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ALPHAVIRUS REPLICON-BASED STRATEGIES FOR VACCINATION

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The cover picture shows the Chikungunya virus nsP2 protein. The picture was generated with data deposited in the Protein Data Bank (doi:10.2210/pdb3trk/pdb) using the Protein Workshop software (Moreland *et al*., BMC Bioinformatics 2005).

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"Far and away the best prize that life has to offer
is the chance to work hard at work worth doing."
Theodore Roosevelt

ABSTRACT

Vaccination has been extremely successful for the control of many infectious diseases. However, efficient vaccines are still not available against diseases such as HIV, hepatitis C, malaria and tuberculosis. For these diseases, the traditional vaccine approaches are not feasible, and thus new vaccine technologies are needed. Some platforms that are being developed for this purpose include virus vectors and DNA vaccines. In addition, combining different vaccine modalities into heterologous prime-boost regimens induces antigen-specific immune responses that are greatly increased compared to homologous prime-boost immunization.

In this thesis, we evaluated the use of alphavirus replicons as a vaccine platform and characterized antigen-specific immune responses induced in mice and rhesus macaques. Alphavirus replicons can be administered either as viral particles (VREP), or as naked DNA (DREP) or RNA (RREP). We used both model antigens (**papers II** and **IV**) and HIV antigens that are in clinical development (**papers I** and **III**).

We show in **paper I** that antigen-specific $CD8^+$ T cell responses induced by DREP can be further increased by delivery with intradermal electroporation. These responses were superior in magnitude to those induced by a conventional DNA vaccine and required lower doses. We also showed in mice and macaques that priming with DREP rather than conventional DNA prior to a heterologous boost with a poxvirus or adenovirus vector resulted in stronger immune responses characterized by multifunctional T cells. In **paper II**, we characterize the kinetics and memory phenotypes of $CD8^+$ T cell responses induced by VREP and DREP. We show how altering factors such as timing and dose affects the magnitude and phenotype of the resulting immune response. In addition, we characterize the phenotypes of T cell responses induced by different heterologous boosters given after a DREP prime. For example, a poxvirus vector boost favored expansion of effector memory T cells. In **paper III**, we expand the heterologous prime-boost studies and explore the outcome of altering the number of DREP prime immunizations prior to a poxvirus and/or protein antigen boost. We demonstrate that a single prime with a low dose of DREP was sufficient for induction of antigen-specific T cells that were expanded by a poxvirus boost and antibody responses boosted by protein antigen. By boosting with poxvirus together with protein, both arms of adaptive immunity were induced. In **paper IV**, we use VREP as an adjuvant for antibody responses against a co-immunized protein antigen. We demonstrate that incorporating the innate immune stimulant flagellin into the replicon enhances its adjuvant potency, resulting in augmented antigen-specific antibody responses.

In conclusion, we have characterized immune responses induced by alphavirus replicons administered as VREP or DREP and shown that DREP is an excellent prime of T cell and antibody responses prior to a heterologous boost immunization. These results strongly support further clinical testing of alphavirus replicon vaccines.

LIST OF SCIENTIFIC PAPERS

I. **Knudsen ML**, Mbewe-Mvula A, Rosario M, Johansson DX, Kakoulidou M, Bridgeman A, Reyes-Sandoval A, Nicosia A, Ljungberg K, Hanke T, Liljeström P. 2012. Superior induction of T cell responses to conserved HIV-1 regions by electroporated alphavirus replicon DNA compared to that with conventional plasmid DNA vaccine. J. Virol. 86:4082–4090.

II. **Knudsen ML***, Ljungberg K*, Kakoulidou M, Kostic L, Hallengärd D, García-Arriaza J, Merits A, Esteban M, Liljeström P. 2014. Kinetic and phenotypic analysis of $CD8^+$ T cell responses after priming with alphavirus replicons and homologous or heterologous booster immunizations. J. Virol. 88:12438-12451.
* These authors contributed equally

III. **Knudsen ML**, Ljungberg K, Tatoud R, Weber J, Esteban M, Liljeström P. Alphavirus replicon DNA expressing HIV antigens is an excellent prime for boosting with recombinant modified vaccinia Ankara (MVA) or with HIV gp140 protein antigen. Submitted manuscript.

