gene [20].

SFV vectors have also been subjected to intratumoral injections in animal tumor models. For instance, administration of SFV particles expressing the interleukin-12 subunits p40 and p35 to a B16 melanoma resulted in significant tumor regression and inhibition of tumor blood vessel formation measured by Doppler ultrasonography [31]. Additional studies in mice implanted with human lung tumors demonstrated that intratumoral injection of recombinant SFV-GFP particles were responsible for a substantial tumor regression [32].

Alphaviral DNA vaccines are very attractive for several reasons: 1) compared to other viral vectors (poxvirus and adenovirus), there is a general lack of pre-existing immunity in the population; 2) their expression is both transient and lytic, which circumvents the biosafety risks of chromosomal integration and the induction of immunological tolerance; 3) the absence of viral structural proteins eliminates both the induction of an otherwise strong immune response towards the vector and the risk of amplification of recombined replication-competent viruses; 4) they can be applied to therapeutic vaccination by the expression of a „self-antigen“ to break tolerance and provide immunity to tumors as well as to viral, bacterial and protozoan organisms, for prophylactic and therapeutic purposes; and 5) they have been reported to be more
efficient than other or conventional DNA vectors, requiring 100 to 1000-fold smaller DNA amounts per immunization [33].
1.3. Tyrosinase-related protein-2 as a tumor antigen

1.3.1. Tyrosinase-related protein-2

The production of melanin pigment in mammals requires tyrosinase, an enzyme which hydroxylates the amino acid tyrosine to DOPA (3,4-dihydroxyphenylalanine), thus allowing the cascade of reactions necessary to synthesize that biopolymer. However, there are other regulatory steps that follow the action of tyrosinase and modulate the quantity and quality of melanin produced. Dopachrome tautomerase is one of such melanogenic enzymes (Fig. 7).

Tyrosinase-related protein-2 (TRP-2, dopachrome tautomerase, DCT) (EC 5.3.2.3) is a metal-containing glycosylated enzyme, catalyzing the conversion of the red compound dopachrome to a colorless 5,6-dihydroxyindole-2-carboxylic acid[34]. It has also been termed dopachrome conversion

Figure 7. Melanogenic pathway from tyrosine. Enzymatic reactions attributed to tyrosinase, TRP-1 and TRP-2 are indicated. (del Marmol et al., FEBS Letters (1996) vol.381)
factor[35], dopachrome oxidoreductase [36, 37] and dopachrome isomerase[38, 39]. The TRP-2 function might be that it protects the melanocyte against cytotoxicity of decarboxylated indolic melanogenic intermediates by limiting their formation [40].

The enzyme is preferentially associated to the pigmented and unpigmented melanosomes, but it is also found in the microsomal and cytosolic fractions of cellular homogenates. [34].

In 1992, the molecular cloning of TRP-2 confirmed the existence of the third member of tyrosinase related protein family [41]. All proteins of this family are well conserved and are dispersed and located on three different chromosomes, with no obvious chromosomal relationships[41], which means that this is a very old family. Dopachrome tautomerase shares about 40 % amino acid identity with tyrosinase or tyrosinase-related protein-1[42].

The TRP-2 protein is encoded by 1.6 kb of coding sequence which is distributed on 8 exons at the TYRP2 locus on chromosome 13q31-32 [43, 44] in human and on 14 chromosome in mice [41, 42] (Fig. 8). The collective absence or low level of polymorphism in the TYRP gene family in the human populations studied argues that differences in normal patterns of melanization are not produced by differences in the encoded catalytic

![Figure 8](image.png)

Figure 8. A. Exon/intron structure of TRP-2 geneas identified in human and mouse. Interruption of phases within introns are indicated by numbers, between codons (=0), and leaving one base (=1)or two bases (=2) of a codon on the 5’ part of an intron. B. Schematic representation of the tyrosinase family protein domains, with N-terminus at the left, inside melanosome, and the C-terminus at the right, in the cytoplasm. (V. Del Marmol, FEBS Letters (1996), vol.381)
activity of these enzymes. This doesn’t rule out the possibility that different TRP protein levels or enzymatic activity within melanosomal complex are responsible for variation in pigmentation. Indeed, such variation is apparent when melanocytes have been cultured from individuals of different skin types [45] and assays performed for each of the three melanogenic enzymes.

1.3.2. Tyrosinase-related protein-2 in antitumor therapy

Tyrosinase-related protein-2 is a well known antigen of spontaneous cytotoxic T-cell responses in melanoma patients. Its frequent expression in metastatic tumors suggests that it might be an ideal candidate antigen for T-cell based immunotherapy.

In a Julia Steitz study, B16 melanoma cells were injected intra-venously into C57/BL6 mice after immunization by injecting adenovirus encoding human TRP-2. As a result mice did not develop any melanoma metastases and mice injected with adenovirus encoding murine TRP-2 showed a reduction in the number of metastases compared to naive mice. Alternatively, B16 cells were injected s.c. in immunized mice. 50% of mice immunized with adenovirus containing human TRP-2 gene completely rejected the s.c. challenge and were tumor free at the end of the experiment. In contrast, all mice immunized with Ad-mTRP-2, developed tumors [46].

Almost complete protection against a lethal challenge (10-fold greater than the minimum tumorigenic dose) was achieved in mice immunized with an eucaryotic expression plasmid pcDNA3 encoding murine TRP-2. Overall the rate of tumor prevention was 86%. In another study of the same group mice were injected with a lethal dose of B16 cells and 5 days later received treatment with vaccinia virus encoding TRP-2. The negative controls died within 3-4 week after tumor challenge. In contrast, 50% of the mice treated with rVV-TRP-2 remained tumor free for 3 months [47].

Also there was made an attepmt to protect from melanoma using an oral vaccine. Mice were immunized by oral gavage of Salmonella typhimurium AroA, harboring the expression plasmid pUb-H and encoding ubiquitin and the human gp-100 and TRP-2 peptide epitopes, mice were challenged with B16 melanoma cells. Mice showed tumor-
protective immunity (two mice rejected tumors, other mice had a marked suppression in tumor growth) [48].
1.4. Anti-tyrosinase antibodies in cancer therapy

Cancer immunotherapy is a difficult challenge both because of the „self“ nature of antigens found on tumors and the ability of cancer to actively evade protective immune responses. One strategy to improve T-cell-based immunotherapy is to combine it with antibodies targeting antigens relevant to a specific tumor type [49, 50].

Antibodies modulate T-cell responses in infectious disease, autoimmunity, and cancer through Fc domain interactions with surface receptors on antigen presenting cells [51]. Dendritic cells pulsed with antigen-antibody complexes (immune complexes) containing ovalbumin are a more effective vaccine against ovalbumine expressing B16 than are dendritic cells pulsed with ovalbumine alone [52].

