Investigation of the Vaccine Potential of Different Semliki Forest Virus Based Vectors

Ieva Vasiliauskaite

Degree project in applied biotechnology, Master of Science (2 years), 2009
Examensarbete i tillämpad bioteknik 45 hp till masterexamen, 2009
Biology Education Centre, Uppsala University, and Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet, 17177 Stockholm, Sweden and the Department of Immunology and Vaccinology, Swedish Institute for Infectious Disease Control, 17182 Stockholm, Sweden.
Supervisors: Prof. Peter Liljeström, Dr. Maria Kakoulidou, and Dr. Daniel Johansson.
SUMMARY

Vaccination is one of the greatest achievements in human medicine and prevents millions of people from potentially fatal infectious diseases. However, the fact that present vaccines are not always effective or safe enough, in addition to the emergence of new dangerous infectious diseases such as human immunodeficiency syndrome, indicates a demand for novel vaccines. Vectors derived from Semliki Forest virus (SFV) are suitable candidates for a future vaccine platform as well as other therapeutic applications due to plenty characteristics such as their replicating nature and stimulation of innate immune responses. In this thesis, two variants of replication-competent SFV vectors were compared for their potential for vaccination purposes: TREP-C which contains an antigen (Ag) gene cloned in frame with the structural viral genes and TREP-E2A which carry an Ag gene under an additional subgenomic promoter. As these vectors encode not only an Ag but also structural viral proteins, the delivery of them as DNA to the cells results in production of infectious viruses that infect new cells. In the first part of the project, the stability of the Ag gene in these vectors was evaluated by propagating TREP virus for several passages in cell culture and checking the cells for Ag expression by flow cytometry analysis. This study revealed that there is no obvious difference between the capacities of these vectors to sustain a functional Ag gene. Moreover, the investigation of mutations causing the loss of Ag expression from TREP vectors showed that the presence of homologous sequences, i.e. duplicated subgenomic promoters, does not necessary lead to homologous recombination. It seems that the loss of Ag expression in both TREP vectors primary occurs due to the imprecise function of the viral replicase which lacks proof-reading ability. Therefore, the lack of homologous sequences in TREP-C vectors does not improve its stability markedly. Though flow cytometry analysis implies that the TREP-C vectors are marginally more stable than the TREP-E2A counterparts, this observed difference might be attributed to the slower replication of the TREP-C vectors. Interestingly, in light of some other experiments carried out in the course of this project it seems that the TREP-C virus might have problems in processing its structural proteins resulting in lower titers of the virus as well as formation of higher numbers of deficient non-infectious viral particles.

In the second part of the project, the TREP-E2A and TREP-C vectors were assessed for their capacities to induce Ag-specific cellular and humoral immune responses. In addition, another SFV- based vector (DREP-E2A) which does not encode the structural viral genes and, therefore, does not form viral particles, was also included to compare the immune responses after immunization with different types of SFV vectors. All mouse vaccination experiments were performed by intradermal injection followed by electroporation. In vivo experiments demonstrated that both TREP vectors induced similar cellular as well as humoral immune responses against the encoded Ag. Meanwhile, the Ag-specific cellular and humoral responses reached significantly higher levels using DREP-E2A vectors. Moreover, bioluminescent imaging revealed that more Ag molecules were expressed in vivo from DREP-E2A vectors in comparison with TREP vectors. The reasons why the DREP-E2A vector is more efficient than the TREP vectors in inducing immune responses are not completely clear. First, the structural features of DREP-E2A and TREP vectors may cause different levels of Ag expression and subsequently differences in immune responses. One more assumption is that type I interferon can play an important role by signaling to cells to enter an anti-viral state and in this way suppress the spread of TREP virus. Moreover, other innate immunity components recognizing the budding virus in the extracellular space might also participate in blocking TREP virus replication. In conclusion, the research performed revealed interesting observations regarding the characteristics of SFV-based vectors that need to be investigated in more detail.
# CONTENTS

CONTENTS ............................................................................................................................... 3
LIST OF ABBREVIATIONS ........................................................................................................... 3
INTRODUCTION .......................................................................................................................... 5
The immune system and its mechanisms of action ................................................................. 5
Semliki Forest Virus ................................................................................................................. 8
SFV vectors .............................................................................................................................. 12
Applications of SFV Vectors ................................................................................................. 15
AIMS ......................................................................................................................................... 18
RESULTS .................................................................................................................................. 19
Construction of SFV vectors with inserted Ag genes ............................................................ 19
Stability studies ...................................................................................................................... 19
Analysis of mutations leading to loss of Ag expression ....................................................... 22
Ag expression kinetics in vitro .............................................................................................. 24
Comparisons of viral titers obtained using qRT-PCR and plaque assay ................................ 26
Immunization studies ............................................................................................................. 26
DISCUSSION ............................................................................................................................. 36
MATERIALS AND METHODS ................................................................................................. 47
Transfection ......................................................................................................................... 47
Transformation of DNA into competent cells of E.coli ....................................................... 47
Propagation and purification of plasmid DNA ...................................................................... 47
Restriction Endonuclease Digestion ..................................................................................... 48
Agarose Gel Electrophoresis ................................................................................................. 48
DNA Extraction from Agarose Gels ................................................................................... 48
DNA quantification .............................................................................................................. 48
DNA Insert Ligation into Vector .......................................................................................... 48
Sequencing ............................................................................................................................ 49
One-step Quantitative Real-time Reverse Transcription Polymerase Chain Reaction .......... 49
cDNA synthesis .................................................................................................................... 50
Cells ....................................................................................................................................... 50
Plaque assay .......................................................................................................................... 51
Flow Cytometry Analysis ..................................................................................................... 51
Mice and immunizations ...................................................................................................... 51
In vivo Bioluminescent Imaging ............................................................................................ 52
ELISPOT ................................................................................................................................. 52
The Direct Enzyme-linked Immunosorbent Assay (ELISA) for EGFP-specific IgG Antibodies ......................................................................................................................... 54
Statistical Analyses .............................................................................................................. 54
APPENDIX I ............................................................................................................................. 55
ACKNOWLEDGEMENTS .......................................................................................................... 57
REFERENCES ........................................................................................................................... 58

Front page illustration: cryo-electron microscopy image of Semliki Forest virus (Mancini et al., 2000). Published with permission from UCSF Chimera image gallery, the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco.
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2A Translational-skip peptide derived from foot-and-mouth disease virus 2A protease</td>
<td></td>
</tr>
<tr>
<td>Ag Antigen</td>
<td></td>
</tr>
<tr>
<td>APC Antigen presenting cell</td>
<td></td>
</tr>
<tr>
<td>BCR B cell receptor</td>
<td></td>
</tr>
<tr>
<td>BHK Baby hamster kidney cells</td>
<td></td>
</tr>
<tr>
<td>C Capsid protein</td>
<td></td>
</tr>
<tr>
<td>CD Cluster of differentiation molecule</td>
<td></td>
</tr>
<tr>
<td>cDNA Complementary DNA</td>
<td></td>
</tr>
<tr>
<td>CMV Cytomegalovirus immediate early promoter</td>
<td></td>
</tr>
<tr>
<td>CTL Cytotoxic T cell</td>
<td></td>
</tr>
<tr>
<td>DC Dendritic cell</td>
<td></td>
</tr>
<tr>
<td>DREP Semliki Forest virus-derived layered DNA-RNA vector</td>
<td></td>
</tr>
<tr>
<td>E Translational enhancer element (N-terminal part of the Semliki Forest virus capsid)</td>
<td></td>
</tr>
<tr>
<td>E1, E2, E3 Envelope proteins</td>
<td></td>
</tr>
<tr>
<td>EGFP Enhanced green fluorescent protein</td>
<td></td>
</tr>
<tr>
<td>ELISA Enzyme-linked immunosorbent assay</td>
<td></td>
</tr>
<tr>
<td>ELISPOT Enzyme-linked immunosorbent spot assay</td>
<td></td>
</tr>
<tr>
<td>Fc Constant region of antibody</td>
<td></td>
</tr>
<tr>
<td>FCS Fetal calf serum</td>
<td></td>
</tr>
<tr>
<td>FMDV Foot-and-mouth disease virus</td>
<td></td>
</tr>
<tr>
<td>P Passage</td>
<td></td>
</tr>
<tr>
<td>HIV Human immunodeficiency virus</td>
<td></td>
</tr>
<tr>
<td>Ig Immunoglobulin</td>
<td></td>
</tr>
<tr>
<td>IFN Interferon</td>
<td></td>
</tr>
<tr>
<td>IL Interleukin</td>
<td></td>
</tr>
<tr>
<td>IU International unit</td>
<td></td>
</tr>
<tr>
<td>MCS Multiple cloning site</td>
<td></td>
</tr>
<tr>
<td>MHC Major histocompatibility complex</td>
<td></td>
</tr>
<tr>
<td>MOI Multiplicity of infection</td>
<td></td>
</tr>
<tr>
<td>Nsp Non-structural protein</td>
<td></td>
</tr>
<tr>
<td>ORF Open reading frame</td>
<td></td>
</tr>
<tr>
<td>OVA Ovalbumin</td>
<td></td>
</tr>
<tr>
<td>PAMP Pathogen-associated molecular pattern</td>
<td></td>
</tr>
<tr>
<td>PBS Phosphate buffered saline</td>
<td></td>
</tr>
<tr>
<td>pfu Plaque-forming unit</td>
<td></td>
</tr>
<tr>
<td>PRR Pattern recognition receptor</td>
<td></td>
</tr>
<tr>
<td>OD Optical density</td>
<td></td>
</tr>
<tr>
<td>qRT-PCR Quantitative real-time reverse transcription polymerase chain reaction</td>
<td></td>
</tr>
<tr>
<td>SFV Semliki Forest virus</td>
<td></td>
</tr>
<tr>
<td>TCR T cell receptor</td>
<td></td>
</tr>
<tr>
<td>Th T helper cell</td>
<td></td>
</tr>
<tr>
<td>TLR Toll-like receptor</td>
<td></td>
</tr>
<tr>
<td>tLuc Recombinant luciferase carrying an N-terminal peptide sequence known to be displayed on major histocompatibility complex molecules</td>
<td></td>
</tr>
<tr>
<td>TREP Replication-competent Semliki Forest virus (“Trojan” replicon)</td>
<td></td>
</tr>
<tr>
<td>VREP Semliki Forest viral replicon</td>
<td></td>
</tr>
</tbody>
</table>
INTRODUCTION

The immune system and its mechanisms of action

Innate immunity

Innate immunity is the first guard of an organism against foreign invaders such as bacteria, viruses and parasites. It consists of several components. The skin protects us from most infectious agents. Only pathogens that pass through this barrier can potentially cause disease. Moreover, the mucus, tears and saliva are liquids that not only can wash out the microorganisms, but also possess some biological compounds such as lysosymes, phospholipases, defensins, and lactoferrins that have detrimental effects for the viability of the invading pathogens. In addition, the natural flora of the skin and other epithelium surfaces also prevents invasion of other microorganisms. The pH of the epithelial surfaces, especially in the gastrointestinal tract, is also unfavourable for the pathogens. If a pathogen, however, succeeds in penetrating the mechanical and chemical barrier of the epithelial layer, the cells and molecules of the innate immune system attack and try to eliminate the invader. Phagocytes, natural killer (NK) cells and other innate immune system cells have evolved to sense different pathogens and destroy them in a local immune reaction before letting them spread into the organism (Aderem & Underhill, 1999; Godaly et al., 2001). Other important cells of the innate immune system are dendritic cells (DCs), a class of professional antigen (Ag)-presenting cells (APCs) (Cao & Liu, 2007). They are able to phagocytose invading pathogens and process foreign proteins into peptides that are presented by the major histocompatibility complex (MHC) class II molecules on the cell-surface. Though macrophages and B cells are also classified as APCs due to their expression of MHC class II molecules, they are less efficient in this process. Some of the DCs bearing the foreign peptide-MHC complexes migrate to lymphoid organs where they present the Ags obtained in the peripheral sites to the CD4+ T cells and activate the adaptive immune system (Savina & Amigorena, 2007). Moreover, DCs can also load the peptides that they generate from exogenously captured pathogens on MHC class I molecules and present them to CD8+ T cells. This process, referred to as cross-presentation, is very helpful in clearing viral infections if the virus does not infect DCs directly (Cresswell et al., 2005; Villadangos & Schnorrer, 2007). Furthermore, there are a number of molecules in the serum that also are important players in the innate immunity. For example, the complement system, collectins, cytokines and acute phase proteins, are all employed in eradicating the pathogen. One important feature of the innate immune system is that it can distinguish the biological compounds of “self-origin” from the ones found on the microorganisms. The microbial components that are recognized by the innate immune system are known as pathogen-associated molecular patterns (PAMPs). Since PAMPs are highly conserved essential molecules of microorganisms, the cells of the innate immune system have evolved to produce pattern-recognition receptors (PRRs) able to detect PAMPs (Akira et al., 2006; Janeway & Medzhitov, 2002; Medzhitov, 2007). When PRRs recognize their ligands, signaling cascades are triggered in the cells, leading to the expression of other molecules that stimulate further immune responses. PRRs include Toll-like receptors (TLRs), complement receptors, collectins, scavenger receptors, CD14 and many others (Meylan et al., 2006). The PRRs are germ-line encoded, and they only interact with conserved molecules of the pathogens, i.e. the PAMPs. However, the infectious organisms have learned how to mask their PAMPs and hence to avoid encounter with the PRRs. The activation of PRRs affects not only the innate immune responses but also the subsequent adaptive immune mechanisms (Iwasaki & Medzhitov, 2004; Pasare & Medzhitov, 2004). Overall, innate immunity is of paramount importance for repressing infections during the
initial phase of infection, since the response of the adaptive immunity takes several days before becoming fully active. Moreover, the types of adaptive immune responses that are formed are highly influenced by the signals that come from the innate immunity. Finally, the cells of the innate immune system are also involved in destruction of the pathogens that are detected by the players of the adaptive immune system (Hoebe et al., 2004).

The adaptive immune responses

Though innate immunity is a powerful gatekeeper watching the safety of our body, it does not always protect the organism from invading pathogens. Many microbes have developed certain mechanisms that help them to avoid recognition by the cells and molecules acting in the innate immunity. For example, they can change their surface molecules, inhibit phagocytosis or the complement system, and impair TLR binding and signalling (Bowie & Unterholzner, 2008; Finlay & McFadden, 2006; Hornef et al., 2002). The adaptive immune response is characterized by high specificity to Ags of infectious agents, and the ability to form immunological memory which allows a rapid and efficient targeting of reencountered pathogens. The adaptive immune system is comprised of two branches, humoral and cellular responses. The key cells mediating the work of adaptive immunity are lymphocytes. They originate from the same progenitor cells, but the lymphocytes that mature in a thymus develop into T cells, whereas the ones that undergo maturation in the bone marrow become B cells. In contrast to the innate immune system, T cells and B cells can recognize enormous number of Ags of non-self origin as the receptors of these cells undergo extensive rearrangement and editing processes at the genetic as well as the protein level (Bassing et al., 2002; Maizels, 2005).

The cellular immune response

The T cells are responsible for the cellular arm of the adaptive immunity. They are crucial for immune responses against intracellular microorganisms such as viruses. T lymphocytes are classified by their surface molecule expression: CD8+ T cells and CD4+ cells. CD8+ T cells are also known as cytotoxic T cells since they can directly kill the infected cells by releasing perforin, granzyme and other cytotoxins. Moreover, CD8+ T cells carry Fas ligand and can induce apoptosis in cells expressing Fas (Nagata & Golstein, 1995). In addition, they release other bioactive molecules, such as cytokines. In order to perform their effector function, the CD8+ T cells have to be activated first. Three activation signals are required: 1) Recognition of the foreign peptide presented on the MHC class I molecule by the specific T cell receptor (TCR). The peptides of 8-10 amino acids that are presented on MHC class I molecules are derived from proteins processed by the proteosome (Rudolph et al., 2006). Almost all cells (except erythrocytes) possess MHC class I molecules on their surface. Cytotoxic T cells are crucial for combating viral infections and other intracellular infections. 2) Costimulatory signals from APCs (e.g. B7 molecules expressed on APCs binding to CD28 found on T cells). Costimulation is essential in order to prevent destruction of self-tissues. The self-peptides are also loaded on MHC molecules, but if the TCR recognizes them without the presence of costimulatory signals, the T cells enter an unresponsive state called anergy (Appleman & Boussiotis, 2003; Bour-Jordan & Blueston, 2002). Importantly, B7 molecules are expressed on APCs only after they have encountered the pathogen. 3) Binding of cytokines (commonly IFN-α/β or IL-12) to the cytokine receptors on CD8+ T cells. The activated CD8+ T cells increase the production of molecules that further provides stimuli for immune cells to expand and differentiate. In this way, the CD8+ T cell enters to a new state defined as clonal expansion, which is characterized by a rapid proliferation of the cells. Interleukin-2 (IL-2),
which is released by the activated CD8$^+$ T cells, is the main molecule in this process. The activated CD8$^+$ T cells also increase their expression of the IL-2 receptor. The proliferation and differentiation of CD8$^+$ T cells continues several days and leads to an enormous increase (up to 100,000-fold) in the number of Ag-specific cells. These cells are fully-competent immune system players that can perform their effector functions without any extra stimulation except Ag recognition. Moreover, the synthesis of adhesion molecules on CD8$^+$ T cells is also changed, allowing the cells to leave the lymphoid system and migrate to the sites of action where they attack infected cells.