IV. **Knudsen ML**, Johansson DX, Kostic L, Nordström EKL, Tegerstedt K, Pasetto A, Applequist SE, Ljungberg K, Sirard J-C, Liljeström P. 2013. The adjuvant activity of alphavirus replicons is enhanced by incorporating the microbial molecule flagellin into the replicon. PLoS One 8:e65964.

CONTENTS

LIST OF ABBREVIATIONS

ADCC	antibody-dependent cellular cytotoxicity
AF	aqueous formulation
ALVAC	attenuated canarypox virus
APC	antigen-presenting cell
cGAS	cyclic GMP-AMP synthase
ChAd	chimpanzee adenovirus
CHIKV	Chikungunya virus
DAI	DNA-dependent activator of IFN-regulatory factors
DAMP	damage-associated molecular pattern
DC	dendritic cell
DREP	DNA replicon
ds	double-stranded
ELISA	enzyme-linked immunosorbent assay
ELISpot	enzyme-linked immunosorbent spot
EP	electroporation
GLA	Glucopyranosyl Lipid Adjuvant
HIV	human immunodeficiency virus
i.d.	intradermal
i.m.	intramuscular
ICS	intracellular cytokine staining
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IRF	interferon regulatory factor
LCMV	lymphocytic choriomeningitis virus
LGP2	laboratory of genetics and physiology 2
LPS	lipopolysaccharide
MDA5	melanoma differentiation factor 5
MHC	major histocompatibility complex
MVA	modified vaccinia Ankara

MyD88	myeloid differentiation primary-response protein 88
NF-κB	nuclear factor kappa B
NLR	Nod-like receptor
nsP	non-structural protein
NYVAC	attenuated New York vaccinia
ORF	open reading frame
PAMP	pathogen-associated molecular pattern
PRR	pattern recognition receptor
rAd	recombinant human adenovirus
RIG-I	retinoic acid inducible gene I
RLR	RIG-I-like receptor
RREP	RNA replicon
SFV	Semliki Forest virus
SIN	Sindbis virus
ss	single-stranded
Tcm	central memory T cells
Tem	effector memory T cells
Th	T helper
TLR	Toll-like receptor
TNF	tumor necrosis factor
VEE	Venezuelan equine encephalitis virus
VREP	viral replicon
WT	wild type

1 INTRODUCTION

1.1 VACCINES

Vaccines are considered one of the greatest medical successes in history and have had a tremendous worldwide impact on reduction of morbidity and mortality of infectious diseases. Some of the earliest documented attempts at immunization stem from 17th century China, where the variolation method was used. This involved inoculation against smallpox (variola) by introducing powdered scabs derived from smallpox pustules into the nose. Variolation was first introduced in Europe in 1721 by Lady Montagu, who had observed the technique being practiced in Constantinople. Although the treatment had a death rate of 2-3%, this was far lower than the 30% case-fatality rates that occurred during smallpox outbreaks.

The first demonstration of vaccination, i.e. controlling an infectious disease without actually transmitting the disease, was performed by Edward Jenner in 1796. Jenner had noticed that milkmaids who had contracted cowpox were immune to smallpox. He then demonstrated that transfer of material from human pustular lesions caused by cowpox into the skin of another person gave protection from subsequent infection with smallpox. This material was named vaccine and the process vaccination from the latin *vacca*, meaning cow. About a century later, Louis Pasteur broadened the definition to include preventive inoculation with other agents.

Today, smallpox has been eradicated from the world. Vaccines have also been successful in controlling numerous other viral diseases. For example, vaccination has reduced the number of cases of poliomyelitis by 99%. This disease is now also targeted for eradication by the World Health Organization. Other viral diseases against which vaccines have been developed include yellow fever, hepatitis B, influenza, measles, mumps, rubella, rabies and rotavirus (1).

Despite the enormous progress that has occurred since Jenner's first demonstration of vaccination, efficient vaccines are still not available against infectious diseases such as human immunodeficiency virus (HIV), hepatitis C, tuberculosis and malaria. For these diseases, the vaccine approaches that are used against other infectious diseases have not been successful or are not feasible. Therefore, new types of vaccines are being developed and characterized in an attempt to rationally target these diseases.