Therapeutic antibodies, such as chimeric, humanized, and fully human antibodies, typically use for its backbone tructure threee human IgG1 constant region. This isotype was chosen primarily because of its capacity to induce strong effector functions in humans, such as antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC).

Antibodies have evolved into classes with specific assigned functions. Within these classes, further subclassification extends immunoglobulin diversity, most strikingly in the four subclasses of IgG of mammals. These subclasses have evolved specialized effector responses, such as cytotoxicity, phagocytosis, and release of inflammatory mediators.

Recent studies have suggested the importance of these effector functions in antibody therapy [53-55]. Apart from amino acid and structural idiosyncrasies, the four isotypes of human IgG differ from each other in the potencies of effector functions and other activities [56-58]. In general, the rank order of potency is IgG1≥IgG3>IgG4≥IgG2 for ADCC [56, 59] and IgG3≥IgG1>IgG2=IgG4 for CDC [56-58].

According to other research, IgG2a resulted in enhanced ADCC in the metastatic melanoma model compared to other antibodies. The hierarchy of activity for the IgG subclasses given by these investigators is thus IgG2a>IgG2b>IgG1>>IgG3 [60].
Several immunotherapeutic approaches have been introduced for melanoma, such as immunization with anti-idiotypic antibodies [61]. Antibodies against high molecular weight melanoma-associated antigen (HMW MAA) have been used by several groups as a target for the production of anti-idiotypic antibodies. Immunization of melanoma patients with these antibodies (mimicking the original antigen) led to the generation of antibodies aimed against HMW MAA, with beneficial results [62-64].

In the study of Yvonne M. Saenger, they defined a new role for mAb TA99 (IgG2a) as an adjuvant for therapeutic DNA vaccination against B16 melanoma. TA99 was combined with DNA vaccines against the target antigen TRP-1 and against distinct melanosomal antigen gp100. Mice receiving combination therapies both with gp100 and TRP-1 had a significant improvement in tumor burden, whereas animals treated with either agent alone did not [65].

Tyrosinase is a melanocyte specific melanosomal protein that is recognized by both CD8+ and CD4+ T cells. The expression of tyrosinase may be a significant cofactor in determining the recognition of melanoma targets by antigen specific T cells [66-68]. As such, it currently is utilized as a target in immunotherapy protocols for metastatic malignant melanoma (MMM) at the National Institutes of Health/ National Cancer Institute (NIH/NCI).

Tyrosinase has many advantages as a target antigen for the immunotherapy of patients with melanoma because it is expressed in nearly all melanoma specimens with a high degree of cellular homogeneity, and its distribution in normal tissues is limited to melanocytes [69]. The study of tyrosinase is not only interesting in the process of pigmentation but it is also studied for recognizing and fighting melanoma: by its antigenic properties as an early marker for metastases and by the specificity of its promoter it can be used for gene therapy.

In contrast to autoimmune diseases, in which the generation of other antibodies causes acceleration of the disease, in melanoma the generation of another antibody, such as antityrosinase, may lead to regression of tumor in some patients [70].
1.5. Interleukin-2 in cancer therapy

The immune system is dedicated to a series of goals, including the generation of rapid innate and adaptive immune responses to invading pathogens, the elimination of autoreactive T cells and the maintenance of a specific memory response to these pathogens. Such immune responses are normally regulated by cytokines. Common cytokine-receptor γ-chain family of cytokines is represented by the interleukin that was first to be discovered – Interleukin-2 (IL-2).

IL-2 has several functions – stimulate the proliferation of T cells; induce the generation of cytotoxic T lymphocytes (CTLs); facilitate the proliferation of, and the synthesis of immunoglobulin by B cells; and induce the generation and persistence of natural killer cells (NK) [71]. Through its unique role in activation-induced cell death (AICD) and its participation in the maintenance of peripheral CD4+CD25+ regulatory T cells – is involved in the elimination of self-reactive T cells, which have a role in the pathogenesis of autoimmune diseases [72, 73]. In addition, the IL-2 has been identified as a valuable molecular target for immunotherapy of leukaemia and autoimmune diseases [74].

IL-2 has also been approved for use in patients with metastatic renal-cell carcinoma and malignant melanoma. The antitumor effect of IL-2 probably results from its ability to expand lymphocyte populations in vivo and to increase the effector functions of these cells, thereby inhibiting tumor growth [75, 76]. However, IL-2 is not optimal for inhibiting tumor growth, because in the presence of IL-2, either the CTLs generated might recognize the tumor as self and undergo AICD or the immune response might be inhibited by IL-2-dependent T_{reg} cells [77].

Intravenous administration of cytokines can enhance pre-existing anti-tumor immunity. However, as monotherapies they are rarely curative unless tumor burden is sufficiently small. This may be because they do not adequately access the larger tumor microenvironment. Furthermore, their toxicities may be problematic[78].

As an alternative strategy targeting this agent directly into the tumor microenvironment by intratumoral (i.t.) injection, catheterization or other approaches [79, 80]. Studies have shown, that i.t. IL-2 generated T cell-mediated curative
regression of small tumors without the deleterious side effects seen with systemic IL-2 administration [81]. When used intravenously the IL-2 therapy was barely effective, unless higher doses were used. Targeting the tumor microenvironment by i.t. injections enabled lower doses to produce highly-effective anti-tumor immunity without serious side effects during treatment. However, the tumors that were bigger than 25 mm\(^2\) were resistant to IL-2 monotherapy [78].
2. Experimental part

2.1. Aims of the study

Malignant melanoma continues to be a serious clinical problem, with a high mortality rate among humans due to the failure of melanoma cells to respond to cytotoxic treatment in the form of radiation and chemotherapy. Thus, metastatic melanoma continues to challenge researchers to find a systemic treatment for cancer [82]. In this study there was made an attempt to find efficacy of treatment of melanoma in mouse model using wild type and less cytotoxic SFV-based vectors encoding mouse tyrosinase-related protein-2, anti-tyrosinase antibodies, interleukin-2 and SFV vector VLPs.

SFV vectors (DNA/RNA layered vector - wild type and with mutated nsP2; RNA vector – wild type and with mutated nsP2) encoding mouse tyrosinase-related protein-2 were cloned, as it was proved by other researchers that it is a very promising antigen used in immunotherapy of melanoma [46-48]. As one of the aims of this study was, to check the effectivity of vaccines based on wild-type SFV and less cytotoxic SFV vector, groups of mice with established tumors were vaccinated with both mutated and wild-type vectors and the tumor growth development monitored.

As it was assumed by other researchers, generation or administration antityrosinase antibodies can lead to the regression of tumor in some patients [70]. So the second aim was to check the ability of antityrosinase antibodies to tumor growth slowdown.