Most things mentioned above also apply to CD4$^+$ cells, but some differences exist. As noted earlier, the APCs capture the pathogens in the peripheral tissues and load the foreign peptides in MHC class II molecules on their surface. In lymphoid organs, MHC class II:peptide complexes interact with TCRs of CD4$^+$ T cells. This event, together with costimulatory signals, activates CD4$^+$ T cells in a similar manner as CD8$^+$ cells. After initial activation, CD4$^+$ T cells have two different routes of differentiation, i.e. they can become T-helper (Th) 1 or Th2 cells. This process is mainly determined by the cytokine environment that is created by the innate immune cells. IL-12 influences the CD4$^+$ T cells to develop into Th1 cells, while IL-4 signalling leads to formation of Th2 cells. As the name of these cells implies, the primary function of both types of Th cells is to provide help and support for the other cells of the immune system in combating the infection (Reiner, 2007). However, the immune responses shaped by Th1 or Th2 cells are different. Th1 cells promote cellular immunity while Th2 cells mainly sustain the humoral arm of the adaptive immune system (Mosmann & Coffman, 1989). Th cells do not have cytotoxic activity, but they are beneficial in clearing the pathogens by other means. Most of the effector functions of Th cells are mediated by cytokines that they secrete, as well as by surface molecules. Other cells of the immune system express receptors for the cytokines and Th effector molecules and in such way get the supporting signals from Th cells. Th1 cells mainly produce bioactive compounds such as IFN-$\gamma$ and TNF-α that improve the action of macrophages. Th2 cells on the other hand produce IL-4 and IL-5, which activate B cells. Moreover, Th cells are in general very important for an efficient B cell response as they drive them to undergo affinity maturation. The TCR of Th cells can recognize Ags presented on MHC class II molecules on B cells. In addition, CD40 and CD40 ligand interaction as well as cytokines signal to B cells to proliferate. Importantly, a binding of CD40 ligand expressed by CD4$^+$ T cells to the CD40 molecule on APCs, results in activating reactions in both of these cells (Schoenberger et al., 1998). The sub-type of immunoglobulins (Igs) that will be secreted by the activated B cell greatly depends on whether the Th1 or Th2 cells interacted with the B cell. Murine Th1 cells stimulate B cells to synthesize IgG2, whereas Th2 cells induce B cells to generate mainly IgG1 (Coffman et al., 1989; Stevens et al., 1988). Moreover, CD4$^+$ T cells have an impact on CD8$^+$ T cells and take part in such processes as priming of naïve CD8$^+$ T cells as well as sustaining the immunological memory of a subset of CD8$^+$ T cells (Janssen et al., 2003).

Actually, there are two more types of relatively recently found CD4$^+$ T cells. Th17 cells seem to be involved in defending the organism from some extracellular pathogens, but they are also linked to autoimmune diseases (Bettelli et al., 2008). One more class of CD4$^+$ cells, regulatory T (Treg) cells, have an immunosuppressive role and control autoreactive T cells (Jutel & Akdis, 2008; Sakaguchi et al., 2008).
Humoral responses

While cellular immune responses primarily are directed to detect and kill intracellular pathogens, the humoral immune response attacks the infectious agents that appear in the extracellular space. The main acting cells in humoral immunity are B cells. As T cells carry highly-specific receptors that recognize only a particular MHC:peptide complex, B cells also express an enormous variety of B cell receptors (BCRs) and, therefore, have a capacity to specifically bind almost every foreign molecule. BCRs are composed of four chains: two heavy (H) chains and two light (L) chains. Variable segments of the H and L chains comprise the Ag-binding site and determine the specificity of the BCR. In addition, the H chain contains a constant (Fc) region. The binding of the specific Ag to a BCR gives activating signals to the B cell. In addition, the bound Ag is internalized and degraded by the B cell and then loaded on MHC class II molecules (Lanzavecchia, 1990). Th cells recognize the Ags on MHC class II molecules and provide co-stimulatory signals for the B cell. The activated B cells differentiate into plasma cells that are characterized by secretion of immunoglobulins, also known as antibodies. The antibodies that are secreted by the activated B cell are almost identical in structure and specificity to the BCR that have recognized a particular Ag and initiated activation of the B cell. Therefore, they are also known as soluble BCRs. As noted above, the support from Th cells is usually required for proper functioning of B cells and, at least in part, determines the properties of the produced antibodies. First, the support from Th cells leads to the clonal expansion of Ag-specific B cells as well as induction of the process known as affinity maturation during which the affinity of the BCRs to the Ag increases even more. In addition, it promotes the isotype-switch, i.e. changing of the Fc region (Stavnezer, 1996). The type of the antibody (IgG, IgM, IgE, IgA or IgD) influences where and how the antibody will work. However, B cells can be activated without contribution of Th cells. For instance, it happens during the event termed cross-linking, when several BCRs on a B cell recognize repetitive foreign structures such as polysacharides. The response to T cell-independent Ags is rapid and results in production of IgM and IgA. However, this kind of Ag-B cell interaction is not likely to lead to immunological memory (Mond et al., 1995; Vos et al., 2000).

There are several ways in which antibodies help to clear an invaded microorganism. Antibodies bind to many different structural patterns on the pathogen thereby covering the unwelcomed guest. This process, referred to as opsonization, leads to phagocytosis as phagocytes bear Fc receptors and recognize the Fc part of antibodies. In addition, the pathogen covered by antibodies can be detected by complement proteins which may destroy the pathogen by activating the complement system cascade. Importantly, the Fc regions of bound antibodies change their conformation thereby allowing interactions with Fc receptors or complement proteins (Brown & Koshland, 1975). The local concentration of antibodies is also important for activation of pathogen-killing events. Both of these conditions help to prevent damage to self-cells. Moreover, the binding of the antibodies to the pathogen also has a neutralizing effect as it can inhibit the entrance of viruses or bacterial toxins to the cells.

Semliki Forest Virus

Semliki Forest Virus belongs to the genus Alphavirus of the family Togaviridae. SFV was first described in 1944 when it was isolated from Aedes abnormalis mosquitoes in Semliki forest in Uganda (Smithburn & Haddow, 1944). SFV is transmitted by mosquitoes to other hosts, mainly rodents and birds. Some alphaviruses can infect humans as well and can be quite pathogenic, sometimes even causing fatal encephalitis. However, SFV is commonly
regarded as avirulent for humans, though it is related with an outbreak of the mild illness in the Central African Republic (Mathiot et al., 1990) and, probably, one death from encephalitis of a German scientist in 1979. In Europe, all laboratory work with SFV is carried out at Biosafety level 2. In the United States, however, SFV is classified as a Biosafety Level 3 virus, although handling of it is allowed at Biosafety Level 2 in most cases. The laboratory strain SFV4 was used to make the infectious clone known as pSP6-SFV4. SFV4 strain is less virulent in mice and most of the mice inoculated peripherally surmount an infection, however, intranasal infection is still lethal. The vectors used in this project are derived from the SFV4 strain.

SFV structure and replication cycle

SFV is a small virus which can be visualized by cryoelectron microscopy as spherical particles of 65-70 nm in diameter (Mancini et al., 2000). An envelope of SFV is composed of 80 glycoprotein spikes embedded in a lipid bilayer which is gained from host-cell membranes during the budding process. Each spike is actually a heterotrimer formed by three envelope proteins, E1, E2 and E3. The envelope surrounds the nucleocapsid which is built from 240 copies of capsid (C) protein arranged to an icosahedral core. A single-stranded positive sense RNA molecule, which represents the SFV genome, is stowed in the nucleocapsid. Genomic RNA is capped with 5’-methylguanylate and polyadenylated at the 3’ end, and consists of approximately 12000 nucleotides.

The genome of SFV contains two open reading frames (ORFs). The first ORF comprises the 5´ two-thirds of the genomic RNA and encodes four nonstructural proteins (nsp1, nsp2, nsp3 and nsp4). They are directly translated to a large polyprotein precursor by the host’s RNA polymerase immediately after release of the viral genome into the cytosol of the host cell (Figure 1). These proteins form a replicase complex which is responsible for the highly efficient viral RNA synthesis. Actually, each of these proteins has its own function in the transcription process. Nsp1 is known as an enzyme capping viral RNAs during transcription, since it has methyltransferase and guanyltransferase activities (Ahola et al., 1999; Laakkonen et al., 1994; Peranen et al., 1995). In addition, it is believed that Nsp1 contributes to an assembly of SFV replication complexes to intracellular membranes (Piver et al., 2006). It has been shown that Nsp2 is capable of binding RNA and embodies NTPase activity in its N-terminal domain; therefore, it is believed to act as an RNA helicase to unwind double stranded RNA during replication and transcription of viral RNA (Rikkonen et al., 1994). In addition, the C-terminal domain acts as a thiol protease and is required for processing of nonstructural polyproteins (Hardy & Strauss, 1989; Strauss et al., 1992). Moreover, Nsp2 has a nuclear localization signal, which explains why about half of the Nsp2 proteins reside in the nucleus. However, it is not known exactly what role Nsp2 plays in the nucleus. From all nonstructural SFV proteins, the least is known about Nsp3. It has been shown that it consists of an N-terminal region conserved among alphaviruses and a non-conserved C-terminal region. No enzymatic activity is assigned to this phosphorylated protein (Lastarza et al., 1994a; LaStarza et al., 1994b; Peranen et al., 1988). Nsp4 has been found to function as a RNA polymerase, due to that it contains a GDD motif, which is also present in other viral RNA polymerases. Mutagenesis studies have also demonstrated that Nsp4 possesses this enzymatic activity.

The second ORF is situated in the 3´ one-third of the genome, and carries the sequence of a structural polyprotein which is translated late in infection after the subgenomic RNAs are generated. This polyprotein is post-translationally processed into five structural proteins, referred to as C, E1, E2, E3 and 6K. The functions of C, E1, E2 and E3 as the structural parts
of the virion have already been mentioned above. X-ray crystallography of the capsid protein revealed that the C-terminal amino acids form a domain that functions as a serine protease, which cuts C from the rest of the structural polyprotein (Choi et al., 1991). This domain interacts with the C-terminal intracellular part of the E2 protein. The N-terminal domain of the C protein points to the inside of the core (Mukhopadhyay et al., 2002). One more glycoprotein, 6K, seems to play a role in events such as processing of envelope proteins, membrane permeabilization and budding of virions. However, virions usually contain only negligible amounts of this small hydrophobic protein. A recent study has shown that 6K actually exists in two forms. The previously unknown form, designated as TF, results from ribosomal frameshifting. It was suggested that TF is important for the stability of virions, since TF rather than 6K is present in the virions. 6K is thought to contribute mostly to the processing of envelope proteins (Firth et al., 2008). Both 6K and TF are suspected to be involved in virus budding. E2 and E3 are synthesized as a larger precursor designated p62, which is cleaved to the mature proteins on its way to the cell surface.

![Diagram of Genome Replication of SFV](image)

**Figure 1.** Genome replication of SFV. The positive strand RNA genome of SFV contains two ORFs, the first one coding for non-structural proteins and the second one coding for the structural proteins. The structural proteins are under the control of a 26S subgenomic promoter. The synthesis of positive sense and negative sense viral genomic RNA occurs at the first stage after infection. Later the production of the subgenomic RNA from the negative sense SFV RNA is initiated.

The SFV genome also includes some other elements that are important for virus replication. One of them is a subgenomic 26S promoter located on the negative strand RNA in front of the ORF encoding the structural proteins (Ou et al., 1982). Viral replicase binds to the 26S promoter with strong affinity, and drives the synthesis of the subgenomic RNA encoding the structural proteins. Another sequence element conserved among alphaviruses comprises 19 nucleotides just in front of the poly A tail. Studies concerning this element suggest that it functions as a promoter for production of the minus-strand genomic RNA (Kuhn et al., 1990; Ou et al., 1981). At the 5' untranslated region of the viral RNA, a sequence forming a stem-loop structure is present. It was demonstrated that this element is essential in minus-strand RNA synthesis. It interacts with a 19 nucleotides sequence at the 3’ end of the genome. This indicates that the 5’ and 3’ ends of the alphavirus genome cooperate in the initiation of minus-strand genomic RNA synthesis (Frolov et al., 2001). The same properties were demonstrated for some other RNA viruses possessing the genome of positive polarity (Barton et al., 2001; Herold & Andino, 2001; You & Padmanabhan, 1999). Moreover, the complementary
sequence of this element at the 3’ end of the minus-strand RNA acts as a promoter for plus-strand RNA synthesis (Levis et al., 1990). One more cis-acting sequence element is located in the Nsp1 protein and spans 51 nucleotides. It is not an essential element, however, it affects the efficiency of genomic RNA replication (Frolov et al., 2001).

SFV infects cells by binding to cell surface receptors. The exact receptor mediating the entry of the virus to the cell is unknown. However, the fact that SFV is capable of infecting many different hosts and types of cells indicates that the receptor should be quite common on the cell surface. Also, it may be that several proteins serve as the receptors (Strauss et al., 1994). Laminin-binding protein is believed to serve as one of the receptors (Wang et al., 1992). It is believed that E2 is employed in attachment to a receptor on the plasma membrane, which leads to the entrance of the virus to the cell by endocytosis. The endocytic pathway, which starts with clathrin mediated endocytosis of the virions and ends with degradation of envelope proteins in the lysosomes, has been investigated in detail. The importance of small GTPases from the Ras GTPase superfamily has been demonstrated in this transport pathway (Vonderheit & Helenius, 2005). The fusion of the viral and endosomal membranes occurs when the vehicles are acidified (White & Helenius, 1980). The low pH in the endosomes causes the conformational changes of the E1/E2/E3 trimer. These conformational changes result in the dissociation of the trimer and the exposure of a hydrophobic loop of E1. This loop is inserted into the target membrane, which must contain cholesterol to promote this action (Smit et al., 1999; Wahlberg et al., 1992). Finally, the genomic RNA contained in the core of the virus is released into the cytoplasm. The nucleocapsids are probably present in a metastable state after entering the cell. They are rapidly disrupted in a process in which ribosomes seem to be involved (Singh & Helenius, 1992; Wengler, 1984; 2009).

The genomic RNA is treated as mRNA by the translational machinery of the cell. Therefore, the nonstructural polypeptide is synthesized from the first ORF in the ribosomes shortly after virus entry of the cell. This polypeptide is cleaved into mature proteins by Nsp2 which possesses protease activity. Then the replication cycle of the virus begins. It has been found that RNA replication occurs at the surface of the special vacuoles termed cytophatic vacuoles type I (Froshauer et al., 1988; Kujala et al., 2001). The nonstructural proteins assemble into a replicase complex which generates full-length negative-sense genomic RNAs. The replicase can produce several types of transcripts. It can bind to the promoter located at the 3’ end of the negative strand RNA, which leads to the production of more genomic RNA or it can bind to the 26S subgenomic promoter and synthesize subgenomic RNAs encoding structural proteins. It was shown that the viral replicase has a higher binding affinity to the 26S subgenomic promoter, which results in high-level replication of subgenomic RNA molecules. Actually, the replicase complex has a slightly different composition in each step of the genome replication. Nsp2 and Nsp4 constitute the replication complex responsible for the synthesis of negative-sense RNA. Later the replicase complex composed of Nsp1, the polypeptide Nsp23 and Nsp4 is formed. It takes part in both negative-sense RNA and positive-sense RNA generation. Finally, the replicase complex made up of the fully processed nonstructural proteins Nsp1, Nsp2, Nsp3 and Nsp4 is set up. It has been demonstrated to be involved in making only positive-sense RNA molecules, i.e. genomic RNA and subgenomic RNA. However, it is not capable of manufacturing negative strands (Lemm et al., 1998; Lemm et al., 1994; Shirako & Strauss, 1994). As mentioned above, the 26S subgenomic RNA encodes the polypeptide C-p62-6K-E1. Since the C-terminal domain of the C protein has protease activity, it releases itself from the growing chain in an autocatalytic reaction. After this cleavage, the N-terminal end of p62 serves as a signal sequence for transportation of the structural polypeptide into the endoplasmatic reticulum (ER). Some important events happen
First, the precursors of the envelope proteins are glycosylated. Second, the polypeptide is cleaved to E1, 6K and p62 (Liljestrom & Garoff, 1991). In addition, E1 and p62 aggregate to form heterodimers (Barth et al., 1995). These heterodimers leaves the ER and travels to the cell membrane through Golgi apparatus. On the way, one more processing step occurs: p62 is cleaved to individual proteins E2 and E3 by ubiquitous protease called furin. The nucleocapsid of the virus is formed when newly synthesized C proteins come into contact with genomic RNA molecules. Only genomic RNA can be packed into nucleocapsids, since the packaging signal is present in the nucleotide sequence encoding Nsp2 (Frolova et al., 1997). The budding of the virus is facilitated by interaction between viral core, or, more precisely, C protein, and the cytoplasmic part of E2 anchored in the cell plasma membrane (Suomalainen et al., 1992). As mentioned above, the function of 6K in budding of the viral particles is not completely revealed. It has been suggested that it can operate as an ion-channel at the budding site or take part in the processing of the envelope proteins in the ER (Garoff et al., 2004; Melton et al., 2002).

SFV infection is always accompanied by inhibition of cellular protein translation. The translational machinery is completely overtaken by the viral protein synthesis. It has been shown that the phosphorylation of eukaryotic translational initiation factor 2a subunit (eIF2α) by double-stranded RNA-activated protein kinase R (PKR) is the most likely reason for the shut-off of host protein production. Phosphorylation of eIF2α results in blocked assembly of GTP-eIF2-tRNAi^{Met} ternary complexes. These complexes are essential for the translation initiation of the majority of the cell’s mRNA as well as SFV RNA. However, SFV evolved to overcome this translational inhibition mechanism. It has been demonstrated that the translational enhancer element consisting of the first 102 nucleotides of the C gene allows the translation of 26S subgenomic RNA even in the absence of GTP-eIF2-tRNAi^{Met} ternary complexes (McInerney et al., 2005).

**SFV vectors**

SFV has been used for many years as a model virus in order to gain more knowledge about viruses in general. Better understanding of the structure and life cycle of SFV had led to the idea that a man could use this virus for his own purposes. The construction of the first expression vector based on SFV in 1991 opened new windows for the use of this virus. Today, SFV vectors can be classified into three different classes: SFV replicons (VREP), layered DNA-RNA vectors and replication-competent vectors. Each of them is shortly described below.