1.2 IMMUNOLOGY OF VACCINATION

The goal of vaccination is to induce long-lived immunity against a disease without causing the disease itself. When the first vaccines were developed, little was understood of the mammalian immune system. Today, we understand more about the components and functions of the immune system and protective mechanisms. Vaccines mimic natural infection in that they stimulate nonspecific innate immune responses that lead to induction of pathogen-specific adaptive immune responses. Innate responses are quick, usually occurring within minutes to hours after initiation of an infection, whereas adaptive immune responses

take several days to form. Adaptive responses contract after clearance of antigen and persist as immunological memory that upon exposure will protect an individual from subsequent infection with that pathogen.

1.2.1 Innate immunity

The innate immune system is the first line of defense that pathogens or vaccines encounter after crossing physical barriers such as skin. Antigen-presenting cells (APCs) such as dendritic cells (DCs) are innate immune cells that patrol the body. These cells are activated upon exposure to a pathogen or vaccine with sufficient "danger signals" and migrate to secondary lymph nodes where they activate antigen-specific T and B cells. Recent insights into the importance of triggering innate immune responses for inducing and shaping adaptive immune responses have led to an interest in exploiting this knowledge for the design of novel vaccine adjuvants (covered in section 1.3.3).

Cells of the innate immune system use pattern recognition receptors (PRRs) to sense conserved structural and functional molecules of pathogens, known as pathogen-associated molecular patterns (PAMPs). In addition, PRRs detect molecules derived from necrotic or traumatized host cells, known as damage-associated molecular patterns (DAMPs). Signaling through PRRs triggers signaling cascades that result in innate responses such as nuclear factor kappa B (NF-κB)-dependent cytokine responses, interferon regulatory factor (IRF)-dependent type I interferon (IFN) responses and inflammasome / caspase-1-dependent interleukin (IL)-1β responses (2). These responses keep the infection under initial control and play a critical role in inducing and shaping adaptive immune responses. Several families of PRRs have been identified, including the transmembrane Toll-like receptors (TLRs) and the cytoplasmic retinoic acid inducible gene I (RIG-I)-like receptors (RLRs), Nod-like receptors (NLRs), and DNA-sensing molecules (**Table 1**).

Table 1. Pattern recognition receptors

PRR family	Examples	Location	Natural ligand
TLRs	TLR1/2/6	Cell surface	Lipoproteins
	TLR3	Endosome	dsRNA
	TLR4	Cell surface	LPS
	TLR5	Cell surface	Flagellin
	TLR7/8	Endosome	ssRNA
	TLR9	Endosome	Unmethylated CpG DNA
RLRs	RIG-I	Cytoplasm	Uncapped ssRNA, short dsRNA
	MDA5	Cytoplasm	Long dsRNA
NLRs	NLRC4	Cytoplasm	Flagellin
DNA sensors	cGAS	Cytoplasm	dsDNA

1.2.1.1 TLRs

TLRs were the first identified and today most well characterized family of PRRs. These receptors recognize distinct PAMPs derived from viruses, bacteria, mycobacteria, fungi and parasites, including lipoproteins (detected by TLR1, TLR2 and TLR6), double-stranded RNA (dsRNA) (TLR3), lipopolysaccharide (LPS) (TLR4), flagellin (TLR5), single-stranded RNA (ssRNA) (TLR7 and TLR8) and unmethylated CpG DNA (TLR9). The TLRs that recognize nucleic acids are located in endosomal compartments, while the other TLRs are located on cell surfaces. TLRs are expressed by innate immune cells such as DCs and macrophages, as well as nonimmune cells such as epithelial cells (3, 4).