Many studies were made on the IL-2 in cancer therapy [75-80]. An attempt to reduce the melanoma growth with i.t. administration of IL-2 together with SFV vector VLPs (encoding mouse TRP-2 as well) was another aspect of our study.
2.2. Materials and methods

2.2.1. Used plasmids and vectors

Four expression plasmids were constructed for expression of mouse tyrosinase-related protein-2. Two of them were based on layered DNA/RNA vectors (DrepE vector, kindly provided by prof. Peter Liljeström from Karolinska Institute, Stockholm, Sweden): one was a wild type vector and the other had a point-mutation in it’s nonstructural protein 2 of SFV replicase (RRR to RRD mutation). Other two were based on SFV 4 vector for preparation of VLPs, one of them was wild type and the other one had the same mutation as the layered vector. Respectively: DE\_wt\_mT2, DE\_RRD\_mT2, pSFV\_wt\_mT2 and pSFV\_RRD\_mT2. DE\_wt\_mT2 and pSFV\_RRD\_mT2 constructs were cloned before (by Nele Jaanson in our laboratory).

DrepE (DE) plasmids are layered DNA/RNA vectors based on Semliki Forest Virus (SFV) replicon genes driven by cytomegalovirus (CMV) immediate early promoter (viide!). Our vectors contain mouse tyrosinase-related protein 2 encoding fragment cloned after the SFV replicase gene, placed under the subgenomic promoter of SFV.

DE\_wt\_mT2 – Drep plasmid (layered DNA/RNA SFV vector) encoding wild-type nsP2 and mouse TRP-2

DE\_RRD\_mT2 – Drep plasmid encoding mutated nsP2 protein and mouse TRP-2

pSFV\_wt\_mT2 – RNA SFV vector encoding wild-type nsP2 and mouse TRP-2

pSFV\_RRD\_mT2 – RNA SFV vector encoding mutated nsP2 and mouse TRP-2
2.2.2. Mice and cell lines

For eukaryotic protein expression BHK-21 cells were used. Cells were grown in GMEM (*Glasgow Modified Eagle Medium*, Gibco, UK), supplemented with 5% FCS, 0.3% Bacto™ Tryptose Phosphate Broth, penicillin 100 IU/ml and streptomycin 100 ng/ml. For infection of cells with VLPs FCS-free serum was used supplemented with bovine serum albumin 10%.

For tumor inoculations B16F0 mouse melanoma cell line (ATCC, Manassas, VA, USA) was used, this is syngeneic to C57 /BL6 mice. After taking up from liquid nitrogen cells were grown few passages (2-3) in RPMI, supplemented with 10% FCS and penicillin 100 IU/ml and streptomycin 100 ng/ml. Before inoculation cells were trypsinized, harvested and centrifuged, then washed two times with pure RPMI (no FCS to avoid innate immune response induced by serum). Cells were dyed with Trypan Blue and counted, also the same time measuring cell viability. Only cells over 95% viability were injected with insulin syringe through 26G needle (Braun, Germany) previously shaved right flank of mice, 50,000 cells per injection in 60 µl of RPMI.

For tumor treatment studies C57BL/6 mice were obtained from the Animal Facility of Institute of Molecular and Cell Biology of University of Tartu and were kept in pathogen free environment of the same institution. All mice entered the study between 8-10 weeks of age. All animal procedures were approved by the Animal Care Committee of the University of Tartu in accordance with the European Communities Directive of 24 November 1986 (86/609/EEC).

2.2.3. Western blotting

Western blot analysis was performed to check the expression of the TRP-2 protein before other tests. The analysis was made by running 10% SDS-polyacrylamide
gel with the lysates of BHK-21 cells transfected with DE_mT2, DE_hT2, DE_RRD_mT2 and DE_RRD_hT2 according to the method of Laemmli. As the molecular mass marker was used Prestained Protein Ladder (Fermentsas). A sheet of PVDF filter was placed against the surface of SDS gel to perform the electrophoretic transfer, using semi-dry buffer and moistened filter paper pads (Whatman paper). The transfer was performed at 15 V for 15 minutes in the semi-dry blotter (Trans-Blot, BioRad).

Protein-binding sites were blocked on the PVDF filter in the buffer made from 5% non-fat dried milk in Western Wash Buffer (WWB: 0,05 M Tris, 0,15 M NaCl, 0,1% Tween-20) by churning the membrane for 1 hour on a shaker at room temperature.

The membrane was incubated in the 2% non-fat dried milk and WWB with the primary antibody for 1 hour at room temperature. As primary antibody mouse monoclonal anti-TRP-2 (Santa Cruz Biotech, California, US) was used in 1:1000 dilution in WWB. The filter was washed then 3 times for 15 minutes with WWB. Then the membrane was incubated in the secondary antibody solution (2% non-fat dried milk and WWB) at a shaker for 1 hour. As secondary antibody was used mouse HRP (goat anti-mouse IgG peroxidase conjugate) in dilution 1:5000. The membrane was washed again 3 times for 15 minutes in WWB. Specific signals were then detected using the Western blotting detection reagents, with Enhanced Chemiluminescence (ECL™, Amersham Biosciences).

2.2.4. In vitro transcription

For in vitro transcription the DNA was linearized by restriction enzymes cutting after the poly-A tail of SFV vector ORF (Fermentsas enzymes, Lithuania, for different vectors different enzymes). Linearized DNA was purified with „JetQuick PCR Purification SpinKit“ (Genomed Gmbh) and eluated in 30 µl of dietylpyrocarbonate-treated water (DEPC).
In vitro transcription solution was prepared on ice: 1.5 µg of linearized and purified DNA, rNTP mix (1 mM ATP, CTP, UTP and 0.5 mM GTP, "Promega"), 5 mM dithiotreitol (DTT), 1 mM Ribon 7G Cap Analog ("Promega"), 10x SP6 buffer (80 mM Hepes-KOH, pH=7.4; 12 mM MgOAc; 4 mM spermidine-HCl) and the volume was up to 50 µl with 0.1% DEPC-water. Then 50 units of recombinant ribonuclease inhibitor (Rnasin® , Promega) and 30 units of SP6 polymerase (Promega) was added. The mixtures were incubated at 37°C for 90 minutes.

2.2.5. RNA electroporation

BHK-21 cells were grown to late log phase (80% confluent) in GMEM medium, washed once with 2 ml PBS, trypsinized with 1 ml of trypsin (Gibco). Trypsinization was stopped by addition of 8 ml of GMEM medium, when the cells began to detach. The cell suspension was centrifuged at 400g for 5 minutes and then washed with PBS, then centrifuged again and PBS was added to get 0.8 ml of cell suspension for one electroporation.

50 µl of in vitro transcribed vector-RNA and 50 µl of helper-RNA were mixed in 0.4 cm cuvetes containing 800 µl PBS-cell suspension. Two pulses were given at 850 V/25 µF in delay of 30 seconds at room temperature.