**The VREP system**

The initial step in the development of VREP vectors, and SFV expression systems in general, was performed by inserting the cDNA of the SFV genome into a bacterial plasmid under the control of the SP6 or T7 polymerase promoter. In the next stage, the structural genes of the virus were excised from the vector and replaced by a multiple cloning site (MCS) right after the 26S subgenomic promoter. These modifications allowed any gene of interest to be cloned into MCS in such a way that the gene is under the control of the 26S subgenomic promoter. Many copies of recombinant RNA molecules resembling a real SFV genome can be produced by simple *in vitro* synthesis with corresponding RNA polymerase. The transcribed RNA can replicate in the transfected cells in the same manner as the SFV genomic RNA, since it retains the nonstructural genes coding for the replicase complex. This means that the subgenomic RNA encoding a foreign gene is transcribed at a high rate resulting in a high expression of the
inserted gene. In vitro prepared recombinant RNA can be imported into animal cells by electroporation or lipofection (Liljestrom & Garoff, 1991). However, these delivery methods do not work well for all kinds of cells. Therefore, it was decided to exploit the natural ability of SFV to infect many types of cells. For this purpose, a helper vector which carries the structural genes of the virus and the 26S subgenomic promoter driving their synthesis was designed (Liljestrom, 1994). The helper vector allowed recombinant RNA molecules encoding the sequence of a foreign protein to be packaged into virions in vitro. This is achieved by mixing in vitro transcribed RNA species from the helper vector with recombinant RNA and transflecting them into baby hamster kidney (BHK) cells. As SFV buds from the cells, the supernatant collected from transfected cells contains SFV virions containing recombinant RNA. These viral particles, designated VREP, can be used to infect new cells in vivo or in vitro. Moreover, only VREP packaged with recombinant RNA is formed, since the packaging signal lies in the nsp2 gene, which is absent in helper RNA. However, new concerns regarding this type of vectors have arisen. The possibility that the viral replicase can switch strand, thus excluding transcription of a cloned sequence, has to be considered. Such events could lead to recombination of the RNA molecules in a way that wild type virus is generated. A couple of strategies were developed to avoid the production of the easily spreading wild type virus. One of them is to mutate the sequence of the helper vector encoding the cleavage site of p62 (Berglund et al., 1993). As noted earlier, the viral envelope proteins play a very important role in the host-infection process. These non-functional forms of the envelope proteins suppress the ability of viral particles to infect the cells. However, treatment of the virions with α-chymotrypsin activates the spike proteins allowing full infection capacity of the virus-like particles. However, it still does not eliminate the chance of generation and spreading of wild type virus, since the cells possess their own proteases that can potentially activate the virions encapsulating wild type genomic RNA. In addition, reversion or suppressor mutations can occur (Tubulekas & Liljestrom, 1998). To increase the safety of VREP vectors, the helper system was divided into two helper plasmids, one carrying the C gene and the other one the remaining structural genes of the virus (Smerdou & Liljestrom, 1999). This almost completely eliminated the risk of forming a wild-type virus as recombination among three RNA molecules is extremely rare. Moreover, a mutation that resulted in a C protein without self-cleavage activity was generated, which would exclude the formation of replicating virus in case of recombination. The expression of the desirable gene from VREP vectors is transient as the formed viral particles have a suicidal nature and/or the infected cells die from apoptosis (Urban et al., 2008).

Layered DNA-RNA vectors

The production and packaging of recombinant SFV RNA is a quite tedious work. To alleviate this step, a new type of SFV vector has been designed. The sequences encoding the replicase and heterologous genes have been placed in a plasmid vector under the eukaryotic cytomegalovirus (CMV) promoter instead of a prokaryotic promoter. When the cells are transfected with such DNA vectors, the sequences cloned downstream of the CMV promoter are transcribed by the host-cell machinery. The proteins constituting the replicase complex are translated from this RNA that start the cycle of RNA multiplication and subsequent expression of the gene of interest as described for VREP. An expression system substituted with layered DNA-RNA helper vectors has also been built (Berglund et al., 1998; DiCiommo & Bremner, 1998). The layered DNA-RNA system has a lot of advantages compared with the VREP system, especially in potential applications for vaccination. First, DNA vectors are not only easily manufactured but also more stable than VREP, which is important for storage and transportation. Furthermore, introduction of DNA into the organism does not produce
neutralizing antibodies to the vector. In contrast, VREP administration is likely to result in anti-VREP immunity since viral proteins can trigger the production of antibodies. The antibodies to viral proteins can reduce the efficiency of subsequent vaccinations for boosting the immune system. The primary concern for this system has been the possibility of foreign DNA integration into the host’s genome, as the vectors enter the nucleus where the transcription from the CMV promoter is initiated. However, it has been demonstrated that the frequency of integration of DNA into chromosomes that can possibly result in cancer-causing mutations is very low and happens approximately 3000 times rarer than spontaneous mutations (Ledwith et al., 2000; Nichols et al., 1995; Wang et al., 2004). Moreover, SFV infection leads to apoptosis of infected cells. So, even if the integration of the vector sequence occurs, it will be eliminated when the cell dies by apoptosis.

The layered DNA-RNA SFV vectors used in this project are designated DREP.

**Replication-competent SFV vectors**

The replication-competent vectors comprise a separate group of SFV vectors. The main difference of these vectors, compared to the other vectors, is that they contain all structural genes of the virus in addition to the cloned heterologous gene. In essence, this system mimics a real SFV infection: the recombinant viral particles budding from transfected cells are infectious and can spread from cell to cell. Several types of such vectors have been engineered. The first variant of replication-proficient SFV vectors has a heterologous gene inserted downstream an extra 26S subgenomic promoter, which is cloned before the native 26S promoter or after the ORF encoding the structural genes (Hahn et al., 1992; Raju & Huang, 1991; Rausalu et al., 2009; Vaha-Koskela et al., 2003). In this case, two subgenomic RNAs are synthesized (one carrying the gene of interest and the other carrying the genes encoding the structural proteins) and translated separately. Another strategy is to clone a foreign gene straight to the region encoding structural genes. Then the cloned gene will be translated together with the structural genes from the same subgenomic RNA. A preferential tactic is to clone the foreign sequence after the C gene (Fragkoudis et al., 2009; Thomas et al., 2003). As mentioned above, C also works as an autoprotease, so the heterologous protein is cleaved off from the fusion with C. The replication-proficient SFV vectors, containing the genes of interest inserted into the ORF of the non-structural viral genes, have also been successfully developed. However, such vectors can be utilized mostly for marker gene expression, which allows tracking alphavirus infections *in vivo* (Bick et al., 2003; Frolova et al., 2006; Tamberg et al., 2007). Such vectors are not suitable for high-level expression of foreign genes, as the amplification of a whole recombinant RNA unit is not efficient in comparison with amplification of the subgenomic RNA. The advantage of the replication-competent SFV vectors is that the expression of the cloned gene lasts longer than from viral replicons. But the expression still comes to an end because of apoptosis of the infected cells and/or clearance by the immune system. Therefore, replication-competent SFV vectors have gained attention for their potential use in treatment of infectious diseases and cancer. One of the drawbacks of the system is that an inserted heterologous gene Ag might be deleted from the recombinant RNA in the replication process.

The replication-competent SFV vectors used in this project are designated TREP (from “Trojan Replicon”).
Translational enhancer element

One important step in the development of SFV-based vectors has been the discovery of the translational enhancer element (E). It consists of the sequence coding for the 34 N-terminal amino acids of the C protein. An additional modification has been made by inserting the sequence coding for the foot and mouth disease virus 2A translational skip-peptide downstream from E. Together, this element is called E2A, and is positioned into vectors immediately after the 26S subgenomic promoter. The expression of the genes cloned in frame with E2A is enhanced around 10 times. During translation, the 2A will not form a peptide bond to the following peptide. This will result in the formation of two polypeptides upon translation: E2A and the protein encoded by the inserted gene (Frolov & Schlesinger, 1994; Sjoberg et al., 1994).

Applications of SFV Vectors

SFV-derived vectors have become a greatly valuable tool in biotechnology. Their potential use as therapeutic agents in treatment of infectious diseases, cancer and central nervous system diseases has also been thoroughly investigated.

Production of Recombinant Proteins using SFV vectors

One of the areas where the SFV expression system has been shown to be extremely effective is a high-scale production of membrane proteins, especially G protein-coupled receptors (Eifler et al., 2007; Hassaine et al., 2006; Lundstrom, 2003). G protein-coupled receptors are of great interest as drug targets; therefore, the synthesis of the amounts sufficient for pharmacological studies is of great significance. Really high yields of these difficult to express proteins are obtained by the SFV expression system: as much as 10 million molecules of receptors can be synthesized on the cell surface. The transferrin receptor, the dopamine transporter, potassium channels, and cyclooxygenase are just several representatives in the list of other proteins successfully expressed by the use of SFV vectors.

The Use of SFV in Neurobiology

As mentioned above, SFV is a neurotropic virus. It was demonstrated that SFV preferentially infects neurons. Moreover, the transduction efficiency of neurons is much higher with SFV vectors in comparison with other viral vectors. Therefore, the SFV system is widely used to transduce neurons and express genes of interest in vivo, in mice models, and in vitro, in hippocampal slice cultures as well as primary cultures. A lot of studies examining the role of different proteins, e.g. the AMPA receptors, Ca\(^{2+}\) channels, and glutamate transporters, in the central nervous system were performed with the help of the SFV expression system (Hennou et al., 2003; Schweitzer et al., 2000; Takamori et al., 2000; 2001; Wittemann et al., 2000). Moreover, alphavirus vectors are very useful because they allow delivery of several genes into neuron cells (Gorrie et al., 1997). To avoid cytotoxicity, SFV vectors, based on avirulent strains or attenuated virus, are used for most neurobiology experiments (Ehrengruber, 2002). Moreover, mice infected by avirulent SFV strains undergo demyelination of the neurons, which has a lot of similarities to demyelination present in multiple sclerosis affected humans. Therefore, SFV infected mice are also used as a model to explore this disease (Atkins et al., 1994; Atkins et al., 1999). In addition, several studies were performed where SFV vectors carrying the genes for IL-10, IFN-β or inhibitor of metalloprotease 2 were intranasally
delivered to treat autoimmune encephalomyelitis in mice. These experiments revealed some promising results that therapeutic compounds can be effectively delivered and expressed in the central nervous system using the SFV system (Jerusalmi et al., 2003; Nygardas et al., 2004; Vaha-Koskela et al., 2007). However, all research has been conducted mainly in mice, so there are still plenty of issues that have to be addressed in order to apply SFV vectors in human therapy.

**Investigations of SFV Vectors as Agents for Cancer Treatment**

SFV vectors are also examined for their potential use in treatment of cancer. There are several ways how SFV vectors can help to fight this in many cases lethal disease. First, the natural ability of SFV to induce apoptosis in infected cells can be used to kill cancer cells. It can be enhanced by administrating SFV vectors with inserted pro-apoptotic genes. Second, SFV vectors can be employed to express specific tumour Ags in a way that the organism builds its own immunity against them and attacks existing tumours. A similar strategy can be used not only to cure established tumours but also to develop immunity in order to prevent certain types of cancer. Finally, it is known that some cytokines and other kinds of proteins can help to stop the growth of tumours, spreading of metastases or have some other beneficial effects. The SFV system can be a choice for expressing them.

The research concerning SFV vectors as instruments in combating oncolytic diseases is still in the stage of animal experiments. It should be noted that SFV vectors are not able to attack tumour cells specifically if inoculated peripherally. However, if injected into a tumour, viral particles are not prone to spread outside the tumour tissue (Rodriguez-Madoz et al., 2007). Therefore, in order to use the strategy based on induction of apoptosis in tumour cells, SFV vectors have to be delivered straight into the tumour. However, the increasing number of reports presenting successful results in animal experiments raises expectations that, in the future, SFV vectors may be used in treatment of certain forms of cancer in humans. It is beyond the scope of this report to review all literature in this field; however, some reports are worth special attention. It has been demonstrated that VREP expressing Ags of papilloma virus 16-transformed tumours resulted in eradication of the tumours and created long-term CTL memory against subsequent challenges with the same tumour. Even mice that had tumours as big as 500 mm$^3$ were completely cured (Daemen et al., 2003; Daemen et al., 2004). Additional studies indicate that co-administration of SFV vectors expressing IL-12, IL-18 or vascular endothelial growth factor receptor 2 can help to achieve even better results if included in cancer treatment (Chikkanna-Gowda et al., 2006; Lyons et al., 2007; Riezebos-Brilman et al., 2009; Rodriguez-Madoz et al., 2005). An SFV vector encoding genes of p53, angiostatin and PTEN resulted in significant regression of established malignant glioblastoma tumours in mice (Lee et al., 2006). Moreover, replication-competent vectors derived from the avirulent SFV strain A7(74) are also under investigation as candidates to treat glioblastoma, lung cancer and other types of tumours (Ketola et al., 2008; Maatta et al., 2007; Vaha-Koskela et al., 2006).

Several trials to use VREP encased in liposomes that allowed exact direction to cancerous tissues have also been performed. Some advantages have been highlighted for this approach. First, it eliminates the need of intratrumoral administration that is not always possible to perform. In addition, it helps to prevent healthy tissues from the damage. Second, it almost completely eliminates the chances of inducing anti-vector immunity that is important in the case if several inoculations are needed. Finally, VREP sheathed in liposomes can stay longer in the organism and increase the period for the activity of the vector (Lundstrom, 2005). One
phase I/II clinical trial with such kind of vectors has even been announced (Ren et al., 2003). Liposomes carrying a SFV vector with an inserted IL-12 gene were intended for treatment of patients with glioblastoma. The same report also described another clinical trial which had used the identical vector for patients with stage III/IV melanoma and renal cell carcinoma. The injections had been well tolerated by the patients and IL-12 levels in the serum were augmented 10 times and remained steady for approximately 3-4 days. However, the results of these clinical trials have not been published to date.

**SFV Vectors for Generation of Novel Vaccines**

Though SFV vectors are auspicious tools for many applications, the most potential application of SFV vectors is vaccination. There are a number of features that make the SFV expression system particularly suitable for inducing effective immunity against foreign Ags. First, SFV vectors are capable to produce large quantities of the protein encoded by the gene inserted into the vector. Second, the infected cells undergo apoptosis and are engulfed by macrophages. As the dead cell contains large amounts of the heterologous protein, efficient cross-priming leading to robust immune response against that protein may occur. Apoptosis also eliminates a fear that virus can persist in an organism. Third, SFV vectors carrying encoded Ag can stimulate the innate as well as the adaptive immune systems. For instance, innate immunity is stimulated by double stranded RNA that forms as intermediates in the SFV replication process and can be recognized by toll-like receptor-3 (TLR-3) (Pichlmair & Reis e Sousa, 2007). Furthermore, the replication of SFV-based vectors happens in the cytoplasm of the cell, so there are no chances that viral sequences will be integrated into host chromosomes. As mentioned above, the probability of such event is too low to consider. One more positive factor is that humans are not the natural hosts for SFV and hence human serum does not contain antibodies targeting SFV that can significantly limit the action of SFV vectors in the organism. For example, this is a huge problem when using adenovirus serotype-5 based vectors (Sumida et al., 2004; Sumida et al., 2005). Taken together, these qualities have made SFV vectors capturing scientific attention. Therefore, extensive research of all types of SFV vectors carrying particular Ags has been performed seeking to create vaccines against viral, bacterial and parasitic infectious diseases. SFV vector-based prototype vaccines that have been tested in animal models are listed in Table 6 under Appendix.

All these studies suggest that SFV vectors can be efficient vehicles for vaccination purposes and can elicit cellular and humoral immune responses. Of course, plenty of work still should be done in order to understand the underlying mechanisms of vaccination with SFV vectors and the optimal design of the vector. Despite this, SFV vectors have a great potential to be used in future vaccines.
AIMS

In order to use SFV vectors for human vaccination, the design and characteristics of SFV expression systems need to be thoroughly investigated. It is not clear yet what kind of SFV vectors has the greatest potential in this field. Therefore, all types of SFV vectors have to be evaluated for their suitability to develop recombinant vaccines. In this thesis, two different variants of replication-competent SFV vectors with cloned Ag genes of interest are compared: TREP-E2A-Ag and TREP-C-Ag (Figure 2). They are examined for the following questions:

1. Does the in vitro stability of the inserted Ag gene differ between the vectors?
   a. Is the stability of the inserted Ag gene dependent on the nature of the Ag gene?
   b. What are the reasons that lead to loss of Ag expression from TREP-E2A-Ag and TREP-C-Ag vectors?
2. Which one of the replicating SFV vectors can elicit better cellular and humoral immune responses towards the encoded heterologous Ag in a mouse model?
3. Are there any differences in cellular and humoral immune responses induced in mice vaccinated by replication-competent SFV vectors and layered DNA-RNA vectors (DREP-E2A) encoding the same Ag?

The Ag genes examined in the project are two model Ags: enhanced green fluorescent protein (EGFP) and recombinant luciferase (tLuc) carrying an N-terminal peptide sequence known to be displayed on MHC molecules.

Figure 2. SFV vectors used in the project. The DNA-RNA layered vector DREP-E2A contains an Ag gene placed under the control of 26 S subgenomic promoter. The replication competent SFV vector TREP-E2A possesses the duplicated 26 S subgenomic promoter which drives the expression of an Ag gene. Replication-competent SFV vector TREP-C contains an Ag gene inserted in frame with the structural viral genes.
RESULTS

Construction of SFV vectors with inserted Ag genes

The TREP-C-EGFP plasmid was digested with XmaI and SpeI to remove the EGFP gene. Then the tLuc gene, which was cut out from the DREP-Shuttle-tLuc vector with AgeI and AvrII, was ligated into the XmaI-SpeI site of the TREP-C vector thereby creating the TREP-C-tLuc vector.

To create DREP-E2A-EGFP, the TREP-E2A-EGFP vector was digested with XmaI and SpeI and a ~700 bp fragment representing the EGFP gene was purified. DREP-E2A-OVA was digested by XmaI and SpeI, thereby removing the OVA gene, and the EGFP gene was inserted in its place.

The sequence of all constructs was confirmed by sequencing.

The other plasmids used in this project were constructed by others.

Stability studies

The ability of TREP vectors to produce infectious viral particles was evaluated in BHK-21 cells. For this purpose, a BHK-21 cell-monolayer was transfected with TREP-E2A and TREP-C vectors carrying the EGFP or tLuc genes. The supernatant containing virus was harvested 18 hours post-transfection and used for the plaque assay. The results obtained from the plaque assay indicated that TREP constructs produce infectious viral particles as plaques were formed in the cell cultures (Figure 3). However, a difference in viral titer as well as plaque-phenotype was revealed for the different TREP vectors. The TREP-C-Ag vectors were produced at lower titers and produced 4 times fewer plaques than the TREP-E2A vectors, independent of the inserted Ag gene. Moreover, TREP-C-Ag formed smaller plaques than TREP-E2A-Ag. This implies that TREP-C-Ag produces infectious particles at a slower rate compared to TREP-E2A-Ag. A difference in titer between the same variants of TREP vectors carrying different Ag genes was also observed. TREP vectors encoding tLuc formed 1.5 times fewer plaques compared with TREP vectors encoding EGFP.
To evaluate the stability of the Ag genes in TREP-E2A and TREP-C vectors, TREP-E2A-EGFP, TREP-C-EGFP, TREP-E2A-tLuc and TREP-C-tLuc viruses were passaged five times at a multiplicity of infection (MOI) of 0.1 in BHK-21 cells. In all cases the cells were harvested 18 h post-infection with the virus. Then the expression of the Ag gene and the viral replicase was evaluated in the cells infected with the TREP viruses from each passage (P) by flow cytometry analysis. For this purpose, TREP-E2A-EGFP and TREP-C-EGFP infected cells were stained for the viral replicase while TREP-E2A-tLuc and TREP-C-tLuc infected cells were stained for tLuc and the viral replicase. The results obtained from the flow cytometry analysis revealed that TREP-E2A constructs possess a lower genetic stability than TREP-C vectors (Figure 4). In the case of TREP-E2A-EGFP, only about half of the cells were EGFP and viral replicase positive even when infected with the virus from P1. The percentage of double-positive cells dropped to negligible levels in cells infected with the virus from P3. A similar pattern was observed in BHK-21 cells infected with TREP-E2A-tLuc virus. The expression of EGFP was also lost in cells infected with the virus from P3 though there were more double-positive cells after infection with the virus collected from P1 and P2. EGFP expression in the cells infected with TREP-C-EGFP virus from different P was relatively more stable. Several percent of the cells infected with the virus from P3 were still expressing EGFP. TREP-C-tLuc appeared to be the most stable TREP construct. 67.5% of BHK-21 cells infected with the TREP-C-tLuc virus from P3 were still EGFP positive. However, the TREP-C-tLuc virus from P4 induced tLuc expression only in ~9% of infected cells.
Figure 4. Percentage of Ag expressing BHK-21 cells infected with TREP-E2A and TREP-C viruses from different Ps as studied by flow cytometry. The cells infected at a MOI of 0.1 with TREP virus from a certain P were incubated at 37°C for 18 h and then harvested. The cells infected with the TREP-E2A-tLuc and TREP-C-tLuc viruses were stained for viral replicase and tLuc with specific fluorescent-labelled antibodies. The cells infected with TREP-E2A-EGFP and TREP-C-EGFP viruses were stained only for the viral replicase.