All TLRs except TLR3 signal through the adaptor molecule myeloid differentiation primary-response protein 88 (MyD88), ultimately leading to the translocation of NF-κB into the nucleus, where it induces production of inflammatory cytokines such as tumor necrosis factor (TNF)-α, IL-6, IL-12 and pro-IL-1β. TLR3 and TLR4 are able to signal through a MyD88-independent pathway, which leads to phosphorylation and activation of transcription factor IRF3, resulting in production of type I IFNs. TLR7 and TLR9 are primarily expressed in plasmacytoid DCs, which have the capacity to produce large amounts of type I IFNs in a MyD88-dependent manner (3). Signaling through TLRs plays an important role in activation of DCs, which then present antigens to lymphocytes, leading to induction of $CD4^+$ T helper (Th) 1 and $CD8^+$ T cell responses (5–7). In addition, TLRs shape antibody responses through intrinsic signaling in B cells (8).

1.2.1.2 RLRs

RLRs are RNA helicases that detect viral RNA in the cytosol. Unlike TLRs, these receptors are expressed by most cell types. The RLR family consists of three members: RIG-I, melanoma differentiation factor 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2). RIG-I recognizes uncapped 5'-triphosphate ssRNA and short dsRNA, whereas MDA5 recognizes longer dsRNA fragments. Signaling through RIG-I and MDA5 leads to activation of NF-κB and IRFs and subsequent production of proinflammatory cytokines and type I IFNs. LGP2 lacks the signaling domain present in RIG-I and MDA5 and exerts inhibitory and co-stimulatory functions, respectively, on these molecules (9–11). RLR signaling promotes $CD8^+$ T cell responses (5, 12).

1.2.1.3 NLRs

The NLRs are a family of cytosolic receptors that sense a wide range of PAMPs and DAMPs (13). For example, NLRC4 recognizes bacterial flagellin (14, 15). Activation of NLRC4 leads to formation of an inflammasome, a large multiprotein complex. This in turn activates caspase-1, which cleaves pro-IL-1β and pro-IL-18 into their mature forms. In addition, caspase-1 induces pyroptosis, a type of cell death characterized by rapid cell lysis with release of proinflammatory intracellular contents. These caspase-1 activities promote formation of Th1 and $CD8^+$ T cell responses (16, 17).

1.2.1.4 DNA sensors

The presence of DNA in the cytosol is a hallmark of certain infections or tissue damage caused by infection and is recognized by several DNA sensors that in turn induce type I IFN production. These sensors have only recently been discovered and are less well characterized. Signaling through many DNA sensors occurs through a central signaling cascade involving adaptor molecule STING, which then activates NF-κB and IRF3, leading to production of type I IFNs (18). So far, eleven different DNA sensors have been proposed with some examples being cyclic GMP-AMP synthase (cGAS) and DNA-dependent activator of IFN-regulatory factors (DAI) (19–22). Cytosolic DNA sensors are important for the immunogenicity of DNA vaccines and their ability to induce T and B cell responses (5, 23). In addition, some DNA sensors including DAI and cyclic GMP-AMP possess adjuvant properties, i.e. they promote immune responses against co-immunized antigen (24, 25).

1.2.1.5 Type I IFNs

Many of the signaling pathways described above lead to the induction of type I IFNs, which are key mediators of antiviral responses and have derived their name for their function to "interfere" in viral replication. Type I IFNs are a group of proteins that includes more than ten different IFN-α, one IFN-β and several other related molecules that all bind to a common receptor, IFN-AR. All nucleated cells can both produce and respond to type I IFNs, although APCs and plasmacytoid DCs are key sources for type I IFN production. Signaling of type I IFNs causes the cell as well as surrounding tissue to enter an antiviral state characterized by the expression and antiviral activity of IFN-stimulatory genes (26, 27). Type I IFNs also have immunomodulatory effects on both innate and adaptive immune cells. In APCs such as DCs, type I IFNs stimulate upregulation of major histocompatibility complex (MHC) and costimulatory molecules, and promote cross-priming of $CD8^+$ T cells (28–30). In addition, type I IFNs can directly activate DCs, T cells and B cells (31–34).

1.2.2 Adaptive immunity

Long-term immunological protection is provided by maintenance of adaptive immune cells that are pathogen-specific (35). Adaptive immune responses are mediated by cell-mediated immunity and humoral immunity. $CD8^+$ T cells limit the spread of infectious agents by killing infected host cells or secreting antiviral cytokines. $CD4^+$ Th cells produce cytokines and provide support for the generation and maintenance of B cells and $CD8^+$ T cells. B cells produce antibodies that can bind to pathogens and prevent or reduce infections.