Afterwards, cells were transferred to Ø10 cm plates supplemented with 9 ml of GMEM medium and incubated for 72 hours at 28°C, collecting the virus like particles every day for wild type and every other day for RRD mutant vector transfected cells.
2.2.6. Harvesting VLPs

Following incubation time collected cells’ covering medium was centrifuged at 18000 rpm for 30 minutes at 4°C to remove the dead cells and the remaining cell debris. Purified medium was then transferred into Beckman ultracentrifuge tubes and ca 5 ml of 20% sucrose solution. Mixture was centrifuged at 25000 rpm for 3 hours at 4°C. The medium and sucrose solution were aspirated and sedimented VLPs were dissolved in the 0.1 ml of 0.9% NaCl (Braun, Melsungen, Germany).

2.2.7. VLP titration

The BHK-21 cells in 6-well plates (Greiner Bio-One, Germany) were grown (using cover slips) to a late log phase in complete GMEM. Cells were washed with 1xPBS 2 times. PBS was aspirated and the dilutions of virus stocks $10^{-2}$, $10^{-3}$, and $10^{-4}$ in serum-free GMEM were added to the cells. The amount of medium was kept as low as possible, since the probability of virus attachment is higher if a small volume of infection medium is used. The cells were then incubated at 37°C for 1 hour and gently shaked in every 10 minutes to increase the efficiency of infection.

When the incubation was completed the infectious medium was replaced with complete GMEM and cells were incubated overnight at 37°C.

2.2.8. Immunofluorescent staining of cells

Infected cells on cover slips were grown for 16-18 hours, after that the medium was aspirated. The cells were fixed by addition of 4%-PFA-PBS solution and incubating for 10-15 minutes (This also inactivates SFV). Then the cells were covered with -20°C
methanol and incubated at -20°C for 10 minutes. The cover slips were washed twice with Dulbecco PBS. After PBS was aspirated the blocking solution (5% FCS-PBS) was added and the cells were incubated at room temperature for 30 minutes.

The blocking solution was removed and the primary antibody (rabbit anti-nsp1 and mouse anti-TRP-2) diluted in 5% FCS-PBS was added. The dishes with cover slips were incubated for 1 hour at room temperature. The liquids were aspirated and the cover slips were washed 3 times with Dulbecco PBS. The secondary antibody (anti-rabbit FITC and anti-mouse rhodamine red) solution in 5% FCS-PBS was added and incubated for 1 hour at room temperature protected from light. The cover slips were washed again 3 times with Dulbecco PBS and once with dd H₂O, then dried and mounted onto the microscope slides in a small drop of mounting medium (Mowiol).

2.2.9. Preparation of DNA/gold cartridges

In a 1.5 ml microfuge tube, 25 mg of gold microcarriers was weight out. The gold microcarriers size was chosen to be 1µm, because it is the right size to carrier enter the skin, but not to go too deep. To the measured gold was added 100 µl of 0.05 M spermidine. Mixture was vortexed and sonicated for 5 seconds using an ultrasonic cleaner to break up gold clumps. To the gold and spermidine mixture was added plasmid DNA in different concentrations – DE_mT2 0.33 µg, DE_mT2 1.0 µg, DE_mT2 3.0 µg and DE 1.0 µg as negative control. One more negative control was used, which didn’t consist any plasmid DNA only gold particles. While vortexing 100 µl of 1M CaCl₂ was added dropwise for DNA to associate to gold microcarriers. Gold was then pelleted and after washing three times with 1 ml of 100% ethanol, resuspended in 200 µ of the ethanol solution containing 0.05 µg/ml of PVP (polyvinylpyrrolidone).

Two cartridges were left overnight in the Tris/EDTA 10/1 mM to check the correct loading of DNA on gold particles by running the gel electrophoresis.
2.2.10. Tumor inoculation

Briefly, mice were anesthetized by isofluorane inhalation and hair was removed with depilatory cream. $5 \times 10^4$ B16F0 cells in 60 µL of serum-free RPMI medium were injected into the right flank of C57/BL mice. Tumors were followed by appearance, measured by caliper length and cross diameters. Tumor volume was calculated by the formula: $\text{width}^2 \times \text{length} / 2$. In survival studies, mice over 75mm$^3$ of tumor volume were considered marked dead. Survival analysis was done by Kaplan-Meier and Log-Rank test. Statistics for tumor volumes were calculated using one-way ANOVA test.

2.2.11. DNA injection

Plasmid DNA was injected to 8-12 weeks old mice by particle bombardment using Helios gene gun (Biorad) according to the manufacturer’s protocol. Briefly, DNA was purified and coated onto 1-µm gold bullets, and mice were anesthesized by isofluorane inhalation. Hair was removed with depilatory cream, exposing abdominal skin of mice. It it postulated that delivery of DNA by gene gun into the abdominal region of mice directly targets antigen presenting cells. In a first pilot experiment, mice were divided into groups of receiving 0.33 µg, 1.0 µg, 3.0 µg of plasmid DNA per injection. Negative control groups received 1.0 µg of empty plasmid DNA or pure gold particles. DNA was injected once a week for a total of three doses. First injection was performed on the third day after receiving B16F0 cells. Mice were then followed for the appearance of tumor. Tumors were noted as positive when visible tumors were seen.

for the appearance of tumor. Tumors were noted as positive when visible tumors were seen.

In a second DNA injection study everything was prepared as described above using DE_mT2 and DE_RRD_mT2 (less cytotoxic SFV vector) plasmids expressing
mouse TRP-2. Mice of both groups received 0.33 µg of plasmid DNA per injection, once per week for total of three doses. Negative control group received pure gold particles. Mice were then followed for the appearance of tumor. Tumors were noted as positive when visible.

2.2.12. Antibody injection

Four groups of mice (8-12 weeks old) were treated with IgG1 type anti-Tyrosinase mouse monoclonal antibody solution – 4F2, 5H12, 5B5 and 5A2 [83]. 400 µg of purified antibody in 100 µL of 0.9% NaCl were injected i.p. First injection was performed on the third day after receiving B16F0 cells and repeated injected once a week for a total of three doses. Mice were then followed for the appearance of tumor. For survival study mice with tumors over 75 mm³ were considered dead. Statistical analysis of tumor treatment was done by one-way ANOVA, and conferred by Bonferroni test, and survival analysis was performed by Kaplan-Meier test.

2.2.13. IL-2 and SFV VLP injection

IL-2 and VLP particles (pSFV_wt_mT2 and pSFV_RRD_mT2) solution was injected intra-tumorally to 3 months old mice who had been developed tumors from 20 to 234 mm³. Solutions were prepared freshly before injection. Mice received only one injection and were monitored then for tumor growth. When the tumors started growing again mice were sacrificed, (4 days after the treatment).