The plaque assays were performed for the viruses collected from each P in order to determine the titers before transferring 0.1 MOI to fresh BHK-21 cells (Table 1). The titers of the viruses from each P and the phenotypes of the plaques also revealed some interesting observations. The titers of TREP-E2A viruses reached ~1×10^10 pfu/ml after P4. It is known that titers of ~1×10^10 pfu/ml are also obtained for wild type SFV virus when it is propagated in BHK-21 cells. The titers of TREP-C viruses also approached ~1×10^10 pfu/ml but remained a little bit below this value. Interestingly, the expression of the Ag gene in cells infected with the viruses of this P was already lost (except for cells infected with TREP-C-tLuc). However, when the titers of the different TREP viruses after the two first Ps were analysed and compared with the results obtained by flow cytometry analysis, a clear tendency can be seen: the earlier the virus reached the titer characteristic for the wild type virus, the earlier it lost the ability to induce the expression of the Ag gene in the infected cells.

The phenotype of the plaques formed by the viruses from different Ps also changed. An increase in the plaque size was observed for all TREP viruses; however, TREP-C viruses still produced smaller plaques than TREP-E2A viruses.
Table 1. The titer of the TREP virus stocks collected after different number of Ps.

<table>
<thead>
<tr>
<th>P</th>
<th>TREP-E2A-EGFP</th>
<th>TREP-C-EGFP</th>
<th>TREP-E2A-tLuc</th>
<th>TREP-C-tLuc</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.7×10^9</td>
<td>3.8×10^7</td>
<td>1.9×10^9</td>
<td>3.2×10^7</td>
</tr>
<tr>
<td>2</td>
<td>4.6×10^9</td>
<td>3.0×10^8</td>
<td>3.0×10^9</td>
<td>3.6×10^7</td>
</tr>
<tr>
<td>3</td>
<td>4.2×10^9</td>
<td>5.0×10^9</td>
<td>8.0×10^9</td>
<td>8.0×10^8</td>
</tr>
<tr>
<td>4</td>
<td>1.4×10^10</td>
<td>8.4×10^9</td>
<td>1.0×10^10</td>
<td>6.0×10^9</td>
</tr>
<tr>
<td>5</td>
<td>8.0×10^9</td>
<td>6.2×10^9</td>
<td>1.6×10^10</td>
<td>5.2×10^9</td>
</tr>
</tbody>
</table>

The stability study of the Ag gene was also evaluated when TREP-E2A-EGFP and TREP-C-EGFP viruses were passaged seven times at a MOI of ~10. It turned out that the EGFP gene was sustained better under such virus propagation. Approximately 10% of the BHK-21 cells infected with the TREP-C-EGFP virus of P6 were expressing EGFP. However, the tendency that TREP-C-EGFP is genetically more stable than TREP-E2A-EGFP remained. As Figure 5 indicates, the TREP-E2A-EGFP virus of the P3 was able to induce EGFP expression only in the several percent of the infected cells.

![Stability study of TREP-E2A-EGFP and TREP-C-EGFP at a high MOI](image)

**Figure 5.** Percentage of EGFP expressing BHK-21 cells infected at a high MOI with TREP-E2A and TREP-C viruses from different Ps as studied by flow cytometry. The cells infected at a MOI of ~10 with TREP-E2A-EGFP and TREP-C-EGFP viruses from a certain P were incubated at 37°C for 18 h and then harvested. Prior to acquisitions by flow cytometry, the collected cells were stained for the viral replicase. P0 represents the transfection with the TREP vector-DNA.

**Analysis of mutations leading to loss of Ag expression**

In order to investigate what mutations lead to loss of Ag expression, viral RNA was isolated from produced TREP particles and a cDNA spanning the the Ag gene and flanking nucleotides was synthesized and cloned into a special vector for cloning of blunt-end PCR
products. Colonies were picked out for sequencing of the cloned cDNA. Viral particles from Ps still partly expressing the Ag were chosen for analysis. In the case of TREP-E2A-EGFP and TREP-E2A-tLuc virus, the P2 viruses were sequenced while viruses from P3 and P4 were chosen as the most suitable ones for TREP-C-EGFP and TREP-C-tLuc, respectively. Results obtained from the sequencing revealed that deletions of the entire Ag gene sequence or at least part of it occurred in the TREP genome (Figure 6).

![Diagram of TREP genomes](image)

**Figure 6.** A schematic representation of mutations that caused the loss of Ag expression from TREP-E2A and TREP-C virus genomes.

All six clones containing cDNA from the TREP-C-EGFP virus population showed that 1/3 of the EGFP gene beginning with the start codon is retained while the rest is deleted. Interestingly, the sequence of the E2A element, which is normally fused to the C-terminus of EGFP, was still present in the mutated genome. In contrast, no Ag sequence was detected in any of the seven sequenced clones of TREP-C-tLuc. The tLuc gene was completely deleted and the wild type virus genomic sequence restored. In the case of TREP-E2A-tLuc (only one clone sequenced), the tLuc sequence was also completely deleted though the first seven amino acids of the E2A element as well as the SpeI cloning site were sustained. Both the native and the artificially inserted 26S subgenomic promoters were present in the genome of the TREP-E2A-tLuc mutant. Several variants of mutated genomic RNA existed for the TREP-E2A-EGFP virus. The sequencing pattern obtained from one clone revealed a reversion to wild type virus. Three other sequences showed that the last amino acids of Nsp4 were deleted in addition to the first 37 amino acids of C. However, the number of removed amino acids in the C-terminus of Nsp4 was different in all three clones (50, 62 or 73). Deletions of the last 62 and 73 amino acids resulted in the fusion of Nsp4 and C. In contrast, the C sequence was out of frame when the deletion of the last 50 amino acids of Nsp4 occurred (Table 2).
Table 2. The mutations that caused the loss of Ag expression from the TREP virus genome. The sequences of the corresponding genes are represented by colours: light green-EGFP, brown-2A, blue-C and violet-Nsp4. Stop codons are marked in red, start codons are marked in yellow and the subgenomic 26 S promoters are marked in grey. A star (*) shows where a fusion site between two sequences occurred. A minus sign (–) means that the sequence is continued.

<table>
<thead>
<tr>
<th>Clone</th>
<th>Description of mutation</th>
<th>Nucleotide sequence detected in the site of mutation</th>
<th>Amino acid sequence detected in the site of mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>TREP-C-EGFP 1-8</td>
<td>The last 2/3 of the EGFP gene is deleted. The 2A peptide is still sustained.</td>
<td>-C.TAC.CCC.GAC.CAC.AT*G.ACT.AGT.AAT.TTT.GA-</td>
<td>-RYPDH<em>M</em>TSNFDDLK-</td>
</tr>
<tr>
<td>TREP-C-tLuc 1-7</td>
<td>Reversion back to wild type sequence.</td>
<td>-GTG.CGT TAATACACAGAAATTC-TGATTATAGCGC ACTATTATAGC-ACC ATG AAT TAA TTG AGA-</td>
<td>-VIHLYGPRPLVR + MNYIPTQTFYGR-</td>
</tr>
<tr>
<td>TREP-E2A-EGFP 1</td>
<td>Last 62 amino acids from Nsp4 and first 37 amino acids from C are deleted.</td>
<td>-CGA.GCA.CTG.AGT.GAC*CAG.GCC.CAG.CAG.ATG-</td>
<td>-EDRRRALSD*QAQQMQQL-</td>
</tr>
<tr>
<td>TREP-E2A-EGFP 2</td>
<td>Last 73 amino acids from Nsp4 and first 37 amino acids from C are deleted.</td>
<td>-A.ACA.GCT.GAA.GAC.AA*C.CAG.GCC.CAG.CAG.ATGCGA-</td>
<td>-KLGKPLTAED<em>N</em>QAQQMQQL-</td>
</tr>
<tr>
<td>TREP-E2A-EGFP 3</td>
<td>Last 50 amino acids from Nsp4 and first 37 amino acids from C are deleted. The C sequence is out of frame.</td>
<td>-TC.CGG.ACA.GGC.TTG.GGG.GC*AG.GCC.CAG.CAG.ATG.CAGCA-</td>
<td>-FRTRGLGA*GPADAA-</td>
</tr>
<tr>
<td>TREP-E2A-EGFP 4</td>
<td>Reversion back to wild type sequence.</td>
<td>-GTG.CGT TAATACACAGAAATTC-TGATTATAGCGC ACTATTATAGC-ACC ATG AAT TAA TTG AGA-</td>
<td>-VIHLYGPRPLVR + MNYIPTQTFYGR-</td>
</tr>
<tr>
<td>TREP-E2A-tLuc 1</td>
<td>tLuc gene deleted after the initial 7 amino acids in the E all the way to the last nucleotide of the stop-codon (SpeI site intact). A new peptide consisting of 22 amino acids is formed. Both subgenomic are 26S promoters retained.</td>
<td>-CTTACAGACTATATTATAGCGC-ACC ATG AAT TAC ATC CTC ACC CGG CAG CAA *AA CTA GT-ATT GAG AGG ACC TGT TAT ATC A.CCT CCA TATAT AGCTTGGTCGTTAATACA CAGAGATT CTGATTATAGCGC ACTATTATAGC ACC ATG AATTACA-</td>
<td>-VIHLYGPRPLVR + MNYIPTQ<em>K</em>LVIERT-CYPLRRS+</td>
</tr>
</tbody>
</table>

Ag expression kinetics in vitro

Ag expression kinetics was measured in BHK-21 cells infected with TREP-E2A-EGFP, TREP-C-EGFP, TREP-E2A-tLuc or TREP-C-tLuc virus. TREP virus stocks collected after transfection with the TREP vector DNA were used to infect the cells at a MOI of 0.1. Subsequently, the cells were harvested at different time points (4, 8, 12, 16, 24 and 32 h) post-infection. The cells were stained for replicase and Ag and analysed using flow cytometry in order to check how rapidly the expression of Ag is induced and develops in the early stages of infection with different TREP virus variants. A big difference of EGFP expression kinetics after infection of the cells with TREP-C-EGFP or TREP-E2A-EGFP virus was not observed, though TREP-C-EGFP virus infected cells seemed to express EGFP slightly more rapidly as
can be judged from the higher level of EGFP expression in the harvested cell culture (Figure 7 (A)). A similar pattern was observed in the case of tLuc expression (Figure 7 (B)). It appears that there were more tLuc expressing cells in the cell culture infected with TREP-C-tLuc virus than in the case of infection with TREP-E2A-tLuc virus. Surprisingly, the level of tLuc expression was much lower than that of EGFP. Less than 20% of the cells expressed tLuc 24 h post-infection with TREP-E2A-tLuc or TREP-C-tLuc virus. In contrast, EGFP expression was detectable in more than half of the cells 24 h post-infection with TREP-E2A-EGFP or TREP-C-EGFP virus.

Figure 7. Ag expression kinetics of different TREP vectors as analyzed by flow cytometry. BHK-21 cells were infected at a MOI of 0.1 with the stock of TREP-E2A-EGFP, TREP-C-EGFP, TREP-E2A-tLuc or TREP-C-tLuc virus collected 24 h post-infection. The infected cells were harvested 4, 8, 12, 16, 24 and 32 h post-infection. The cells infected with TREP-E2A-tLuc and TREP-C-tLuc viruses were stained for the viral replicase and tLuc with specific fluorescent-labelled antibodies (B). The cells infected with TREP-E2A-EGFP and TREP-C-EGFP viruses were stained only for the viral replicase. Only the cells infected with the virus, i.e. positive for the viral replicase, were included in the analyses. Due to technical reasons, data for tLuc expression 8 h and 32 h post-infection with TREP-C-tLuc virus is missing.
Comparisons of viral titers obtained using qRT-PCR and plaque assay

Since it takes a long time to get the viral titers using the plaque assay, a qRT-PCR was set up to establish an alternative method to calculate the titer of the virus. One-step fast mode protocol for 10µl-reaction was established that allows detecting the titer of the virus in 40 min (excluding the time for the viral RNA purification). RNA purified from wild type virus was used for the standard curve which also allowed correlation of qRT-PCR data with viral titer determined by plaque assay.

The plaque assay is based on infectious viral particles, while the qRT-PCR quantifies RNA copies, not necessarily infectious particles. To eliminate the problem of detecting free viral RNA in the supernatant collected for qRT-PCR, an additional step was introduced. The supernatant was first filtered to get rid of any possible remnants of cells. Then it was treated with a mixture of RNase A and RNase T1 for 30 min. The mixture of Rnases was chosen to ensure complete digestion of viral RNA. RNaseA is known to degrade RNA at C and U residues while RNase T1 hydrolyzes RNA at G residues. As the RNA packed in viral particles is protected, it should remain intact after RNase treatment. To avoid interference of RNases with the subsequent viral RNA purification process, the RNases were degraded by a 30 min incubation with proteinase K. These modifications of the primary protocol revealed that the titer of RNase treated TREP virus detected by qRT-PCR is 1.5-fold lower compared to untreated TREP virus. This indicates that free viral RNA is really present in the supernatant collected from the virus infected cells. However, a direct correlation between TREP virus titer detectable by the plaque assay and the one detectable by qRT-PCR was not achieved. Moreover, it was observed that the ratio of the titer estimated by qRT-PCR to the one estimated by plaque assay is different for TREP-E2A and TREP-C viruses. In the case of TREP-C virus, the titer detectable by qRT-PCR was overestimated more than the titer of TREP-E2A virus (Table 3).

Table 3. Comparison of the titers detected by qRT-PCR versus the titers detected by the plaque assay. The titers for some different batches of the TREP virus were detected by qRT-PCR and the plaque assay. The most representative results obtained after the optimization of RNA qRT-PCR protocol are shown.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Titer detected by the plaque assay</th>
<th>Titer detected by qRT-PCR</th>
<th>Ratio of the titers (qRT-PCR/Plaque assay)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TREP-E2A-EGFP</td>
<td>9.80E+08</td>
<td>5.61E+09</td>
<td>5.72</td>
</tr>
<tr>
<td></td>
<td>1.80E+09</td>
<td>7.94E+09</td>
<td>4.41</td>
</tr>
<tr>
<td>TREP-C-EGFP</td>
<td>8.80E+05</td>
<td>2.19E+07</td>
<td>24.9</td>
</tr>
<tr>
<td></td>
<td>7.00E+05</td>
<td>1.28E+07</td>
<td>18.3</td>
</tr>
</tbody>
</table>

Immunization studies

Determination of the cellular immune response kinetics after immunization of mice with TREP vectors

All animal experiments have been performed using Balb/c mice. Therefore, we have chosen peptides that have been reported to be H2-K$^d$ restricted immunodominant cytotoxic T cell epitopes (Gambotto 2000 and Takahashi 1988 PNAS). The concentrations of the peptides HYLSTQSAL and RGPGRAFVTI for the anti-EGFP and anti-tLuc response, respectively,
were first tried out (data not shown). For both peptides, a final concentration of 2 µg/ml was selected for all further experiments.

To investigate when the cellular immune response peaks after immunizations with TREP-E2A-EGFP and TREP-C-EGFP, Balb/c mice were immunized by i.d. injection of 20 µg of the corresponding DNA followed by electroporation. Spleens were taken out on days 8-14 after immunization, and IFN-γ ELISPOT was performed with the splenocytes in order to evaluate the cellular immune response towards the Ag. The anti-EGFP cellular immune responses were already detectable 8 days after immunizations, and reached a plateau at day 11-14 for TREP-C-EGFP, and day 12 or 13 for TREP-E2A-EGFP (Figure 8).

![Figure 8](image_url)

**Figure 8.** The kinetics of the cellular immune response after immunization of Balb/c mice with TREP-C-EGFP and TREP-E2A-EGFP vectors. CD8+ T cell responses were assessed by IFN-γ ELISPOT. The results are pooled from two independent experiments. The horizontal line represents the median.

In order to find out which TREP variant elicits more efficient cellular immune response to EGFP, comparisons of the IFN-γ responses induced by TREP-E2A-EGFP and TREP-C-EGFP immunized at the same day were also performed (Figure 9).
Figure 9. Comparisons of the cellular immune responses against EGFP at different days post-immunization with TREP-E2A-EGFP and TREP-C-EGFP. CD8+ T cell responses were assessed by IFN-γ ELISPOT. The results for day 10, 11 and 12 are pooled from two independent experiments. The horizontal lines represent the medians for each group.
**Cellular immune responses after delivering different doses of TREP-E2A-EGFP**

To compare the cellular immune response upon immunization with various doses of TREP vector-DNA, female Balb/c mice were injected i.d. with 0.2, 1, 3, 8, 20 and 36 µg of TREP-E2A-EGFP and electroporated. All mice responded even to immunization with the lowest dose, i.e. 0.2 µg of DNA (Figure 10). The cellular immune response was elicited to a similar level when mice were immunized with 3, 8, 20 and 36 µg of TREP vector-DNA.

![Figure 10](image-url)

**Figure 10.** The cellular immune response of Balb/c mice immunized with different doses of TREP-E2A-EGFP. CD8⁺ T cell responses were assessed by IFN-γ ELISPOT performed with lymphocytes isolated from the spleens of mice on day 14 post-immunization. The horizontal lines represent the medians for each group.