Most of the currently licensed vaccines confer protection by induction of neutralizing antibodies (35, 36). However, control of HIV, malaria, hepatitis C virus and tuberculosis depends largely on $CD4^+$ and $CD8^+$ T cells, and thus future vaccines against these pathogens will rely on induction of potent and durable T cell responses as well as neutralizing antibodies (37–39).

1.2.2.1 T cell responses

T cell responses are generated when DCs capture antigen in peripheral tissue, mature into an activated state and migrate to draining lymph nodes, where they present antigen-derived peptides on MHC molecules to T cells. The general notion is that $CD8^+$ T cells recognize short peptide fragments 8-11 amino acids in length presented on MHC class I molecules, whereas $CD4^+$ T cells recognize fragments 10-18 amino acids in length presented on MHC class II molecules. Peptides presented on MHC class I molecules are derived from endogenous proteins in the cytosol or from exogenous proteins in a process knows as cross-presentation. MHC class II molecules present peptides derived from exogenous proteins. Recognition of peptide:MHC complexes by T cell receptors results in activation of the T cell if costimulation and cytokines such as type I IFN or IL-12 are simultaneously provided by the DC.

Naïve T cells are present at low frequencies and expand to frequencies as high as 100,000-fold higher after infection or vaccination (40) (**Fig. 1**). This "clonal expansion" phase lasts for 7-10 days and is followed by a "clonal contraction" phase, which occurs after removal of the antigen, 2-4 weeks after vaccination, resulting in elimination of around 90% of effector cells. This is followed by the "maintenance of memory" phase, where a subset of effector T cells survives and is maintained as long-lasting memory T cells.

$CD4^+$ T cells can differentiate into several subsets of effector cells upon activation, such as Th1 or Th2, depending on the cytokines produced at the site of activation (35). In general, Th1 cells promote cell-mediated immunity in response to intracellular infection with viruses or some bacteria, whereas Th2 cells promote humoral immunity in response to extracellular pathogens such as parasites. Th1 cells secrete IFN-γ and TNF-α, which activate macrophages and support $CD8^+$ T cell differentiation as well as maintenance of memory $CD8^+$ T cells. Th2 cells produce cytokines such as IL-4, which stimulates B cells and the production of neutralizing antibodies. More recently, other subsets such as Th17, Th9 and Th22 have been described. In addition, T follicular helper cells are a subset of $CD4^+$ T cells present in germinal centers of lymph nodes that are crucial for development of B cell memory and long-lived plasma cells (41).

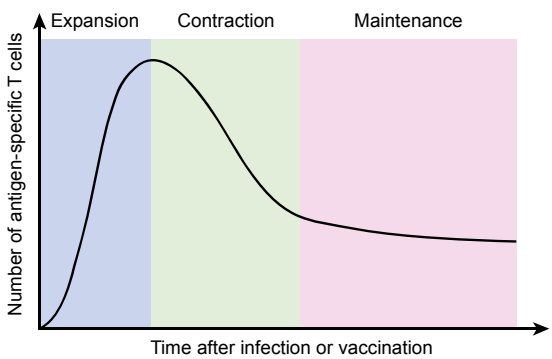

Figure 1. Kinetics of antigen-specific T cell responses after infection or vaccination. Upon exposure to a pathogen or vaccine, T cells undergo massive expansion. After clearance of the antigen, around 90% of effector T cells die. Remaining T cells are maintained as a memory pool.

Activated $CD8^+$ T cells differentiate into cytotoxic T cells that travel to sites of infection and mediate the killing of infected cells. Killing is mainly mediated by exocytosis of granules that contain granzymes and perforin that induce apoptosis of the target cell. In addition, effector $CD8^+$ T cells secrete IFN-γ. $CD8^+$ T cell responses correlate strongly with control of disease progression in viral diseases such as HIV infection. Although a clear correlate has not yet been defined, it appears that the magnitude of the HIV-specific $CD8^+$ T cell response alone is not an effective measure of protective immunity. Instead, quality measures such as the ability of $CD8^+$ T cells to produce multiple cytokines, such as IFN-γ, IL-2 and TNF-α, as well as cytotoxic function are important parameters in correlation with viral control (42–45).