2.2.14. Enzyme-linked immunosorbent assay
Coating of ELISA plates (Nunc-Immuno™ plates 96-well plate, Sigma-Aldrich) was made by addition of poly-D-lysine in each well. The plates were left for 2 hours to incubate. The plates were washed with 1×PBS and cultured B16F0 cell suspension was added 150 000 cells per each well. Cells were incubated overnight at 37°C. Next day wells were washed 3 times with PBS to remove unbound cells and culture media, the cells were fixed by adding of ice-cold methanol to the wells and kept at -20°C for 20 minutes, then washed again 3 times with 1×PBS. Cells were blocked with 2%-BSA-PBS, and incubated for 1 hour at room temperature to reduce non-specific binding sites. 3 times with 1×PBS. 50 µl of test sera was then added into each well in different dilutions : 1/50, 1/150, 1/450 and 1/1350 in 2%-BSA-PBS and incubated for 1 hour at room temperature on a shaker. The plates were washed again 3 times with 1×PBS and next the secondary anti-mouse HRP conjugated antibody solution was added in dilution 1:500 in 2%-BSA-PBS and incubated for 1 hour. Wells were washed again with 1×PBS and 100 µl of TMB+ Substrate-chromogen (DAKO) reagent was added to develop the color reaction. To stop the reaction 100 µl of 0,2M H$_2$SO$_4$ was added after 30 minutes. The color reaction was quantitated on the microplate reader Sunrise™ (Tecan) at 450 nm. Statistical analyses was done by one-way ANOVA test.

2.2.15. ELISpot

The preparation of ELISpot plates was done by the instructions of the producer. For Mabtech ELISpot plates prewetting with 50 µl of 70% ethanol per well was performed for 2 minutes. The plates were washed with sterile water 5 times. The coating with capture IFN-γ antibody-PBS solution was done over-night. Then the antibody solution was removed and the plates were washed 5 times with sterile PBS. 200 µl of RPMI medium was then added to each well and plates were incubated for 30 minutes at room temperature. The medium was removed and the 250 000 spleen cells per well and culture medium including stimulatory agents (conA solution as positive control and peptide solutions in two replicates) was added into each well. Plates were incubated at
37°C for 24 hours. The same was done with RD plates (RD Systems) with an exception that prewetting and precoating with capture antibody was already done by the manufacturer.

After incubation plates were washed 5 times with PBS. The IFN-γ detection antibody diluted in 0,5% FCS-PBS to 1µg/ml was added and incubated for 2 hours at room temperature. The plates were then washed again. Next the diluted streptavidin-ALP (1:1000) in 0,5% FCS-PBS was added to each well. The plates were incubated for 1 hour at room temperature. The plates were then washed again and the developing substrate solution (BCIP/NBT) was added to wells. The plates were incubated for 1 hour at room temperature protected from light. The chromogen solution was then discarded and the plates were rinsed with ddH₂O. After that plates were dried for 30 minutes at 37°C. Automatic scanning reading of spots was done by service (CTL Europe GmbH, Bonn, Germany).

Statistical analysis was performed by one-way ANOVA and Dunn’s test. Tumor volumes correlations with IFN-γ was done by Spearman rank correlation test.
3. Results

3.1. Western blot

The Western blot analysis was performed with DE_mT2, DE_RRD_mT2, to check whether the cloned plasmids express the encoded protein. The analysis was made by running SDS-PAGE gel with the lysates of BHK-21 cells transfected by these plasmids. As primary antibody mouse anti-TRP-2 was used (1:1000). As secondary antibody I used anti-mouse HRP (1:5000). As negative control BHK-21 mock lysate was used.

Analysis confirmed that these plasmids after being transected into the cells express the encoded mouse TRP-2 protein (Fig. 9).

3.1. The lowest dose of DNA SFV vector gives the better result

This test was performed to check which concentration of DNA is the best for treating tumor for the next studies. Five groups of mice were treated by injection of plasmid DNA that was based on Semliki Forest Virus (SFV) replicon genes driven by cytomegalovirus (CMV) immediateearly promoter and contained mouse tyrosinase-related protein 2 encoding fragment placed under subgenomic promoter of SFV (DE_mT2). DNA was injected in three different concentrations - 0,33 µg, 1,0 µg and 3,0 µg (Fig 10).
The analysis showed the best DNA concentration for the next experiments with
mice. As seen in figure 10, the best concentration is $0.33 \, \mu g$ of plasmid DNA, as the tumor sizes were the smallest compared to other groups. Important is also the fact that these mice started developing tumors a lot later than other groups (Data 2).

Statistical analysis of this study showed that until the day 15 there were no differences in the tumor growth speed between the groups of vaccinated mice. The only difference was that mice vaccinated with $0.33 \, \mu g$ of $DE_{mT2}$ had smaller tumors (Fig. 11). As this was a pilot study, we only wanted to see the difference between the groups vaccinated with different amounts of DNA. If we would like to have more precise results we would have to repeat this study with at least twice bigger groups.

![Figure 12](image.png)

**Figure 12.** Survival analysis of the vaccinated groups. The right graph compares the best result ($DE_{mT2} 0.33$) to the negative control.

The survival analysis showed the differences in the tumor protection between the vaccinated groups. According to this test $DE_{mT2} 0.33$ mice had the smallest tumors (Fig. 12). But according to Kaplan-Meier’s test and the Log-rank test there was no statistical difference between the groups (Fig. 13).
Figure 13. Kaplan-Meier’s test and Log-rank tests results. Pairwise comparisons between all of the groups yielded no significant differences between the vaccine groups.

<table>
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<th>Percent Censored</th>
<th>Median Time</th>
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<td>3</td>
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<td>0</td>
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Log-Rank Test:

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<td>4</td>
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3.2. Tumor specific antibody 5A2 gives the best result

This test was performed to check which antibody gives the best protection from tumor or reduces the tumor growth. Four groups of mice were treated with 400 µg of different antibodies aimed against human tyrosinase – 4F2, 5H12, 5B5 and 5A2. All of them have been checked in our lab to detect mouse tyrosinase as well.

The analysis showed the best antibody for the next experiments. As seen in figure 12, the best result was shown by 5A2 antibody. Mice treated with this antibody didn’t have tumors at all or had very small tumors compared to other groups even after 17 days (Data 3). Important fact is that mice treated with 5A2 antibody developed tumors later than other mice.