**Kinetics of tLuc expression and cellular immune responses in mice immunized with SFV vectors containing tLuc gene**

As luciferase is a bioluminescent enzyme it can be employed as a marker protein *in vivo*. To find out tLuc expression kinetics from TREP-E2A, TREP-C and DREP-E2A vectors *in vivo*, the mice immunized with 20 µg of corresponding DNA and tLuc expression in the animals was followed for 9 days. It was figured out that tLuc expression from DREP-E2A vector is the strongest ~48 h post-immunization (Figure 11 (A)). tLuc expression from TREP vectors peaked a little bit later and the highest level of tLuc was detected ~72-96 h post-immunization (Figure 11 (B)). In all vaccinated mice tLuc activity was observed only around injection sites (Figure 12). Longer exposure times were usually required to detect the tLuc signal in mice vaccinated with TREP vectors. Interestingly, the expression of tLuc from DREP-E2A vector was significantly more efficient as can be indicated by the estimated tLuc activity in the immunized mice. In addition, it appeared that tLuc activity depends on the time when the measurement is done after injection of its substrate, i.e. luciferin. The highest tLuc activity was detected when the measurement was performed ~19 min post-injection of luciferin and dropped relatively quickly afterwards. This fact most likely explains a drop in tLuc activity observed 48 h post-immunization with TREP vectors (Figure 11 (B)), since the measurement at this time-point was performed a little bit later after the luciferin injection.
Figure 11. tLuc expression kinetics. The mice were immunized with 20 µg DREP-E2A-tLuc (A) or 20 µg TREP-E2A-tLuc and TREP-C-tLuc (B). The determination of tLuc activity was performed under a cooled CCD camera (IVIS 100) at different time points post-immunization. tLuc activity was measured as photon emission/s in the regions of interest (ROI). The mice immunized with DREP-E2A-tLuc were exposed for 5 s, while the mice immunized with TREP-E2A-tLuc either TREP-C-tLuc were exposed for 20 s. A drop in tLuc activity 48 h post-immunization with TREP vectors is most likely the outcome of doing the measurements at different time-points post-luciferin injection. The different colours including the numbers in the legend represent individual mice.
Figure 12. Biodistribution of tLuc activity in mice inoculated with DREP-E2A-tLuc (A), TREP-E2A-tLuc (B) and TREP-C-tLuc (C). All mice were exposed for 20 s under a cooled CCD camera (IVIS 100) 72 h post-immunization with the corresponding DNA. tLuc activity measured as photon emission/s in the regions of interest (ROI) is also shown in the pictures.

The mice that were used in the tLuc expression kinetics were also analysed for the cellular immune responses. tLuc specific IFN-γ ELISPOT revealed that significantly stronger CD8⁺ T cell responses are induced in the mice vaccinated with DREP-E2A-tLuc in comparison with the animals immunized with TREP-E2A-tLuc or TREP-C-tLuc (Figure 12). No clear difference was observed in the immune responses of mice immunized with TREP-E2A-tLuc or TREP-C-tLuc.

To determine if higher tLuc expression in a mouse also results in a more efficient cellular immune response against tLuc, the data obtained for TREP immunizations were correlated against the IFN-γ ELISPOT results. There was a good correlation (Spearman r = 0.9856) of the cellular immune response and tLuc expression level in the animals (Figure 14).
Figure 13. The cellular immune response in mice immunized with SFV vectors containing the tLuc gene. The cellular immune response was measured by tLuc specific IFN-γ ELISPOT performed with the lymphocytes isolated from the spleens of mice on day 15 post-immunization. The horizontal lines represent the medians for each group.

Figure 14. The correlation of tLuc activity and T cell response in mice immunized with TREP vectors carrying the tLuc gene. Balb/c mice were vaccinated with 20 µg of TREP-E2A-tLuc or TREP-C-tLuc DNA. The graph represents Spearman correlations of tLuc activity, measured 72 h post-injection (20 s exposure time), and the IFN-γ response of the lymphocytes isolated from the spleens of mice on day 15 after administration of DNA. tLuc activity was measured in vivo as photon emission/s in the regions of interest (ROI) with a cooled CCD camera (IVIS 100) and analyzed using Living Image 2.50.1 software.
Humoral responses in mice after immunization with TREP-C-EGFP, TREP-E2A-EGFP and DREP-E2A-EGFP vectors

In the second part of the *in vivo* experiments, the ability of the TREP-E2A-EGFP and TREP-C-EGFP vectors to induce humoral immune responses to EGFP was evaluated. To compare if the replication-competent SFV vectors are able to elicit more efficient humoral responses than layered DNA-RNA vectors, the DREP-E2A-EGFP vector was also included in the study. With this aim, female Balb/c mice were immunized intradermally and then electroporated with 20 µg of the corresponding plasmid DNA. All mice received homologous booster immunizations three weeks after priming.

The serum samples were obtained from all mice two weeks after the initial immunization as well as two weeks after the booster immunization and analysed for the presence of anti-EGFP IgGs (Figure 15). A high level of EGFP-specific IgGs was induced in mice already after the first inoculation with DREP-E2A-EGFP. However, the mice immunized with TREP-E2A-EGFP or TREP-C-EGFP displayed only weak anti-EGFP antibody response after priming which was significantly lower in comparison with DREP-E2A-EGFP (P<0.05 and P<0.001, respectively). The booster immunizations resulted in increased EGFP-specific antibody titers in all three groups. Nevertheless, the level of Ag-specific antibody titers still remained significantly higher in the animals immunized with DREP-E2A-EGFP (P<0.05). Interestingly, the level of the anti-EGFP IgGs rose substantially in the animals after the second homologous immunization with TREP-E2A-EGFP or TREP-C-EGFP (P=0.0391, dilution 1/800). In addition, a homologous booster immunization with DREP-E2A-EGFP resulted in an augmentation of the EGFP-specific antibody titers (P=0.0078, dilution 1/800). The obtained results indicate that DREP-E2A-EGFP is apparently better at inducing anti-EGFP antibody response in comparison with TREP-E2A-EGFP and TREP-C-EGFP (Figure 16). No significant difference in the magnitude of the total IgG responses was determined in mice immunized with TREP-E2A-EGFP or TREP-C-EGFP neither post-prime nor post-boost.
Figure 15. The humoral response in mice immunized with DREP-E2A-EGFP (A), TREP-E2A-EGFP (B) either TREP-C-EGFP (C). Balb/c mice were primed with 20 µg of the corresponding DNA. All animals got a homologous booster immunization with the same dose of DNA three weeks later. The titers of the EGFP-specific IgGs were measured in the serum samples collected two weeks post-prime and two weeks post-boost.
Figure 16. The comparison of total IgG response in mice immunized with DREP-E2A-EGFP, TREP-E2A-EGFP either TREP-C-EGFP. Balb/c mice were primed with 20 µg of the corresponding DNA. All animals got a homologous booster immunization with the same dose of DNA three weeks after priming. The titers of the EGFP-specific IgGs were measured in the serum samples collected two weeks post-prime (A) and two weeks post-boost (B). The OD values of a 1:800 dilution of the serum samples are displayed.
DISCUSSION

The replication-competent SFV vectors can potentially be used for therapeutic applications, particularly vaccination. The ability of these vectors to produce infectious viral particles carrying the Ag of interest may result in several rounds of infection of the cells *in vivo* and longer persistence of the Ag in an organism. It is known that the duration of Ag exposure to the immune system is an important factor influencing the host’s immune response to the Ag. Therefore, vaccines incorporating replicating SFV vectors carrying an Ag gene from a pathogen may elicit protective immunity.

The most important requirement for such kind of vectors is their stability, i.e. the vectors have to maintain the Ag gene when undergoing replication cycles. However, it has been demonstrated that replicating alphavirus-based vectors including SFV vectors are prone to loose the Ag gene, resulting in the formation of a wild-type viral genome (Caley *et al.*, 1999; Pugachev *et al.*, 1995; Rausalu *et al.*, 2009; Thomas *et al.*, 2003). Consequently, the genetic instability of the replication-competent vectors may impair not only their efficiency as a vaccine platform but also lead to the reversion to potentially harmful viruses. Therefore, the construction of the genetically stable replicating SFV vectors is the first and most crucial step in order to use them for *in vivo* applications.

A common way to design the replication-competent alphaviruses is to insert an Ag gene under a second subgenomic promoter. TREP-E2A-EGFP and TREP-E2A-tLuc, which were used in this project, represent such kind of vectors. Previously, it has been shown that an Ag gene placed under an additional subgenomic promoter is easily deleted from the replicating vector (Caley *et al.*, 1999; Pugachev *et al.*, 1995; Pugachev *et al.*, 2000; Thomas *et al.*, 2003). It has been supposed that it most likely happens due to homologous recombination of the duplicated subgenomic promoter (Pugachev *et al.*, 2000; Rausalu *et al.*, 2009). Some other strategies have therefore been employed to engineer genetically stable replication-proficient vectors. One of them is to insert the transgene under a picornavirus internal ribosome entry site element (IRES) (Pugachev *et al.*, 2000). Such kind of vectors increase the stability of replication-competent vectors based on rubella virus which also belongs to the *Togaviridae* family. However, a recent study revealed that IRES elements do not function in the case of SFV replicating viruses (Rausalu *et al.*, 2009). Alternatively, an Ag gene can be cloned in frame with the structural viral genes. One strategy is to place the Ag-2A gene right after the C gene, as translated C will release itself from the Ag-2A via autoproteolytic activity and the 2A will not be bound to the rest of the viral proteins because of its translational skip-properties. Moreover, such design of the vector results in a better Ag stability in the vector during multiple replication cycles (Thomas *et al.*, 2003). The TREP-C-EGFP and TREP-C-tLuc vectors, which have the above described structure, were constructed as an alternative to TREP-E2A vectors with the aim to get a genetically more stable replication-competent SFV vector for vaccination studies.

In this project, the genetic stability of two different replication-competent SFV vectors, TREP-C and TREP-E2A, containing the genes for the model Ags EGFP or tLuc was compared. TREP-E2A and TREP-C viruses were propagated in BHK-21 cells for five Ps by infecting the fresh cells with the virus from a previous P at a MOI of 0.1. The flow cytometry analysis performed on the cells infected with the recombinant virus of different Ps demonstrated that all TREPs gradually lost their ability to induce the expression of the Ag gene in the infected cells (Figure 4). The TREP-E2A-EGFP vector appears to display the poorest genetic stability. Less than half of the cells infected with the TREP-E2A-EGFP virus
from P1 were positive for EGFP. This means that the viral stock of P1 already contained TREP-E2A-EGFP mutants that did not express EGFP. These non-expressing EGFP mutants rapidly accumulated in further Ps and overtook non-mutated TREP-E2A-EGFP already in P3. A similar pattern was observed for the TREP-E2A-tLuc vector. The instability of the TREP-E2A vectors is not surprising considering the structural properties of the vectors. These vectors contain two subgenomic 26S promoters that produce two separate RNA units. As mentioned above, one hypothesis is that the subgenomic 26S promoter sequences are prone to homologous recombination at the level of positive or negative sense RNA of TREP-E2A vector. The recombination may lead to deletion of the Ag gene, which results in formation of the genome of the virus used for construction of the vector. TREP-E2A virus that has lost the Ag gene has several properties that allow it to overrun the viruses still containing the Ag gene. First, deletion of the Ag gene means that the genome becomes shorter and can be replicated faster. Second, a shorter genome can more easily be packaged into viral particles. In addition, the absence of the second subgenomic promoter facilitates more efficient synthesis of the structural viral genes as all replicase complexes bind and initiate transcription from only one subgenomic promoter. All these reasons lead to higher titers of the virus with the deleted Ag gene from its genome. Therefore, instead of the virus still carrying the Ag gene, the mutated virus starts to dominate in the population after several replication cycles. However, the expression of Ag from TREP-E2A vectors can be lost due to a different scenario. It is known that the viral replicase do not have proof-reading ability, and therefore the transcripts synthesized by these polymerases usually contain various mutations. In the case of a wild type virus, the mutations do not negatively influence virus population a lot. Though some mutations impair the formation of functional virus, there are a significant number of viable fully replication competent viral particles in the population. Unfortunately, the absence of proof-reading ability has a highly negative impact on a proper functioning of the replication-competent vectors. A single point mutation in the foreign gene can result in an inactive product. Therefore, the loss of Ag expression from TREP-E2A vectors might be a consequence of the mutations within the sequence of the Ag gene. However, such mutants are less likely to start to dominate compared to mutants with deleted Ag genes. For example, point-mutations in the Ag gene do not change the size of the genome and probably do not influence the rate of replication. However, mutations can also arise in the 26S subgenomic promoter, which is controlling the synthesis of the Ag RNA. If the mutations in the sequence of the 26S subgenomic promoter impair the replicase binding, more replicase will be directed to the native 26S subgenomic promoter. This may lead to an increased production of the viral particles and, subsequently, enrichment of a viral population with the mutant virus. All these considerations suggest that a higher rate of replication gives an advantage of the mutant virus against the one expressing the Ag. This hypothesis correlates with the results obtained from the plaque assay for TREP virus stocks collected 18 h post-infection. The titers of all investigated TREP viruses increase after each P, and TREP-E2A virus even reaches a titer characteristic for wild type SFV. This implies that fast-growing mutants start to dominate after several Ps of the virus. The flow cytometry analysis shows that the majority of the cells do not express Ag when infected with the TREP virus from the P in which the virus was able to grow to a titer similar to wild type SFV. Moreover, the size of the plaques that are produced by the TREP-E2A virus after three or more Ps is increased in comparison with the size of the plaques formed by the primary stock of TREP-E2A virus. This phenotypic change in the size of plaques also indicates that the virus is able to replicate at a higher rate and most likely has eliminated the Ag gene from its genome.

In this study, the TREP viruses were propagated at a low MOI for five Ps in order to create selective pressure for the virus population. In this case, an individual cell is commonly
infected only by one viral particle. Moreover, budding virus can infect other, yet uninfected, cells in the culture. So, the spreading of the virus is facilitated when it is propagated at a low MOI that creates suitable conditions for the most efficient viral variants to grow. If the cells are infected at a high MOI, several viruses can enter the same cells. As the primary stock of the virus contains mainly Ag expressing virus, it is the Ag carrying virus that primarily infects the cells. The relatively low number of mutant viruses usually infects the cells that are already under the control of original, i.e. Ag expressing virus. In these conditions, the ability to replicate and spread fast becomes of less importance. If the replication-competent vector is propagated at a high MOI, an Ag gene is more likely to be sustained over time. Such behavior was observed for TREP vectors as well. When TREP-E2A-EGFP and TREP-C-EGFP viruses were used to infect cells at a MOI of ~10, the genetic stability of these vectors was obviously higher (Figure 5). A small amount of cells infected with TREP-E2A-EGFP virus of P4 still expressed EGFP. Moreover, the TREP-C-EGFP virus of P6 was still capable of inducing EGFP expression in ~10% of the cells.

Concerning the structural features of TREP-E2A vectors probably causing the instability of inserted Ag gene, a new type of TREP vector, named TREP-C, has been constructed. The expectations of a higher genetic stability of this vector have been based on the following considerations. First of all, the TREP-C vector does not contain the duplicated sequences and should not be susceptible to homologous recombination. Second, the mutations introducing changes to the reading frame of the Ag gene such as nonsense or frameshift mutations would be lethal as the viral structural genes downstream the mutation site would also be translated incorrectly. Therefore, TREP-C virus that contains a functional unit of the Ag gene should dominate in a viral population. Surprisingly, TREP-C-EGFP and TREP-C-tLuc vectors were only slightly more stable in comparison with their TREP-E2A counterparts. For example, ~5% of the cells infected with TREP-C-EGFP virus of P3 expressed EGFP while the cells infected with TREP-E2A-EGFP virus of P3 did not produce significant levels of EGFP any more. The situation was a little bit better in the case of the TREP-C-tLuc vector. The expression of tLuc was almost gone in the cells infected with TREP-E2A-tLuc virus of P3. In the case of the TREP-C-tLuc vector, the expression of tLuc disappeared in the cells infected with the virus of P5. Interestingly, TREP-C viruses grew to lower titers in all Ps in comparison with TREP-E2A viruses and did not reach so high titers even when they were not capable of inducing detectable expression of Ag in the infected cells. However, the increase in titers also happened during the Ps, indicating that the population of the virus became enriched with fast-growing mutants.

It is more difficult to foresee the reasons of genetic instability for TREP-C vectors. One of the hypotheses is that the most likely event causing the loss of Ag expression from TREP-C vectors is miss-sense mutations in frame of Ag gene that renders the expressed protein nonfunctional. Also, it is possible that compensatory mutations in the Ag gene downstream the site of the miss-sense or frame-shift mutation can occur. This would restore the normal reading frame of the viral proteins, but the Ag would remain nonfunctional due to the fact that a major part of its sequence is altered. In addition, the loss of the functional Ag gene from the TREP-C vector may be a consequence of non-homologous recombination or replicase switching of the strand.

Unexpectedly, TREP vectors appeared to sustain the tLuc gene better than the EGFP gene. Taking into consideration the fact that tLuc is a bigger gene (~1650 bp) than the EGFP gene (700 bp), the reverse outcome was predicted. The titers of TREP-E2A-EGFP, TREP-E2A-tLuc, TREP-C-EGFP, and TREP-C-tLuc viruses have been determined in the course of the
project. We found that the tLuc gene containing TREP viruses reached a 1.5-fold lower titer than the EGFP gene containing TREP viruses. We attributed this difference in titers to the length of the Ag gene. EGFP is shorter and, therefore, it should replicate faster. Moreover, the tLuc carrying genomes might be more difficult to pack into TREP viral particles due to the longer RNA molecule. However, this might not be a problem if the sequence of tLuc facilitates the more compact secondary and tertiary structure of the vector RNA. The toxicity of the gene may also have an influence on the virus replication and reduce the titer. As previously mentioned, the ability to replicate fast is an especially advantageous property when virus is propagated at a low MOI. Certainly, the virus that has lost the Ag gene replicates at the highest rate, followed by the replication of the virus carrying the EGFP gene, and last the virus carrying the tLuc gene. As the virus containing the EGFP gene can replicate faster than the one containing the tLuc gene, we believed that it should compete better with the virus that deleted the Ag gene from the genome. Therefore, EGFP expression from TREP vectors should also be retained over more Ps than tLuc expression from these vectors. Moreover, the vectors carrying tLuc are more likely to accumulate mutations since the viral replicase might introduce more errors in a longer sequence. So, tLuc should more easily be inactivated due to mutations in the reading frame than EGFP. All these factors imply that the genetic stability of TREP vectors has to be higher if an Ag is not tLuc but EGFP. However, the flow cytometry analysis of the cells infected with the TREP viruses of different Ps did not support this hypothesis. There are several possible explanations of such results. EGFP is a fluorescent protein and does not require to be stained with fluorescent antibodies for flow cytometry. However, tLuc is detected in the cells after staining with specific fluorescent-labeled antibodies. In the case of EGFP, a single miss-sense mutation can lead to loss of fluorescence. Automatically, the cells producing the mutated EGFP will be regarded as if they had lost their Ag expression. In contrast, since tLuc is stained with antibodies, it can contain miss-sense or other kinds of mutations. As a result, the cells expressing the mutated variants of tLuc are regarded as Ag expressing cells. This could be one of the reasons why there are almost 13-fold more tLuc positive cells than EGFP positive ones after infection with the TREP-C virus of P3.