Protective immunity is also based on the phenotype of the memory T cell population that persists after the initial response has contracted. Memory T cells can be categorized into functionally different subpopulations based on phenotypic markers, the most well characterized subsets being central memory T (Tcm) and effector memory T (Tem) cells (46–51). Tcm reside in secondary lymphoid organs and rapidly proliferate upon antigen encounter, whereas Tem migrate through nonlymphoid tissue and exert immediate effector functions against invading pathogens. The optimal type of response depends on the specific pathogen. For example, Tcm are important for control of systemic infections such as lymphocytic choriomeningitis virus (LCMV), which replicates in lymphoid organs (52–54). For chronic infections such as HIV, which replicates in the periphery, early control of viral spread by Tem is important (38, 55). More recently, resident memory T cells have been described as another memory subpopulation. These cells reside in nonlymphoid peripheral tissues such as lung, gut and skin and provide frontline protection against invading pathogens.

Although Tem and Tcm are the most studied subsets of memory T cells, some other classifications have been proposed, such as that based on expression of CD27 and CD43 (56). Antigen-specific $CD8^+$ T cells that are $CD27^+$ $CD43^+$ display a high proliferation rate, but this population disappears over time. Instead, a $CD27^+$ $CD43^-$ population persists, characterized by a high recall capacity and the ability to migrate to mucosal sites. This $CD27^+$ $CD43^-$ T cell phenotype has been associated with increased cytotoxic potential and control of infection with LCMV (57, 58).

1.2.2.2 Antibody responses

When B cells are stimulated with antigen in the presence of Th cells in lymphatic organs, they develop into high-affinity B cells through an affinity maturation process. These cells can then become antibody-producing cells or migrate to the bone marrow to become long-lived plasma cells or memory B cells. Most current vaccines mediate protection by induction of highly specific serum immunoglobulin G (IgG) antibodies that are neutralizing (35, 36, 59).

Antibodies have several effector functions. Neutralizing antibodies bind to and block pathogens, rendering them unable to infect cells. Antibodies can also coat pathogens and promote their ingestion by phagocytes, a process called opsonization. This occurs through the

binding of antibodies to Fc receptors on phagocytes. Natural killer cells also express Fc receptors and can destroy infected cells coated with antibodies in a process called antibody-dependent cellular cytotoxicity (ADCC). Coating of infected cells or pathogens by antibodies also renders them susceptible to lysis mediated by the complement system.

Induction of broadly neutralizing antibodies that block viral entry into host cells will be an important feature in future vaccines against pathogens such as HIV (45). In addition, recent studies have implied an important role for non-neutralizing IgG antibodies that bind to HIV antigens on the surface of HIV-infected cells and recruit innate immune cells that have an Fc receptor such as natural killer cells, thereby mediating ADCC (60).

In both humans and mice, there are four different subclasses of IgG, each contributing to humoral immunity in different ways. In mice, the IgG1 subclass is associated with a Th2-type response, whereas IgG2a is associated with a Th1 profile, including ADCC and complement activation (61, 62).

1.3 NEW VACCINE STRATEGIES

The first vaccines were prepared by attenuation or inactivation of an isolated infectious agent, without much knowledge on the correlates of protective immunity. Several other vaccine approaches have since then emerged, including the use of toxoid/protein, polysaccharide, glycoconjugate, recombinant protein and blood cell infusion vaccines. This has led to the development of vaccines against several different viruses and bacteria (63). Currently licensed vaccines are summarized in **Table 2**.