One-way ANOVA statistical analysis on tumor final volumes (p=0.052) showed that in mice that developed tumors there was no statistical difference between the groups and only 5A2 antibody shows trend of reduction of tumor volume (Fig. 14).
The survival analysis showed the difference between the groups. Only mice treated with 5A2 antibody showed statistically significant difference in survival, as only 2 mice out of 7 developed tumors at the end of the study. Mice vaccinated with other

Fig 14. Tumor sizes in days after treating mice with inoculated tumors with injection of 400 µg of different antibodies per week. A) Linear graph of tumor average sizes in groups. B) Mean values of the tumors in mAb treated mice.

<table>
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Figure 15. Kaplan-Meier Survival Analysis: Log-Rank Test.
antibodies developed tumors almost as quickly as the negative control mice (Fig. 15).

With Kaplan-Meier’s survival analysis we found the median day when tumor size was so big ($75 \text{ mm}^2$) that there is no regression possible. According to the data (Fig. 15) all the groups except the 5A2 group had almost the same median time value; the 5A2 group doesn’t have the median time value as the tumors were too small.

Figure 13. Effect of antibody therapy on survival of tumor-bearing mice.
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Figure 15. Bonferroni correction t-test.

According to the Bonferroni correction t-test, statistically significant difference compared to all other groups had only 5A2 mAb treatment.

### 3.3. Vaccination with wild-type and mutated SFV vectors

The second analysis was performed to compare the efficacy of the plasmid DNA vaccination using particle bombardment with mutated SFV-based vector and wild-type SFV-based vector encoding mouse TRP-2 protein. The concentration used was also 0.33 µg (Fig. 16).
Figure 16. Tumor growth curves in all vaccinated groups and final tumor volumes and means.

Figure 17. Survival analysis of groups vaccinated with wild type and mutated SFV vector and a Kaplan-Meier’s test.

As we can see tumor sizes of mice vaccinated with mutated SFV vector...
Encoding mouse TRP-2 (DE_RRD_mT2) are even less than in case of vaccinating with the same amount of wild-type SFV vector encoding the same antigen (DE_mT2).

The survival analysis showed that the DE_RRD_mT2 mice were better protected and developed smaller tumors (Fig 17). According to the Kaplan-Meier’s test the survival was higher in mice vaccinated with DE_RRD_mT2 than in DE_mT2 vaccinated mice (Fig. 17). Herefore, we can suggest that RRD mutant vector gives better results in this treatment strategy, although it needs to be repeated in bigger group of treatment.

According to the one-way ANOVA test results there was no statistical difference in the tumor growth in all groups as well as in doubling time of tumors (Fig. 18). Dunn’s test was performed to compare vaccinated groups to controls and it showed that there was statistically significant difference in the tumors sizes, which means such treatment had its reducing effect on tumor growth.

<table>
<thead>
<tr>
<th>Vaccine Group</th>
<th>Final Tumor Volume mm³ (mean, SEM)</th>
<th>Tumor Growth Rate</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>K</td>
<td>Doubling time (days)</td>
</tr>
<tr>
<td>NK</td>
<td>1432 ± 288</td>
<td>0.29 ± 0.02</td>
<td>2.44 ± 0.13</td>
</tr>
<tr>
<td>DE_mT2</td>
<td>575 ± 141</td>
<td>0.26 ± 0.03</td>
<td>2.39 ± 0.38</td>
</tr>
<tr>
<td>DE_RRD_mT2</td>
<td>240 ± 114</td>
<td>0.22 ± 0.05</td>
<td>3.01 ± 0.72</td>
</tr>
</tbody>
</table>

Figure 18. One-way ANOVA on final tumor volumes (p=0.015). Dunn’s test versus controls (p=0.682). K – specific growth rate constant.
3.4. Vaccination by i.t. administration of VLPs and IL-2 induces temporary tumor lysis and boosts up innate immune response

This pilot-study was performed to check the efficacy of the IL-2 and VLPs impact on the established B16 tumors. Mice with established tumors in different sizes were vaccinated once i.t. with the solution of IL-2 and pSFV_wt_mT2 or pSFV_RRD_mT2 VLPs (Fig. 19).

![Graphs showing tumor growth](image)

Figure 19. Tumor growth in days before (days -4,-2) and after (days 2, 4) the treatment with pSFV_wt_mT2+IL-2 and pSFV_RRD_mT2+IL-2.

In result, one mouse vaccinated with pSFV_RRD_mT2 with IL-2 had a pause in tumor growth, other (depending on the tumor size) had a slowdown in the development of tumor. Only one mouse did not respond to the treatment at all.

In group of mice vaccinated with pSFV_wt_mT2 and IL-2 the result seemed to be better. As 2 mice had a pause in the tumor growth and all others had a major slowdown in the tumor development. An interesting fenomen was followed that day - after treatment tumors became soft and floppy inside of it, probably because of the local massive apoptosis triggered by VLPs and IL-2.

As the therapy was performed only once, the tumors started growing again in a normal way when the treatment stopped affecting directly the tumor cells. This study must be repeated with the larger groups of mice and with the same-sized tumors to
confirm the results, and definitely there is needed several administrations to get more pronounced results.

3.5. Antibodies are not produced in high titres in vaccinated mice

The ELISA analysis was done for detecting possible tyrosinase-related protein-2 specific antibody titers in vaccinated mice sera. In both analyses, as negative control non-vaccinated mice sera was used. Goat anti-mouse peroxidase conjugate was used as secondary antibody for all samples.

The analysis showed that all vaccinated mice produced antibodies but in different titers – the ELISA results of sera (Fig. 20) of vaccinated mice are higher than the negative control.

In the first vaccination, the highest titer (the best is to compare the data in the 1/150 or 1/450 dilutions) was obtained in mice vaccinated with 0.33 µg of DE_mT2 (Fig. 120). This result correlated with the tumor growth results, when DE_mT2 0.33 µg group had the smallest and the slowest growing tumors (Data 2). The plasmid...
vaccination in other concentrations also gave an antibody titer was different from negative control (DE empty vector vaccinated mice and mice who received pure gold particles).

In the second vaccination, the lowest titer (the best is to compare the data in the 1/150 dilution) of anti-TRP-2 antibodies was obtained in group of mice vaccinated once with i.t. injection of pSFV_wt_mT2+IL-2 and pSFV_RRD_mT2+IL-2 – this titer was even lower than negative control was (Fig. 20). But as it was only a pilot-study, we analysed only 4 mice out of 12. So this analysis must be repeated with the other mice sera to confirm these results. The highest titer was obtained in the group of mice vaccinated with DE_RRD_mT2 plasmid injection and it was even higher than the titer of the DE_mT2 vaccinated mice, suggesting the better stimulation of antibody production. These results also associate with the results of tumor growth results, where DE_RRD_mT2 group had smaller and slower growing tumors than DE_mT2 group had.

According to the one-way ANOVA test there was no difference between the DE_mT2 (in different concentrations) vaccinated groups in antibody titration. The same analysis performed to compare the DE_mT2 and DE_RRD_mT2 groups showed that DE_RRD_mT2 is significantly different from other groups (Fig. 22).