In addition, the difference in kinetics for tLuc and EGFP carrying vectors may also lead to the observed differences in the stability of the Ag gene. The fact that TREP virus with an inserted tLuc gene produces lower titers means that it replicates slower. Therefore, its genome is involved in less replication cycles and, consequently, accumulates fewer mutations during the period of time than viral genomes carrying the EGFP gene. The same thing applies to TREP-E2A and TREP-C vectors independent of which Ag gene they contain. As can be concluded from the titers, TREP-C vectors take part in fewer replication cycles and have fewer chances to experience events leading to the instability. This can create the illusion that TREP-C vectors are more stable than TREP-E2A vectors. Probably, the Ag expression from these vectors would be lost in the same pattern if they replicate for the same number of cycles after each P.

However, the results obtained from the Ag expression kinetics for TREP-E2A and TREP-C viruses leads to new observations indicating that the data should be interpreted in another way. In this experiment, the levels of Ag expressing cells were evaluated at different time points post-infection with TREP-E2A-EGFP, TREP-C-EGFP, TREP-E2A-tLuc, and TREP-C-tLuc virus (Figure 7). The number of Ag positive cells over a span of time represents how rapidly TREP virus containing that Ag gene induces expression of the Ag gene in the infected cells. The data acquired by flow cytometry of the harvested cells showed that infection with TREP-C viruses leads to a slightly higher rate of Ag expression in the cells. It is a quite
unexpected result considering the fact that TREP-E2A viruses grow to a higher titer than TREP-C viruses. A higher titer is believed to reflect an ability of a virus to replicate faster. Therefore, it was considered that the capacity of the TREP-E2A virus to replicate faster should lead to production of more Ag in the infected cells during a certain period of time. However, TREP-E2A virus behaves in a different way and is not so efficient in inducing expression of Ag in the infected cells as its slower growing TREP-C counterpart. This contrary might be explained in several ways. The first stage of SFV replication in the infected cells is the formation of the replicase complexes that production of the negative sense genomic RNA. A few hours later the replicase complexes bind to the 26S subgenomic promoter and transcription of the structural polyprotein genes encoded in the subgenomic RNA starts. These processes are identical for TREP vectors as well. Flow cytometry of the cells harvested 4 h post-infection demonstrates high levels of the viral replicase in the infected cells though Ag expression representing the translation of the subgenomic RNA species can barely be detected. The rate of the replicase synthesis and the amount of the replicase should not differ for the cells infected with TREP-E2A or TREP-C viruses as the cells were infected with 0.1 MOI of each virus. In addition, TREP-E2A and TREP-C vectors have almost the same size of their genome if they carry the identical Ag gene. Therefore, the differences in the availability of the replicase complexes can not be the reason for the Ag expression discrepancy between the TREP-E2A and TREP-C viruses. However, it is important to assess the structural differences of the TREP-E2A and TREP-C vectors. As noted above, TREP-E2A vectors possess two 26S subgenomic promoters while the TREP-C vector has only one. It means that the replicase complexes are distributed between two 26S subgenomic promoters on TREP-E2A virus negative sense RNA. This distribution has two consequences. First, less subgenomic RNA for translation of Ag is transcribed and, second, less subgenomic RNA encoding the structural genes is produced. Naturally, the levels of Ag as well as production of structural proteins are diminished. In the case of TREP-C vectors, the replicase complexes do not have to compete between two 26S subgenomic promoters as only one subgenomic promoter is present. Moreover, the transcription of an Ag gene as well as the viral structural genes is controlled by this 26S subgenomic RNA as an Ag gene is cloned in frame with the structural viral genes. Taking into account these facts, it is not a surprise that the kinetics of the Ag expression is greater after infection with TREP-C virus. However, the question why TREP-E2A virus grows to a higher titer though it produces less structural proteins than TREP-C virus remains unanswered. It can be of several reasons why such kind of pattern is observed. Probably, the replicase complexes have a preference of binding to the native subgenomic 26S promoter than to the one controlling the expression of the Ag gene. However, this seems not to be the case since previous research on the TREP-E2A vector showed that the replicase synthesizes more subgenomic RNA encoding the Ag gene than the RNA species encoding structural genes. On the other hand, there is no guarantee that loss of the Ag gene does not occur already after transfection with TREP-E2A vector DNA. Therefore, it is hard to say if the stocks of virus used in this experiment are really homogenous and do not contain any mutants. The presence of mutants not carrying the Ag gene in the TREP-E2A virus stock could be the reason why the cells infected with TREP-E2A but not TREP-C virus express less Ag but grow to a higher titer. However, the most likely explanation why the titers and Ag expression from TREP-E2A and TREP-C vectors do not correlate resides in the structural features of the TREP-C vector. The Ag gene is inserted between the capsid gene and the genes of spike proteins in the TREP-C vector and is translated as one large polyprotein. This polyprotein is processed post-translationally to form functional proteins. It may be that the heterologous Ag sequence that is present in this polyprotein interferes with the proper processing of the polyprotein. First, the auto-cleavage of the capsid from the rest of the polyprotein after translation could be affected. The Ag
sequence following after the capsid sequence can form a secondary and tertiary structure unfavourable for the cleavage reaction. Alternatively, the proteolytic reaction of 2A which is fused to the C-terminus of the Ag might not work efficiently enough. Consequently, the Ag would not be removed from E3. In both cases, the formation of the functional viral structural proteins would be impeded and this would inhibit the formation of the viral particles or result in the production of deficient non-infectious viral particles. Indeed, it would lead to a lower titer detectable by the plaque assay technique. The difficulties in the production of the functional structural proteins of TREP-C virus may be so significant that TREP-E2A virus forms more viral particles and reaches higher titers though it theoretically synthesizes less structural proteins.

Ag expression kinetics analysis also revealed that tLuc is produced much slower than EGFP in the cells infected with TREP containing the corresponding Ag gene. The slow tLuc expression kinetics is supposed to be the outcome of the large size of this protein. tLuc has more than twice as long sequence as EGFP. Therefore, it probably takes more time for the viral replicase to transcribe RNA encoding the tLuc gene, and it takes more time for the translational machinery to synthesize it.

The formation of a relatively high number of deficient viral particles for TREP-C virus is in agreement with the qRT-PCR data obtained. The plaque assay technique is quite tedious and it takes at least two full days work to detect the titer of the virus. qRT-PCR on the other hand takes only 40 min (excluding the time for the viral RNA purification). Therefore, qRT-PCR would be a very valuable method and could replace the commonly used plaque assay. A functional qRT-PCR was set up successfully. However, one difficulty appeared when comparing the titer of the virus calculated by qRT-PCR with the one estimated by the plaque assay. Certainly, one can not expect to get exactly the same value of the titer detected by the two different methods. Therefore, it is important to attain the ratio that allows converting the result obtained by one method to the result determined by the other technique. The titer detected by the plaque assay represents the titer of infectious virus as only the viral particles that infect the cells are able to form plaques. In contrast, the titer estimated by qRT-PCR includes not only infectious viral particles but also the deficient ones, as it is based on detection of the number of viral genomic RNA copies. Moreover, it is known that the infected cells undergo apoptosis, which means that unpacked viral RNA produced in the cytoplasm of the cell is released into the medium.

The titer obtained by qRT-PCR for TREP-C was more overestimated than the titer for TREP-E2A, when compared to the plaque assay results. It is normal that the titer quantified by qRT-PCR is slightly higher in comparison with the titer detectable by the plaque assay. First, qRT-PCR is a much more sensitive method. Next, the plaque assay includes a 1h incubation step in which the supernatant containing the virus is allowed to infect the cells. Then the supernatant is removed from the cell culture. Presumably, it still contains some infectious viral particles that did not infect any cells. However, the loss of the virus in this stage should be the same for TREP-E2A and TREP-C virus and would not explain why the TREP-C virus is detectable by qRT-PCR more efficiently than TREP-E2A. The most likely reason why the ratio of the titer estimated by qRT-PCR to the one estimated by the plaque assay is different for the TREP-E2A and TREP-C viruses resides in the quality of the viral particles formed by these two variants of TREP virus. As previously mentioned, the processing of the viral structural genes can be imperfect leading to the formation of deficient viral particles unable to infect new cells. Such viral particles would not give rise to plaques and hence not be included in the titer obtained from the plaque assay; however, they would be included in the titer as measured by
qRT-PCR. In conclusion, the formation of the deficient non-infectious viral particles for TREP-C virus is strongly supported by Ag expression kinetics analysis as well as from the qRT-PCR experiments.

To verify that the above discussed events that may lead to loss of Ag expression from TREP vectors are really happening, the sequence analysis of TREP viruses from the Ps containing mostly mutated variants was performed. The results revealed that the mutations causing the loss of Ag expression are more complex than thought before (Table 2). Though too few samples were sequenced for making statistic evaluation, some kind of tendencies clearly emerged. For example, all sequenced clones demonstrated that the TREP-C-tLuc virus mutated back to the wild type virus genome. However, TREP-C-EGFP virus mutated due to deletion of 2/3 of the EGFP gene. In the case of TREP-E2A-tLuc virus, a mutated variant which completely lost the tLuc gene together with the major part of the E2A element was detected. Several different mutants were identified for the TREP-E2A-EGFP virus. One mutant had restored the genomic sequence to the wild type virus. Other variants of mutants indicated that not only the EGFP gene was missing, but also the first 111 nucleotides of the C gene and the end-part of the Nsp4 gene. It is obvious that homologous recombination is not the main reason causing the instability of TREP-E2A vectors. Only one of the sequenced clones showed reversion of the TREP-E2A sequence to wild type genome. In other cases, the deletions of the Ag gene occurred not as the consequence of homologous recombination between the 26S subgenomic promoters. Interestingly, the fusion of truncated Nsp4 and C occurred in most of the TREP-E2A-EGFP mutants. The number of amino acids deleted in the C-terminus of Nsp4 varied for the mutants. In contrast, exactly 37 amino acids in the N-terminus of C were removed in all these mutants. It is not completely clear why the fusion of truncated Nsp4 takes place at the 38th amino acid of C. It is possible that these mutants are viable if the deletions of the ends of Nsp4 and C do not interfere with the function of these proteins. In such case, nonstructural and structural viral proteins would be synthesized as one large polyprotein. It may be that it gives some advantages to the virus and results in an increased production of replicase complexes and/or structural proteins. However, the coordination of normal protein synthesis can be too much distorted in such mutants, which means that they are not able to produce offspring. Mutants of this type can be generated because the sequences at the C-terminus of Nsp4 and the N-terminus of C are potentially more prone to recombine due to RNA structural properties. This hypothesis is supported by the fact that Nsp4-C-fusion resulted in a frameshift in the C gene in one mutant. In addition, one of the mutated variant of TREP-E2A-EGFP had the wild type virus sequence. Therefore, most likely this one starts to dominate in subsequent Ps. Moreover, another pattern of deletion of the Ag gene was observed for TREP-E2A-tLuc. This means that the recombination that removes the Ag gene from the viral genome is highly dependent on the secondary structure of RNA which may differ according to the inserted Ag gene. Unexpectedly, TREP-C virus, at least the one with the cloned tLuc gene, seems to revert to the wild type virus more easily than TREP-E2A virus. However, this is not a characteristic for TREP-C-EGFP virus, where a part of EGFP was deleted instead. Considering these mutation patterns, it is apparent that RNA homologous recombination does not occur as easily as it does in DNA. In fact, reports regarding RNA recombination are very limited. It could happen, but it is hard to say that it is actually the main cause of the loss of Ag expression. More likely the mutations in all types of TREP vectors happen due to the improper functioning of the viral replicase. It may be that the replicase skips the foreign sequences or switch the RNA strand when it encounters a sequence that is not familiar. These processes can be mainly influenced by the secondary structure of the RNA. Probably, an RNA structure containing an Ag sequence is too complex or unfavourable to be transcribed by the viral replicase. Therefore, it looses the binding affinity.
or simply can not surmount the sophisticated RNA structure formed by the Ag gene. These findings indicate that the stability issues of TREP vectors are more complex. Therefore, the strategy to increase the stability of the vector by avoiding homologous recombination might be inefficient. Though TREP-C vectors seem to be slightly more stable than TREP-E2A vectors, it can be the result of other differences in these vectors such as the rate of growth or the level and quality of the expressed proteins. The comparison of some properties of these two vectors in vitro indicates that TREP-C vectors do not have a clear superiority against TREP-E2A vectors. Probably one of the advantages of the TREP-C vectors is a higher Ag expression kinetics. However, it can be surpassed by the capacity of the TREP-E2A vectors to grow to a higher titer. Presumably the efforts to improve the stability of the TREP vectors should be directed to the improvement of the functioning of the viral replicase. Perhaps it would be possible to introduce proof-reading ability to the viral replicase without impairing other properties of this enzyme.

As the properties of the vectors can be different in vitro and in vivo, TREP-E2A and TREP-C vectors were also compared for their capacity to induce immune responses against the encoded Ag in the vaccinated mice. There was no clear difference in the cellular immune responses elicited in mice whether they got an Ag in the form of the TREP-E2A or TREP-C vector. This tendency was observed independently from the Ag (i.e. EGFP or tLuc) cloned into TREP-E2A and TREP-C vectors. Taking into account the fact that TREP-C vectors do not possess a big superiority in stability over TREP-E2A vectors, it is not a surprise that they both induce similar levels of the cellular responses against an Ag. Though TREP-C vectors may have slightly faster Ag expression kinetics not only in vitro but also in vivo, it is likely not to be significant enough to cause stronger immune responses. Moreover, it might be counterbalanced by production of more viral particles competent to infect the cells in the next round. Actually, the data indicating that type I IFN may restrain the spread and replication of TREP virus accumulates (Barry et al., 2009; Fragkoudis et al., 2007). Therefore, TREP virus spreading in the organism may be limited to only a few infection cycles. Then the stability issues become less important since the TREP virus may take part in too few replication cycles to mutate. More exactly, even if some viruses in the population loose the Ag expression, they do not dominate the population, and the Ag is successfully produced from the genomes of their non-mutated counterparts. Also, the probability that the stability of the vectors in vivo may show a different pattern can not be completely ignored.

All immunizations of mice were performed in a way of intradermal infection of the vector DNA followed by electroporation. It has been shown that this method of DNA administration is highly efficient and can increase the Ag expression as much as 1000 times (Dobano et al., 2007; Roos et al., 2008). Therefore, we wanted to investigate how low dose of the TREP vector expressing the Ag can be used for immunization still resulting in a productive immune response. In addition, this experiment could help us find the optimal dose for induction of an efficient immune response. Interestingly, a dose of only 3 µg was enough to induce a significant cellular immune response that was not increased even by delivering 36 µg of vector DNA (Figure 8). From the results it seems that a plateau in the cellular immune response is reached already after immunization with 3 µg of TREP vector-DNA. There are several speculations why the cellular immune response was not increased with increasing the dose in this experiment. It is known that the Langerhans cells existing in the skin are the cells that first react to Ag delivered by intradermal electroporation (Peachman et al., 2003). However, the number of these cells that are present in the sites of immunization is limited. Supposedly, the relatively low doses of Ag are enough to saturate these cells with Ag. It might be that the immune responses would be amplified if the DNA was delivered to more
sites of the body. Another explanation is that the Ag level can reach a threshold beyond which the immune response remains the same, since the saturation of MHC complexes on the Langerhans cells or other APCs is reached. In addition, the type I IFN response might limit the spread of the virus, and therefore a higher dose will not influence the immune response to a great extent.

TREP-E2A-EGFP and TREP-C-EGFP vectors were also investigated for their capacity to induce a serological response to EGFP (Figure 15). None of these two TREP vector variants displayed superiority over the other in generating EGFP-specific antibodies. However, DREP-E2A-EGFP immunized mice produced significantly higher titers of IgGs than the TREP variants (Figure 16). Interestingly, a homologous booster immunization yielded noticeably higher titers in mice inoculated with TREP-E2A and TREP-C vectors as well as in mice boosted with DREP-E2A-EGFP. Nevertheless, the level of anti-EGFP IgGs still remained significantly higher in the animals immunized with DREP-E2A even post-boost. Our data suggest that the humoral immune responses follow a pattern similar to the cellular immune responses. For example, DREP-E2A was also more efficient at inducing cellular immune responses in comparison with TREP-E2A or TREP-C encoding the same Ag (Figure 13). This trend is not a surprise since both arms of the adaptive immunity interact and cooperate closely by delivering stimulating signals to each other.