Although the traditional approaches using live attenuated or inactivated organisms have been extremely effective against many diseases, they may not be feasible for pathogens such as HIV and hepatitis C virus. These pathogens differ from the viruses for which effective vaccines have been developed in that they cause chronic infection and have complex immune

Table 2. Licensed vaccines for human use, by type

Type of vaccine	Licensed vaccines
Live attenuated	Smallpox, rabies, tuberculosis, yellow fever, polio (oral polio vaccine), measles, mumps, rubella, typhoid, varicella, rotavirus, influenza (cold adapted), zoster
Inactivated whole organism	Typhoid, cholera, plague, pertussis, influenza, typhus, polio (inactivated polio vaccine), rabies, Japanese encephalitis, tick-borne encephalitis, hepatitis A
Toxoid/protein	Diphteria, tetanus, acellular pertussis, anthrax, influenza subunit
Polysaccharide	Pneumococcus, meningococcus, *Haemophilus influenza*e B, typhoid
Glycoconjugate	*Haemophilus* influenzae B, pneumococcus, meningococcus
Recombinant	Hepatitis B, human papillomavirus, hepatitis E, cholera toxin B, meningococcus
Blood cell infusion	Prostate cancer

Modified from (63).

evasion strategies as well as high genetic variability and mutation rates. Vaccination with live attenuated forms would be too risky with these pathogens due to the risk of establishing persistent infections. In addition, since infection with the wild type (WT) virus of these pathogens does not result in protective immunity, the immune responses that would be induced using the traditional approaches might not be desirable.

Therefore, new vaccine technologies are being developed with the purpose of eliciting the type of immunity that is believed to be important for protection against the pathogen. With the recent increased knowledge of innate immune signaling and its effect on adaptive immune responses, vaccines can be rationally designed to induce strong T cell and antibody responses. New technologies include using viral or nucleic acid vectors for intracellular delivery of immunogens as well as novel adjuvants that trigger specific PRRs. Additionally, these platforms can be combined into heterologous prime-boost regimens to increase the magnitude and tailor the phenotype of immune responses.

1.3.1 Viral vectored vaccines

Recombinant viral vectored vaccines involve the use of safe and well-studied viruses as vectors with inserted antigen genes. The rationale is that the immune system will react to the vaccine as a natural virus infection due to the nature of the vector, which will then result in a strong response against the antigen. Viral vectors used in vaccines have often been attenuated by genetic deletions or by passaging through cell lines. Infection with viral vectors induces innate immune responses that are translated to potent adaptive immune responses. One potential limitation with viral vectors is the development of antivector immunity that can interfere with the effect of immunization. Antivector immunity can either be a result of previous natural infection with the virus that the vector is based on, or it can have developed from prior immunization with the same vector.

Numerous different viruses have been developed for use as vaccine vectors. Some of these vectors and their attributes are listed in **Table 3**. The viruses that have been most studied in preclinical and clinical studies as vectors are poxviruses and adenoviruses. Both of these vectors were used in this thesis and will be discussed below. Alphavirus vectors were a main focus of this thesis and will be covered in section 1.4.

1.3.1.1 Poxvirus vectors

Poxviruses are dsDNA viruses that hold several advantages for use as vaccine vectors and have been widely tested in preclinical and clinical studies over the past two decades (64, 65). First, safety in humans has been tested extensively due to the mass vaccination against smallpox. Since smallpox vaccination ceased in the 1970s after eradication of smallpox was declared, prevalence of pre-existing antivector immunity is low in the population born after this time. In addition, clinical studies have shown that antivector immunity does not reduce immune responses elicited by poxvirus vectors (66). Poxvirus vectors can carry large gene inserts (up to 25 kb of foreign DNA), and as replication occurs in the cytoplasm, there is no risk of integration of viral DNA into the host genome.

Table 3. Examples of viral vaccine vectors

Viral vector	Advantages	Limitations
Poxvirus	Known clinical safety (MVA) Large insert size No integration	Possible pre-existing immunity
Adenovirus	Infects dividing and nondividing cells, including DCs Many strains available	Pre-existing immunity (rAd5)
Alphavirus	High expression No pre-existing immunity No integration	Safety concerns (VEE)
Adeno-associated virus	Physically stable, potential for oral use Non-pathogenic	Possible integration Limited insert size
Flavivirus	Known clinical safety Possibility of chimeric viruses No integration	Pre-existing immunity
Herpesvirus	Broad tropism	Pre-existing immunity Safety concerns
Measles virus	Mucosal delivery possible Infects DCs and macrophages No integration	Pre-existing immunity
Vesicular stomatitis virus	High expression Mucosal delivery possible No pre-existing immunity No integration	Potentially neurovirulent

One of the most studied poxvirus vectors is modified vaccinia Ankara (MVA). MVA was obtained by passaging vaccinia virus in primary chicken embryo fibroblasts more than 570 times. Through this process, MVA was highly attenuated and lost about 30 kb of DNA in deletions, mostly of genes encoding proteins with immunomodulatory properties that counteract host immune responses (67, 68). Thus, MVA has limited replication in host cells, and no infectious particles are formed after infection. In the 1970s, MVA was tested as a smallpox vaccine in >120,000 humans and was demonstrated to have a high safety profile (69).