<table>
<thead>
<tr>
<th>Group Name</th>
<th>N</th>
<th>Mean</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>DE_mT2, 0.33 μg</td>
<td>4</td>
<td>-0.697</td>
<td>0.0118</td>
</tr>
<tr>
<td>DE_mT2, 1 μg</td>
<td>4</td>
<td>-0.852</td>
<td>0.0782</td>
</tr>
<tr>
<td>DE_mT2, 3 μg</td>
<td>5</td>
<td>-0.89</td>
<td>0.0594</td>
</tr>
<tr>
<td>DE</td>
<td>5</td>
<td>-0.96</td>
<td>0.0829</td>
</tr>
<tr>
<td>NK</td>
<td>4</td>
<td>-0.826</td>
<td>0.0434</td>
</tr>
</tbody>
</table>

Figure 21. One-way ANOVA (p=0.104)
Figure 23. ELISpot analysis data. IFN-$\gamma$-positive cells, spots counted per $10^6$ spleen cells.

<table>
<thead>
<tr>
<th>Group Name</th>
<th>N</th>
<th>Mean</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>NK</td>
<td>5</td>
<td>-0.0000434</td>
<td>0.0000144</td>
</tr>
<tr>
<td>DE_mT2</td>
<td>16</td>
<td>-0.0000733</td>
<td>0.0000167</td>
</tr>
<tr>
<td>DE_RRD_mT2</td>
<td>7</td>
<td>-0.000165</td>
<td>0.0000115</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Diff of Means</th>
<th>P</th>
<th>P&lt;0.050</th>
</tr>
</thead>
<tbody>
<tr>
<td>NK vs. DE_RRD_mT2</td>
<td>0.000121</td>
<td>0.006</td>
<td>Yes</td>
</tr>
<tr>
<td>NK vs. DE_mT2</td>
<td>0.0000299</td>
<td>0.610</td>
<td>No</td>
</tr>
<tr>
<td>DE_mT2 vs. DE_RRD_mT2</td>
<td>0.0000914</td>
<td>0.004</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Figure 22. One-way ANOVA results. (p=0.002)

3.6. **ELISpot results are inversely correlated with tumor size**

The mouse IFN-$\gamma$ ELISpot assay is designed for the detection of IFN-$\gamma$ secreting cells at the individual single cell level. ELISpot analysis was performed to monitor immune response in mice to the therapies that were done.

As samples every mouse (that received treatment) spleen cells were used. The tests...
showed that vaccinated mice started secreting IFN-γ (Fig. 23), as all groups had IFN-γ titer higher than negative control group, that received pure gold particles instead of plasmids. The best result was achieved in mice vaccinated by i.t. injection of IL-2 and SFV vectors VLPs, but as it was only a pilot-study and only 4 mice out of 12 were analysed we cannot confirm them.

Mice vaccinated with DE-mT2 and DE_RRD_mT2 had almost the same number of IFN-γ secreting cells. A little higher spot count of DE-mT2 can be explained by higher cytotoxicity of wild type vector, as this induces the faster cell death and IFN-γ response. DE_RRD_mT2 apparently did not have high spot count because it was not cytotoxic enough to induce IFN-γ response to massive cell apoptosis.

According to the one-way ANOVA and Dunn’s test performed on the data from ELISpot there is a difference in IFN-γ positive dots only in DE_mT2 vaccinated group comparing to the negative controls. DE_RRD_mT2 vaccination did not give any results (Fig. 24).

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Median</th>
<th>25%</th>
<th>75%</th>
</tr>
</thead>
<tbody>
<tr>
<td>NK</td>
<td>5</td>
<td>20.000</td>
<td>6.000</td>
<td>20.000</td>
</tr>
<tr>
<td>DE_mT2</td>
<td>16</td>
<td>68.000</td>
<td>40.000</td>
<td>84.000</td>
</tr>
<tr>
<td>DE_RRD_mT2</td>
<td>7</td>
<td>36.000</td>
<td>30.000</td>
<td>42.000</td>
</tr>
</tbody>
</table>

<table>
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<tr>
<th>Comparison</th>
<th>Diff of Ranks</th>
<th>P&lt;0.05</th>
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</thead>
<tbody>
<tr>
<td>DE_mT2 vs NK</td>
<td>14.500</td>
<td>Yes</td>
</tr>
<tr>
<td>DE_RRD_mT2 vs NK</td>
<td>8.857</td>
<td>No</td>
</tr>
</tbody>
</table>

Figure 24. One-way ANOVA on the ELISpot results. Dunn’s test on ranks of one-way ANOVA.
The Spearman rank correlation results show the inversed correlation between tumor size and IFN-γ spots counted - the smaller the tumor was the more spots there were counted in the ELISpot test. It means that the smallest tumors were in those mice that developed an immune response after the therapy and could reject the tumor (Fig. 25).

<table>
<thead>
<tr>
<th>R</th>
<th>Final Tumor Volume</th>
<th>Slope of Log antibody titration</th>
<th>Elispot count</th>
</tr>
</thead>
<tbody>
<tr>
<td>P</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>33</td>
<td>30</td>
<td>33</td>
</tr>
</tbody>
</table>

Figure 25. Spearman rank correlation on the results from ELISpot of DE_mT2, DE_RRD_mT2 and NK. R – is correlation coefficient, p – the significance level, n – number of samples.
4. Discussion

Melanoma is a very serious problem nowadays, as well as any other cancer. A lot of scientists are searching for the better treatment to prevent or to cure this type of cancer, but none has yet succeeded and there is still no cure or vaccine that would work 100% guaranteed.

A first step toward generating an efficacious vaccine in humans is to test it in preclinical model, C57/Bl6 mice bearing established B16 melanoma, a very aggressive tumor. A vaccine that is an effective prophylactic usually fails in antitumor bearing host. This happens because the tumor itself alters the immunologic milieu, crippling nascent antitumor immune responses.

Many attempts were made to regress tumor using different melanosomal antigens, such as tyrosinase, tyrosinase-related protein 1 and 2 and others. The administration of adenovirus vector encoding human TRP-2 showed great results, reducing the number of metastases in mice. This vector also showed good results in a challenge study, when vaccinated mice received B16 cells s.c. and 50% of mice completely rejected this challenge. The same study was performed with mouse TRP-2 and all mice developed tumors in result [46].