The underlying reason why DREP vectors exhibit better capacity to establish protective immunity than TREP vectors perhaps reside in their difference in expression levels of an Ag. As in vivo bioluminescent imaging revealed, more tLuc is produced in animals immunized with DREP-E2A-tLuc than in the ones immunized with TREP-E2A-tLuc or TREP-C-tLuc (Figure 11 and Figure 12). Regarding the fact that TREP vectors are able to produce infectious virus and spread to new cells, we expected the reverse result. It is not completely clear why in vivo expression of Ag from DREP-E2A vector is more efficient. First, the design of these vectors should be considered. DREP-E2A contains only one subgenomic 26S promoter that is devoted completely for the transcription of the Ag gene which is placed under the control of this element. This structural feature of the DREP-E2A vector allows high-level expression of the Ag. In contrast, TREP-E2A vectors have two subgenomic 26S promoters. One of them is also responsible for the production of the Ag while the other one drives the synthesis of the structural polyprotein. As a consequence, the functional replicase complexes are utilized to produce two separate transcripts resulting in a decreased number of RNA species encoding the Ag. In the case of TREP-C vectors, only one subgenomic 26S promoter is present, however, the expression of the Ag may be lower due to the longer transcription unit incorporating the Ag gene as well as the structural viral genes. Furthermore, it costs more for a cell to transcribe and translate a larger gene or several genes that also slows down the synthesis of Ag from the TREP-E2A and TREP-C vectors. The impact of these factors can be validated by conducting certain in vitro experiments. However, it is much more difficult to evaluate the factors that can influence the Ag expression from DREP and TREP vectors in vivo. It is known that the type I IFN response is induced when certain innate system components (TLR3, TLR7, TLR8 and RIG-I-like receptors) detect single-stranded RNA, unmethylated CpG DNA or double-stranded RNA which is formed during viral replication (Honda et al., 2005; Medzhitov & Janeway, 2002). The secreted IFN signals to other cells that an intruder is present in the organism and that an anti-viral state needs to be switched on. Both DREP and TREP vectors should be capable of inducing a type I IFN response. First, both of them are delivered as DNA. Moreover, both of them replicate in the cytoplasm of the cell and form double-stranded RNA. Immunization with TREP vector-DNA should result in the formation of TREP-virus which buds from the originally transfected cells to potentially infect
new cells. However, if the anti-viral state is quickly induced in the adjacent cells the spread of the virus can be suppressed. Then the expression of Ag would be limited mainly to transfected cells. TREP vectors seem to be less efficient in producing Ag compared to DREP vectors due to the additional structural features. Therefore, if TREP virus spreading is inhibited, DREP produces more Ag and subsequently induces better cellular and humoral responses. Moreover, it could be that TREP viruses trigger the production of much more type I IFN than DREP. In such case, the autocrine action of a type I IFN can become of great importance as it can switch off the replication of TREP already in the originally transfected cells. However, it could be that other innate immune system signals also play a significant role. TREP virus starts to bud from the cells relatively early (3-4 h after infection in vitro). It may be that the viral particles released to the extracellular space or the changes in the cell membrane curvature or composition due to the budding are recognized as danger signals by some unknown receptors of the innate immunity. In this way, the replication of the TREP genome could be further suppressed. In contrast, immunization with DREP vectors does not result in a budding virus and, potentially, does not trigger this danger signal. This hypothesis is supported by the fact that certain artificial adjuvants resembling the shape of viral particles (e.g. ISCOM) are particularly effective in elevating an immune response to an Ag (Morein et al., 1984; Ronnberg et al., 1995; Voeten et al., 2000). To our knowledge no specific innate immunity molecule recognizing the budding SFV virus or viral particles in the extracellular space has yet been identified. However, complement components might constitute PRRs involved in the recognition of SFV particles. For instance, mannan-binding lectin was demonstrated to bind to the virions of influenza virus and hepatitis virus (Brown, 2006; Reading et al., 1995). In addition, it was revealed that complement molecules also neutralize some viruses (Ji et al., 2005; Kase et al., 1999). However, it was also suggested that viruses of Togaviridae family can incorporate some complement inhibiting molecules that allows them to avoid an encounter with the complement components (Favoreel et al., 2003). For example, Sindbis virus gains sialic acid residues from its host cells that can help it to escape from the attack of the alternative complement pathway component C3b (Hirsch et al., 1981; 1983). Interestingly, we observed that the immune responses in mice immunized with TREP vectors usually vary markedly among animals. At first, we considered that it is a result of not equally well performed immunizations since intradermal injections should be done with precision. Surprisingly, the immune responses in mice vaccinated with DREP-E2A vectors appeared to be less scattered. It could be that the outcome of immunization with TREP vectors depends on how efficiently the anti-viral state is established in the originally transfected cell and/or adjacent cells. Moreover, it can be influenced by how successfully the virus escapes from the recognition of the innate immunity components such as complement molecules. As in a real infection, sometimes the immune system clears a pathogen quickly and efficiently but sometimes an invader overtakes its host.

In conclusion, no obvious difference was detected between TREP-E2A or TREP-C vectors with respect to the stability of the Ag gene or the induction of the Ag specific cellular or humoral responses. However, DREP-E2A vectors were able to induce significantly higher cellular and humoral responses towards the encoded Ag. Though the DREP vector was not included in the study of dose titration, an independent experiment showed that even a dose as low as 0.08 µg induced a high Ag-specific CD8⁺ T cell response. Though the obtained results indicate that the DREP-E2A vector is more efficient than TREP vectors in inducing the primary immune response against encoded Ag, it does not mean that DREP-E2A is necessarily more suitable for vaccination purposes. It might be that immunization with TREP vectors results in a better immunological memory as it mimics a real infection. Some studies claim that the immunological memory correlates with the primary immune response, i.e. the
better primary immune response, the better memory is induced. Nevertheless, new facts reveal that this is not a strict rule (Naslund et al., 2007). Therefore, the next step in this project would be to evaluate and compare the immunological memory which is formed after vaccination with TREP and DREP vectors. Also, in order to understand the impact of type I IFN on the effectiveness of TREP vectors, it would be interesting to analyze immune responses in mice deficient in the receptor for type I IFN. Furthermore, the developing of the viral replicase resistant to type I IFN might be another strategy.

The investigation of mutations that caused the loss of Ag expression from TREP-E2A and TREP-C vectors denied the hypothesis that TREP-E2A vectors are less stable due to the probability of homologous recombination. The events leading to the loss of Ag expression from TREP vectors seem to be more complicated and diverse and might depend on various factors such as the size of the inserted gene and the secondary structure of the RNA. Nevertheless, this study implies that the stability of the TREP vectors seems not to be the main reason why they are less efficient than DREP vectors. Probably, it is connected with the lower expression of the Ag and triggering of additional innate immunity signals that leads to establishment of an anti-viral state in the cells. However, the stability of these vectors should still be addressed because of biosafety reasons. Though the spread of TREP virus in an organism appears to be very limited or even do not occur at all, the probability of formation of wild type virus may hinder the approval of this vector for human vaccination. A recent study demonstrated that the double-subgenomic SFV vectors display an increased stability when the second promoter is inserted 3’ of the ORF of the structural genes (Rausalu et al., 2009). Therefore, the construction of such kind of TREP vectors for further studies to use this vector as a vaccine platform should be considered. A lot of work still needs to be done in order to develop effective and safe vaccines. It requires not only a better understanding of the mechanisms of immunity formation but also a suitable design of a vaccine. The different variants of SFV vectors remain potential candidates for future vaccines.
MATERIALS AND METHODS

Transfection

Delivery of the plasmid DNA vectors for a transient gene expression in BHK-21 cells was performed with Lipofectamine Plus Reagent (Invitrogen, Carsbad, California) or FuGENE HD Transfection Reagent (Roche Diagnostics GmbH, Mannheim, Germany), according to the instructions provided by the manufacturers. Typically, cells were 80-90 % confluent in a six-well culture dish at the time of transfection. When Lipofectamine Plus reagent was applied for transfection of the cells cultured in a 6-well plate, 1 µg plasmid DNA, 6 µl Plus Reagent and 4 µl Lipofectamine per well were used. If transfection was performed with FuGENE HD Transfection Reagent, 6 µl of the reagent combined with 2 µg plasmid DNA was added to the cells. In both protocols, Opti-MEM I Reduced Serum Medium (Invitrogen) was used for transfection-complex formation.

Transformation of DNA into competent cells of E.coli

Subcloning Efficiency™ DH5α™ Competent Cells (Invitrogen) were used for routine subcloning into plasmid vectors. The procedure was performed according to the guidelines recommended by the manufacturer.

Propagation and purification of plasmid DNA

Propagation and purification of plasmid DNA for immunization

For the immunizations of mice, the plasmids were purified with EndoFree Plasmid Mega kit (Qiagen, Hilden, Germany) following the manufacturer’s instructions. A few slight modifications of the protocol were done. The first change was to grow E.coli transformed with the TREP-C-Ag plasmid for a longer time since bacteria transformed with this construct grew very slowly. A colony of E.coli transformed with TREP-C-Ag DNA was grown in 5 ml Luria-Bertani medium (LB – 10 g/L Bactro-tryptone, 5 g/L yeast extract, 10 g/L NaCl, pH 7.5) supplemented with 50 µg/ml kanamycin for 8 hours with agitation at 37°C. Then it was transferred into 50 ml LB+50 µg/ml kanamycin and grown for an additional period of 8 h. Finally, the 50 ml culture was transferred into 500 ml LB+50 µg/ml kanamycin and grown for an additional 16-18 hours. The second change of the protocol, which applied to all plasmid purifications, was dissolving plasmid DNA in sterile phosphate-buffered saline (PBS) without Mg²⁺ or Ca²⁺ instead of endotoxin free water supplied in the kit. As stated by the manufacturer, plasmid DNA purified with this kit contains endotoxin levels <0.1EU/µg DNA.

Propagation and purification of plasmid DNA for cloning procedures

Typically, one colony of the transformed DH5α cells was inoculated into 5 ml of LB supplemented with 50 µg/ml kanamycin and grown with agitation for 16-18 h at 37°C. Plasmids were extracted with QIAprep Spin Miniprep kit (Qiagen) as described in the kit manual.
Restriction Endonuclease Digestion

0.2-3 µg of DNA was digested with 5-10 u/µg DNA of restriction endonuclease in the total volume of 10-50 µl. 10×buffer from the NEBuffer system which suited the particular restriction enzyme pair best was added to the reaction mix. All restriction enzymes and 10× buffers were purchased from New England BioLabs (Ipswich, MA). The reactions were carried out for at least 1 hour at 37 °C. The restriction enzymes were inactivated by heat treatment (at 65°C or 80°C for 20 min), if further reactions of the digested DNA were performed. Restriction analysis was carried out with the Vector NTI Suite 7 software.

Agarose Gel Electrophoresis

Agarose gels (0.7-1%) were prepared by dissolving the appropriate amount of agarose (Invitrogen) in 1×Tris-acetate-EDTA (TAE) buffer. After melting the agarose, ethidium bromide was added to the solution to a final concentration of 0.5 µg/µl. The agarose solution was poured into a gel tray and combs inserted. When the gel had solidified it was immersed into the electrophoresis tank (HE 33 Mini Horizontal Submarine Unit; Amersham asBioSciences, Uppsalas, Sweden) filled with 1×Tris-acetate-EDTA (TAE). Subsequently, DNA samples were mixed with 10× Orange G loading buffer (50% glycerol, 50 mM Tris-HCl pH 8.0, 50 mM EDTA, 1% w/v Orange G) and samples applied to the wells of the gel. 5 µl of a 2-log DNA ladder (New England BioLabs) was also loaded on the gel for analysis of the size of DNA fragments. The gel was run at 8-10 V/cm until a desired separation of the DNA fragments was obtained. DNA bands were visualized by illumination of a gel under (312 nm) UV light with Variable-intensity transilluminator (Spectronics Corporation, Westbury, NY).

DNA Extraction from Agarose Gels

Specific DNA fragments obtained after the restriction enzyme digestions were excised quickly under low-strength UV light, and DNA was extracted from the gel with Qiaquick Gel Extraction Kit (Qiagen) in accordance with the manufacturer’s instructions.

DNA quantification

Plasmid DNA concentration was determined by measuring the absorbance at 260 nm with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) following the instructions of the manufacturer.

DNA Insert Ligation into Vector

50-400 ng linear vector DNA and an appropriate ammount of insert DNA (3:1 molar ratio of insert DNA termini to vector DNA) were ligated in a 20 µl reaction containing 1-2 U T4 DNA Ligase and 2 µl of 10x ligation buffer for T4 DNA Ligase (all from New England BioLabs). Prior to transformation, DNA was precipitated by adding 1/10 volume of 3 M NaAc (pH 5.2) and 2.5 volumes of 99% EtOH, and incubated at -80 °C for 20 min. The pellets were collected by centrifugation at 14000×g for 45 min at 4 °C and washed with 70% EtOH by centrifugation at 14000×g for 15 min at 4 °C. Subsequently, DNA was air-dried for 10-15 min and dissolved in TE buffer.
Sequencing

100-150 ng/µl of plasmid was mixed with 15 pmol of primer and diluted in water to 15 µl. Then the tubes were sent for sequencing to MWG Biotech AG, Martinsried, Germany.

Table 4. The primers used for sequencing or PCR amplifications.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Primer name</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>DREP-E2A-EGFP</td>
<td>repFwd17</td>
<td>5’-CGGATGCCTCAAAGTACACG-3’</td>
</tr>
<tr>
<td></td>
<td>3’UTR-rev</td>
<td>5’-CGGATGCCTCAAAGTACACG-3’</td>
</tr>
<tr>
<td>TREP-C-EGFP</td>
<td>seqFP1-SP, 2A p62 overhang rp</td>
<td>5’-CGGATGCCTCAAAGTACACG-3’</td>
</tr>
<tr>
<td>TREP-E2A-Ag</td>
<td>repFwd17, seqSFV.cap rp</td>
<td>5’-CGGATGCCTCAAAGTACACG-3’</td>
</tr>
<tr>
<td>TREP-C-Ag</td>
<td>seqFp1sp, seqSFV.E3 rp</td>
<td>5’-CGGATGCCTCAAAGTACACG-3’</td>
</tr>
</tbody>
</table>

One-step Quantitative Real-time Reverse Transcription Polymerase Chain Reaction

The viral titer in the cell supernatant was determined by estimating viral RNA copy number by quantitative real time reverse transcription polymerase chain reaction (qRT-PCR). First, cell supernatants were filtered through Millex® Syringe Driven 0.22 µm Filter Unit (Millipore Corporation, Billerica, MA) to remove the cell debris. Then 200 µl of supernatant was treated with 100 µg RNase A (Qiagen) and 1836 U RNase T1 (Invitrogen) for 30 min at 37 ºC order to destroy all free RNA that is not encased in viral particles. Subsequently, RNases were digested by incubating the samples with 50 U Proteinase K (Finnzymes, Espoo, Finland) for 30 min at 37°C. Then the viral RNA was isolated using Dynabeads® SILANE viral NA kit (Invitrogen) according to the manufacturer’s instructions, i.e. 200 µl of the supernatant containing the virus and 50 µl Dynabeads MyOne™ SILANE suspension was used. Purified RNA was frozen in -80 ºC until use.

One step qRT-PCR was performed using EXPRESS One-Step SuperScript® qRT-PCR SuperMix Kit (Invitrogen). The set of primers (forward primer 5’- ctgccttgttcgctaagacc -3’ and reverse primer 5’- gtgtgtttctgctggagt -3’) as well as TaqMan probe (5’-FAM-tccgctgaggctgccctag- BHQ1-3’) targeting a region in nsp4 were designed using the software Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and purchased from Eurofins (Nantes, Belgium). All sample s were pipetted in MicroAmp® Fast Optical 96-well Reaction Plate (Applied Biosystems, Foster City, CA) in triplicates, and qRT-PCR assays were carried out using the ABI 7900HT instrument (Applied Biosystems). Amplifications were performed in a total volume of 10 µl, containing 500 nM ROX Reference Dye, 500 nM of each primer and 200 nM of probe. Each run also included “No-RT controls” (EXPRESS SuperScript® Mix for One-Step qPCR was not added) to check for DNA contamination of RNA samples, as well as “No-template controls” (template RNA was not added) to examine if the reaction mixture was contaminated with RNA or DNA. The cycling profile consisted of reverse transcription for 15 min at 50 ºC, followed by denaturation for 20 s at 95ºC. Subsequently, 40 cycles of amplification were performed, with each cycle consisting of two steps: 1 s at 95 ºC and 20 s at 60 ºC. Amplification data were analysed with the SDS 2.3 software (Applied Biosystems). Quantification of the RNA copies in qRT-PCR
was based on the standard curve generated by a 10-fold serial dilution of RNA purified from wild type SFV. The RNA of the standard was prepared in the same way as for the samples (described above). The wild type SFV titer was determined by plaque assay. To assess the effectiveness of qRT-PCR, the data obtained from qRT-PCR were compared with plaque assay results.

To avoid cross contamination with RNA and DNA, two separate areas for preparing qRT-PCR assays were set up. A master mix of common components for qRT-PCR reactions was prepared and pipetted in the plate in the special area devoted only for this purpose, while sample RNA was diluted and loaded on the plate inside a laminar flow hood in the other room. To remove RNase contamination, bench tops and pipettors were treated with RNase Away® reagent (Sigma-Aldrich Inc., St. Louis, MO). In addition, UltraPure™ DNase/RNase-Free Distilled Water (Invitrogen) and RNase-free microcentrifuge tubes (Ambion, Austin, TX) were used for all dilutions and reactions.

**cDNA synthesis**

The first-strand synthesis of cDNA for TREP-E2A-Ag and TREP-C-Ag RNA was performed using M-MLV Reverse Transcriptase (Invitrogen). The specific primer seqSFV.cap rp was added in the case of TREP-E2A-Ag and the specific primer seqSFV.E3 rp was added for TREP-C-Ag first-strand synthesis. The reaction conditions recommended by the vendors were used. 2 µl from the first-strand reaction were used for amplification with Dynazyme EXT DNA Polymerase (1 U/µl) (Finnzymes) in 50 µl PCR reaction. The PCR reaction was set up according to the manufacturer’s instructions. To obtain the desired product from cDNA of TREP-E2A-Ag, the primers seqSFV.cap rp and repFwd17 were added to the reaction mixture. The primers seqSFV.E3 rp and seqFp1sp were used to amplify the desired product from cDNA of TREP-C-Ag. The sequences of the primers are listed in Table 4. The cycling profile of the PCR reaction is described in Table 5. The obtained PCR fragments were cloned into pCR2.1 vector which is included into Zero Blunt® PCR Cloning Kit (Invitrogen) as described in a manual of the kit.

**Table 5.** Thermal cycling profile used for amplification of specific products from the cDNA of TREP-E2A-Ag and TREP-C-Ag.

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature (ºC)</th>
<th>Time</th>
<th>No. of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>94</td>
<td>2 min</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94</td>
<td>40 s</td>
<td>20</td>
</tr>
<tr>
<td>Anealing</td>
<td>52</td>
<td>40 s</td>
<td></td>
</tr>
<tr>
<td>Elongation</td>
<td>72</td>
<td>2 min</td>
<td></td>
</tr>
<tr>
<td>Final elongation</td>
<td>72</td>
<td>5 min</td>
<td>1</td>
</tr>
<tr>
<td>Cooling</td>
<td>4</td>
<td>∞</td>
<td>1</td>
</tr>
</tbody>
</table>

**Cells**

BHK-21 cells were cultured in Glasgow’s Minimal Essential Medium (Invitrogen) containing 5% foetal calf serum (FCS), 10% tryptose phosphate broth (Karolinska university hospital laboratory, Stockholm, Sweden), 2 mM L-glutamine (Invitrogen), 100 IU/mL penicillin, 100 µg/ml streptomycin (Sigma, St. Louis, MO) and 10 mM HEPES (Invitrogen) in a humidified 5% CO₂ cell culture incubator at 37 ºC.
Plaque assay

Titters of replication-competent recombinant SFV were determined by the plaque assay technique. The exact protocol used in this project is described below.

First, the BHK-21 cells were grown in a 6-well plate until they formed a monolayer of cells. Then $10^{-1}$-$10^{-9}$ dilutions of the sample containing unknown amount of infectious viral particles were made in Earle’s minimal essential Medium (Gibco/Invitrogen) supplemented with 0.2% (w/v) BSA, 2 mM glutamine and 10 mM Hepes. After washing the cells with PBS, 0.5 ml from the $10^{-4}$-$10^{-9}$ dilutions of virus was added to different wells containing BHK-21 cells. The plate was then incubated for 1h at 37°C in a cell culture incubator with gentle manual shaking every 10 min. Afterwards, the cells were overlaid with a semi-solid growth medium (50% MEM containing 1.9% melted agarose, 50% BHK complete medium). Once the overlay medium solidified, the plate was placed in the 37°C cell culture incubator for approximately 48 h. To visualize the plaques the cells were fixed with 10% formaldehyde in PBS for 30 min. The overlay medium was then carefully removed from all the wells. Next, the viable cells were stained with 0.1 % crystal violet in 20% methanol for 30 min. Finally, the dye was washed out with tap water and the plaques (empty spots) were counted manually. The titer of the original sample was counted as plaque forming units per millilitre (pfu/ml) according to the following formula:

\[
\text{Titer (pfu/ml)} = \frac{\text{number of plaques in a well}}{2 \times \text{dilution factor}}
\]

Flow Cytometry Analysis

For flow cytometry ~0.5×10^6 cells were harvested by trypsination and transferred to fluorescent-activated cell sorting (FACS) tubes. First, the cells were washed 1-2 times in FACS buffer (0.1% BSA in PBS) by centrifugating at 350×g for 3 min. Subsequently, the cells were fixed with 100 µl BD Cytofix/Cytoperm Fixation and Permeabilization Solution (BD Biosciences, San Diego, CA) for 20 min at room temperature. Then 100 µl of 0.1 M glycine was added to neutralize formaldehyde in the buffer. The cells were thereafter permabilized with BD Perm/Wash Buffer (BD Biosciences) and centrifuged at 350×g for 3 min. When the cells were stained for viral replicase, monoclonal mouse anti-SFV replicase antibodies were added to a final dilution of 1:400. The cells were incubated with the antibodies for 30 min at 4 °C with periodical shaking. After staining with the primary antibodies, two washing steps with BD Perm/Wash Buffer followed. Then the cells were stained with the secondary antibodies R-phycoerythrin-conjugated polyclonal goat anti-mouse IgGs (Dako Denmark A/S, Glostrup, Denmark) at a final dilution of 1:100 for 30 min at 4 °C. In case the cells were stained for luciferase, fluorescein-conjugated polyclonal goat anti-luciferase IgG (Rockland, Gilbertsville, PA) to a final dilution of 1:4000 was used, and the cells were incubated for 30 min at 4 °C. Stained cells were washed twice with BD Perm/Wash Buffer and once with FACS buffer. In the end, the cells were resuspended in 200-500 µl of FACS buffer (depending on the number of cells) and stored at 4 °C in the dark until analysed. Flow cytometry samples were acquired using a FACScan instrument (BD Biosciences) and analysed using the BD CellQuest™ Pro Software.

Mice and immunizations

Balb/c mice were bred and kept under pathogen-free environment at the animal house at the Department of Microbiology, Tumor and Cell Biology (MTC), Karolinska Institute. All animal experiments were performed according with the guidelines approved by the
Committee for Animal Ethics in Stockholm, Sweden, ethical application number N374/08 + N209/08.

For immunizations, first, mice were anesthetized in a chamber filled with 4% isoflurane in oxygen. During the vaccination procedure, mice were maintained anesthetized with the help of a breathing mask. The anesthesia equipment Univentor 400 (Zejtun, Malta) was used during the procedure. Mice were injected intradermally to each flank in the lower part of the back with 20 µl of plasmid DNA diluted in PBS and immediately electroporated with Derma Vax™ Clinical DNA Vaccine Delivery System (Cyto Pulse Sciences, Inc. Glen Burnie, MD) over the intradermal injection sites. Electroporation consisted of 2 pulses of 1.125 V/cm for 50 µs, and 8 pulses of 275 V/cm for 10 ms. The interval between the two 1.125 V/cm pulses was 300 ms, and the intervals between the eight 275 V/cm pulses were set to 300 ms. The needle-array electrodes (NE-4-4) with two parallel rows of four 2-mm pins (1.5×4 mm gaps) (Cyto Pulse Sciences) were used for electroporation.

**In vivo Bioluminescent Imaging**

Luciferase activity in mice was assessed by measuring photon emission in an IVIS (In vivo Imaging System) 100 camera (Caliper Life Sciences, Hopkinton, MA). The mice were injected intraperitoneally with 1.5 mg D-luciferin (Caliper Life Sciences, Hopkinton, MA) in 100 µl PBS. Subsequently, to keep mice immobile, they were anesthetized in a gas chamber with 4 % isoflurane. Then they were moved to gas masks in a light-tight chamber and imaged with a sensitive charge-coupled device (CCD) camera. 15-45 min post-administration of luciferin, mice were exposed from 1 s to 10 min using either the “medium sensitivity” or the “high sensitivity” setting depending on the strength of signal. The images were analyzed with the Living Image 2.50.1 software. Signal intensity was verified by measuring maximum photon flux within regions of interest (ROI).

**ELISPOT**

The frequency of T-cell responses was measured by the IFN-γ enzyme-linked immunosorbent spot (ELISPOT) assay.

**Coating**

First, the wells of the the protein binding membranes in the 96-well MultiScreen IP plates (Millipore, Billerica, MA) were pre-wetted with 50 µl 70% ethanol for 1 min. The plates were immediately washed with 200 µl sterile PBS four times. Then 100 µl of 5 µg/ml anti-mouse-IFN-γ monoclonal antibody (AN18; Mabtech AB, Nacka strand, Sweden) was added per well and the plates were incubated overnight at 4°C.

**Blocking of ELISPOT plates**

After coating the ELISPOT plates with anti-mouse-IFN-γ monoclonal antibody, the excess of antibody was removed and the plates were washed 5 times with sterile PBS (200 µl/well). Then the plates were blocked with complete RPMI 1640 medium (200 µl/well) for at least 2 h in a 37 °C cell culture incubator.
Preparation of a single-cell suspension from spleens

Single-cell suspensions were obtained by mashing the mice spleens through 70 μm nylon cell strainers (Falcon; BD Biosciences, San Diego, CA). The cells were then resuspended in 5 ml of complete RPMI 1640 medium, containing 5% FCS, 2 mM-L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin (Sigma), and 20 mM HEPES (Invitrogen) and centrifuged at 350×g for 7 min. The red blood cells (RBC) were then lysed with 1 ml of RBC lysing buffer (Sigma) for 1-2 min at room temperature, followed by a washing step with 5 ml of complete RPMI 1640 medium. Finally, the cells were resuspended in 5 ml RPMI 1640 medium and the concentration of the cells determined by counting in a Bürker chamber.

In vitro stimulation of cells

The blocking medium was removed and 75 μl of stimulatory agents (final concentrations: Concanavalin A (ConA, Sigma-Aldrich, St. Louis, MO): 2 μg/ml; EGFP peptide (HYLSTQSAL, Proimmune, Oxford, UK): 2 μg/ml; and p18 peptide (RGPGRAFVTI, Proimmune): 2 μg/ml) or medium alone were added in triplicates. Then 2×10^5 splenocytes from individual mice were added in triplicates to the wells containing the stimuli of interest (media, peptide and ConA). The final volume in each well was 150 μl. The ELISPOT plates were incubated for 18-22 h at 37 °C in a cell culture incubator.

Detection of spots

After the overnight incubation, the plates were washed six times with PBS and 100 μl of 1 μg/ml biotinylated anti-IFN-γ detecting antibody (R4-6A2; MabTech, Nacka, Sweden) diluted in PBS containing 0.5% filtered FCS (PBS-FCS) was added to each well. The plates were then incubated for 2 h at room temperature. Next, the plates were washed with PBS as described above and 100 μl of 1 μg/ml streptavidin-ALP diluted in PBS-FCS was added per well. After incubation for 1-2 h at room temperature, the plates were washed with PBS again and 100 μl of BCIP/NBT-plus (MabTech) filtered through a 45 μm filter was added to each well. The plates were developed for 7 min until distinct spots emerged. To stop the colour reaction, the plates were extensively rinsed with tap water. The plates were left overnight to dry, and the spots were then counted using an ELISPOT reader (ImmunoSpot, Cellular Technology Ltd., OH).

Data analysis

Results were expressed as spot forming cells (SFC) per 10^6 splenocytes. An assay was regarded valid if the mean of the negative control (unstimulated cells) was below 50 SFU/10^6 splenocytes and the mean of the positive control (cells stimulated with ConA) was above 250 SFU. For the response to be considered positive, the mean value of specific response had to be >55 spots/10^6 splenocytes. Moreover, the ratio between mean specific SFC and mean SFC of immunized controls had to be above 1.5, and the ratio between mean SFU of specific stimulation and mean SFU of media control had to be above 4.
The Direct Enzyme-linked Immunosorbent Assay (ELISA) for EGFP-specific IgG Antibodies

ELISA plates (Immunosorp, Nunc, Denmark) were coated with 50 µl/well of 1 µg/ml EGFP (BioVision Research Products, Mountain View, CA) in 0.1 M carbonate buffer. The covered plates were incubated at +4°C over night. Next day, the plates were washed 5× with PBS-Tween (0.05%), and blocked with 1% BSA-PBS (100 µl/well) for 1 h at room temperature. Afterwards, 50 µl/well of mouse serum samples serially diluted in PBS-Tween (0.05%) were added, and the plates were incubated at room temperature for 2 h. The dilutions of the serum used in the assay were 1/50, 1/200, 1/800, 1/3200 and 1/12800. The serum from a naïve mouse (negative control) was also included on each plate. After washing the plates 5× with PBS-Tween (0.05%), 50 µl of horse radish peroxidise conjugated anti-mouse total IgG (Southern Biotech, Birmingham, AL) diluted 1/5000 in PBS-Tween (0.05%) was added to each well. Then the plates were incubated for 1.5 h at room temperature. Subsequently, the plates were washed as described above. The bound antibodies were detected by adding 50 µl/well of TMB (3,3´,5,5´-tetramethylbenzidine) solution prepared according to the instructions included in BD OptEIA TMB substrate Reagent Set (BD Biosciences). The reaction was stopped after 4 min with 50 µl 2M H₂SO₄. The absorbance at 450 nm and 570 nm was measured by VICTOR² 1420 Multilabel Counter (PerkinElmer, Waltham, MA).

Statistical Analyses

GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA) was used for all statistical calculations. The Kruskal–Wallis test with Dunn's post-test was used to identify any differences among groups for all comparisons, except when two groups were compared. For comparisons of two groups, the Mann-Whitney test was applied. The Wilcoxon matched pairs test was used to compare antibody levels before and after boost. The Spearman rank correlation test was used to correlate luciferase activity to IFN-γ T cell responses. A p-value of 0.05 or less was considered significant for all tests performed.
### APPENDIX

**Table 6.** The representative examples of the prototyeps of SFV vector-derived vaccines.

<table>
<thead>
<tr>
<th>Type of SFV vector</th>
<th>Pathogen</th>
<th>Immunogen used</th>
<th>Comments</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>VREP</td>
<td>Influenza A virus</td>
<td>Influenza A nucleoprotein and hemagglutinin</td>
<td>Strong and durable humoral as well as cellular immune responses were elicited in mice. All vaccinated mice were protected against the challenge with the virus.</td>
<td>(Berglund et al., 1999)</td>
</tr>
<tr>
<td></td>
<td>Layered DNA-RNA vector</td>
<td>Foot and mouth disease virus</td>
<td>The protective humoral and cellular immune response was obtained after immunization. The vaccinated mice cleared the virus after challenge.</td>
<td>(Yu et al., 2006)</td>
</tr>
</tbody>
</table>
| VREP               | Louping ill virus               | Spike precursor prME and the nonstructural protein | 1. Protective humoral response in mice was generated.  
2. Efficient immune response was triggered by vaccination that protected sheep against subcutaneous challenge with the virus.                                      | 1. (Fleeton et al., 2000)  
2. (Morris-Downes et al., 2001) |
| VREP               | Respiratory syncytial virus (RSV) | RSV fusion and attachment protein       | Intranasal vaccination of mice induced broad and strong protective immune responses against the virus.                                                                                                  | (Chen et al., 2002)                           |
| VREP               | Infectious bronchitis virus     | Nucleocapsid polypeptides              | A robust cellular immune response was induced in immunized chickens that resulted in protection against the virus.                                                                                   | (Seo et al., 1997)                            |
| VREP               | Infectious bursal disease virus | VP2 protein or VP2/VP4/VP3 structural polyprotein | The neutralizing antibodies against the virus were elicited in vaccinated chickens.                                                                                                                    | (Phenix et al., 2001)                         |
| VREP               | Rubella virus                   | E1 and E2 envelope proteins            | Efficient humoral response against viral envelope proteins was elicited in mice. However, co-immunization with SFV vectors encoding mumps and measles Ags diminished the immunity. | (Callagy et al., 2007)                        |
| VREP               | Murray Valley encephalitis      | Membrane proteins prM and E            | The vaccinated mice built an effective humoral response and were protected against virus challenge.                                                                                                      | (Colombage et al., 1998)                      |
| VREP               | Brucella abortus                | Cu,Zn superoxide dismutase gene  
2. Brucella abortus translation initiation factor 3 (IF3) | 1. Induced cellular immune responses in mice and cattle.  
2. Induced similar level of protection against the bacteria in mice as live attenuated vaccine.                                                                                               | 1. (Onate et al., 2005; Saez et al., 2008)  
2.(Cabrera et al., 2009) |
| Layered DNA-RNA vector | Swine vesicular disease virus | Capsid protein                          | Half of the vaccinated swine generated protective immunity and survived the challenge with the virus.                                                                                                   | (Sun et al., 2007)                            |
| VREP or DNA-RNA layered vector | Plasmodium falciparum | 1.Pf332 Ag 2. Erythrocyte membrane protein 1 (PfEMP1) | 1. Immunization of mice resulted in immunological memory but low humoral response. 2. The sequestration of infected erythrocytes was significantly depressed in vaccinated rats and cynomolgus macaques. | (Andersson et al., 2001) 2. (Moll et al., 2007) |
| Layered DNA-RNA vector | Clostridium botulinum | Hc domain of C. botulinum neurotoxin serotype A | The protective humoral and cellular immune responses were induced against the challenge with botulinum toxin A in mice. | (Yu et al., 2007) |
| VREP | Chlamydia pneumoniae | Major outer membrane protein and outer membrane protein 2 | The vaccinated mice were partially protected against challenge with the bacteria. | (Penttila et al., 2004) |
| Layered DNA-RNA vector | Classical swine fever virus | Envelope glycoprotein E2 | The pigs developed antibody responses after immunization and survived subsequent lethal challenge with the virus. | (Li et al., 2007) |
| VREP | Hepatitis C virus | Hepatitis C virus non-structural protein 3 | A specific T cell response was detected in mice after immunization. | (Brinster et al., 2002) |
| Layered DNA-RNA vector | Pseudorabies virus | Glycoprotein C | Strong cellular immune response but relatively low humoral response was induced in immunized mice. All vaccinated mice survived the infection with the virus. | (Xiao et al., 2004) |
| Layered DNA-RNA vector | Bovine circovirus | NS3 protein | A virus-specific antibody response as well as a cellular immune response was induced in vaccinated mice. | (Reddy et al., 1999) |
| VREP or DNA-RNA layered vector | Human immunodeficiency virus (HIV) | Various structural and non-structural proteins of HIV | SFV vectors encoding various HIV Ags where examined for their capacity to induce anti-HIV immunity in mice and non-human primates when administered in different routes and regimes. The outcomes of the studies show promising results as considerable protection was achieved in some cases. | (Berglund et al., 1997; Forsell et al., 2007; Gomez et al., 2007; Hanke et al., 2003; Morner et al., 2009; Sundback et al., 2005) |
| VREP or DNA-RNA layered vector | Simian immunodeficiency virus (SIV) | Various structural and non-structural proteins of SIV | SFV vectors encoding various SIV Ags where evaluated for their capacity to induce anti-SIV immunity in non-human primates when administered in different routes and regimes. The outcomes of the studies are promising as partial protection was obtained in some cases. | (Koopman et al., 2004; Maggioralla et al., 2007; Martinon et al., 2008; Michelini et al., 2004; Negri et al., 2004; Nilsson et al., 2001; Stolte-Leeb et al., 2006) |
ACKNOWLEDGEMENTS

I would like to thank Prof. Peter Liljestrom for accepting me as a student to his research group and providing an opportunity to work on a fascinating project as well as all useful comments regarding my exam work. Also, I wish to express my sincere gratitude to Prof. Staffan Svard for coordination of my master’s thesis as well as guidance throughout this entire master’s programme. Thanks for being a great teacher and sparking my interest in infectious biology.

I was really happy to have two wonderful supervisors, Maria Kakoulidou and Daniel Johansson, during the course of my exam work. I wish to express my deep gratitude to Maria for taking care of me from the first minute I joined the group as well as teaching me so much in immunology and science in general. In addition, I thank her for stimulating optimism and being so helpful, warm and lovely person. I am equally greatful for Daniel for sharing his knowledge and brilliant ideas with me, for all inspiring scientific discussion and interesting non-scientific conversations as well as regular assistance and valuable lessons during the project.

Also, I woul like to thank for all other members of Peter Liljestrom’s research group for the warm atmosphere in the laboratory, for their support and all nice and relaxing moments together.

Moreover, I owe a great debt of gratitude to a lot of teachers at Vilnius University and Biotechnology Institute as well as Prof. Erik Fries at Uppsala University for building my theoretical basis as well as my practical skills so preparing me to this thesis.

In addition, I send a special thank for all my friends in Lithuania, Sweden and all over the world for filling my life with joy.

I would like to end with my immense gratitude for my closest family, i.e. my parents, my brother and his family and my grandparents, as without their help my studying in Sweden would be impossible. But in the first place, I thank them for their endless love, support and belief in me that always give me so much strength in everything I do. Therefore, with my greatest love I would like to dedicate this thesis to my darling family.
REFERENCES