Our results showed that mouse TRP-2 administration in SFV vector is effective in smaller doses (such as 0.33 µg), in higher doses (1.0 and 3.0 µg) treatment was less effective. The dependence of the vaccine efficacy from the amount of DNA used can be explained by the cytotoxicity of the vector. SFV vector is cytotoxic to the host cell and if there is too much of the virus administered at a time there can be serious consequences – total shut off the host protein synthesis, including highly important cytokines, and also too fast cell death. With the smaller amounts there is less possibility that few plasmid molecules enter the same cell at the same time, thus lowering the cytotoxicity level in the host cell. But according to the statistical analysis there was no
difference in the tumor growth speed between these groups, the difference was only in the smaller size of tumors in DE_mT2 group. Also there was no significant difference in antibody titers between these groups.

As the cytotoxicity of SFV vector was a serious problem, new vectors have been designed\[84, 85\], that encode the mutated nsP2 protein which is considered to be responsible for the cytotoxicity of the SFV virus. These mutations make the vector less cytotoxic and it can express the encoded protein for longer than with wild type vector.

In our study, we vaccinated mice with wild type vector and with mutated vector encoding the same protein and compared the results. According to our results, the mutated vector was more efficacious than wild type vector. It is apparently due to the lower cytotoxicity, thus the longer life of the host cell and the longer expression of the encoded protein, thus the better effect of the vaccine. Statistical analysis showed that the DE_RRD_mT2 group was significantly better protected from tumor (the growth speed was the same but the sizes were much smaller) than other groups. According, to the ELISA test results DE_RRD_mT2 mice had higher titer of antibodies that any other group. But the statistical analysis of ELISpot test showed that DE_RRD_mT2 vaccination does not activate IFN-γ response. thus, the effect of treatment might be INF-γ independent, involving other cytokines, maybe connected with innate immunity.

There have been studies that showed very powerful effect of IL-2 on tumor regression \[78, 81\]. Our pilot study (administration of IL-2 and VLPs i.t.) showed also that this therapy has effect on the tumor development. Study showed that this vaccine even when administrated only once resulted tumor growth slowdown in established tumors. ELISpot results showed that this vaccination activated IFN-γ responses at levels higher than other vaccination strategies. However, the results showed that it did activate also innate immunity as the negative control wells loaded with culture medium and spleen cells only had also high IFN-γ spot count.
Acknowledgements

The author thanks supervisors – Nele Jaanson and Andres Merits; Eva Zusinaite and Margus Varjak for advisory on the SFV vector methods; Many thanks to M. Terence O'Reilly for doing statistical analysis of our data.
Conclusion

Ideally, vaccines should be: safe, highly immunogenic, non-integrating, easy to manipulate, genetically stable and inexpensive to produce. Also, a therapeutic vaccine must not be compromised by pre-existing immunity of the patient against the vaccine vehicle. While „conventional“ DNA vaccines are frequently hampered by low efficacy, replicase-based vaccines may significantly improve efficacy. „Self-replicating“ genetic vaccines may be effective in the fight against diseases that have so far successfully resisted conventional vaccination strategies using recombinant viruses, proteins or bacteria.

The research that we have made is only a part of a bigger project. As previously found in our lab from small pilot studies using anti-tyrosinase mAbs and DNA vaccination for treating established melanomas in C57/bl mice, some of these mAbs and vaccines inhibited tumor growth (unpublished data). The aim of my work was to further analyse which vaccines have a better effect in tumor growth regression.

As a result of a number of different tests we performed it is now clear that vaccination of mice with SFV vector encoding mouse tyrosinase-related protein-2 gives good results in tumor growth slowdown. Even better result were achieved when vaccinated with mutated (point mutation in nsP2 protein) SFV vector encoding the same protein, as it is less cytotoxic and apparently does not kill the host cell so fast, thus allowing the vaccine to function longer.

The second thing, which was to be proven is that some of the mentioned antibodies actually have an tumor growth slowing effect. According to the study that we have performed, at least one of the antibodies has a great effect of tumor growth slowdown and tumor regression.
One more aspect was viewed: is the IL-2 and VLPs therapy effective against melanoma tumor. The results of a small pilot-study showed that apparently IL-2 and VLPs therapy is slowing down the tumor growth.

As it was already said, this was only a part in a bigger project and we hope to study melanoma antigens and their antibodies further too. In the near future, there are plan to work with antibody engineering and using the combination of vaccination and mAb treatment of melanoma.
Semliki Forest Viiruse põhiste vektorite, melanoomispetsiifiliste antikehadate ja IL-2/VLP-de kasutamine melanoomi immuunutraapis

Kiira Trussova

Resüümee

Vaktsiinid peaksid olema: ohutud, kõrgelt immunogeensed, kergelt manipuleeritavad, geneetiliselt stabiilsed ja odavad. Samuti ei tohiks terapeutilise vaktsiini vektorsüsteemi vastu olema eelnevat immunsust. Kui tavalistel DNA vaktsiinidel on puuduseks see et nad on väheeffektiivsed, siis isereplitseeruvad vaktsiinid võivad vaktsiini effektiivsust tõsta. Isereplitseeruvad vaktsiinid võivad olla effektiivsed selliste haiguste ravis, mis siiamaani suutsid vaktsineerimisele vastu võidelda.

See uurimistöö on ainult väike osa suuremast projektist. Nagu on varem leitud meie laboris, türosinaasi vastaste antikehade ja DNA vaktsineerimise eelkatsetest, mõned nendest antikehadest ja DNA vektoritest inhibeerisid tuumori kasvu C57/BL hiirtes. Selle töö eesmärgiks oli uurida lähemalt neid vaktsineerimise võimalusi ja välja selgitada mis töötavad paremini.

Tehtud analüüside põhjal võib öelda, et hiirte vaktsineerimine hiire TRP-2-ga annab tulemusi tuumori kasvu aeglustamises. Isegi paremad tulemused on saavutatud muteeritud (nsp2 valgus) SFV vektoriga sama antigeeniga vaktsineerimisel, sest see on vähem tsütotoksiline ja tänulele et rakk ei sure nii kiiresti valgu ekspressioon toimub kauem.
Teiste katsete põhjal jõudsime järeldusele, et mõned türosinaasi vastased antikehad pidurdavad tuumori kasvu. Tulemused näitasid, et üks uuritud antikehadest mõjutas tuumori arengut ja kasvu kiirust.

Veel üks aspekt oli uuritud: IL-2 ja VLP-de terapia melanoomi tuumori vastu. Selle piloot-katse tulemused näitasid, et see kombinatsioon pärssis juba tekkinud tuumori kasvu.

Nagu juba öeldud see oli ainult osa meie suuremast projektist ja me loodame ka edaspidi tegeleda melanoomi antigeenide ja antikehade uuendamisega. Lähitulevikus aga, on planeeritud katsetada uut vaksineerimise strateegiat – kombineerida antikehade ja DNA vaktsiini manustamist.
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Data 1. Constructed plasmids.
Data 2. Tumor sizes in days in groups of mice treated with different concentrations of DNA.
Data 3. Tumor sizes in days in groups of mice treated with different antibodies.