MVA induces a type I IFN response through multiple innate signaling pathways, including TLR2, TLR6, MDA5, NLR inflammasome formation and the newly discovered DNA sensor cGAS (70, 71). In preclinical and clinical studies, MVA has been shown to induce $CD4^+$ and $CD8^+$ T cell responses. A veterinary vaccine using MVA to deliver a rabies antigen has been licensed for use in wild animals, and MVA is currently in clinical trials for use in human vaccines against HIV, tuberculosis and malaria (64, 72–75).

Other poxvirus vectors that are being evaluated in clinical trials include attenuated New York vaccinia (NYVAC) and canarypox virus (ALVAC). NYVAC was derived from the Copenhagen vaccinia strain by deletion of 18 open reading frames (ORFs) involved in

virulence and host range. Clinical trials have assessed NYVAC as an HIV vaccine and shown that it especially works well as a boosting agent after a DNA vaccine prime, resulting in induction of polyfunctional T cell responses (76). ALVAC was derived from a canarypox virus that had been passaged 200 times in chicken embryo fibroblasts, and was the priming component of the recent phase III RV144 trial, which for the first time showed modest but transient protective efficacy of an HIV vaccine (77).

Since poxvirus vectors have a restrictive replication capacity and have thus far shown limited efficacy in clinical trials, new poxvirus vector variants are being developed with the purpose of enhancing their immunogenicity (78). For example, expression of cytokines such as IL-12 or IFN-γ from MVA induces augmented cellular immune responses against antigen expressed from co-immunized MVA (79). Another approach that is being evaluated is deletion of immunomodulatory genes still present in the poxvirus vector that can interfere with induction of immune responses. For instance, deletion of a gene encoding a TLR antagonist (*A46R*) in NYVAC resulted in increased magnitudes of $CD4^+$ and $CD8^+$ T cell responses against HIV antigens (80). Also, an MVA vector with deletions in genes involved in inhibition of type I IFN signaling (*C6L* and *K7R*) similarly induced enhanced HIV-specific T cell responses (81). An MVA vector with deletions in *A46R*, *C6L* and *K7R* was used to create an MVA-vectored Chikungunya virus (CHIKV) vaccine candidate that induced protection in mice after only one immunization (82). Administration of this vaccine candidate after a DNA replicon prime resulted in massive expansion of CHIKV-specific T cell responses (83). In **paper II**, we investigate the phenotype of these responses.

A different strategy is to insert optimized antigens into the poxvirus vector rather than modifying the vector backbone itself. For example, NYVAC was constructed to express optimized HIV antigens. In one construct, Env was expressed and secreted as a trimer, while another construct expressed a Gag-Pol-Nef of which Gag induced formation of virus-like particles in infected cells (84). These constructs induced enhanced innate immune responses in human cells and antigen-specific immune responses in mice.

1.3.1.2 Adenovirus vectors

Adenovirus is a well-characterized dsDNA virus with several features that make it an attractive vector. It can infect many nondividing and dividing cell types including DCs, has a high safety profile and exhibits high transgene expression (85, 86). Adenoviruses induce type I IFNs via TLR9 and cytoplasmic PRRs including cGAS (87, 88), resulting in strong T cell responses (85, 89, 90). Several strains have been used as vectors in preclinical and clinical studies, with recombinant human adenovirus (rAd) serotype 5 (rAd5) being the most widely studied strain. One limitation with the use of adenoviruses is the prevalence of pre-existing immunity due to natural adenovirus infection. For example, neutralizing antibodies against rAd5 can be detected in 60-70% of people in Europe and the USA, and in 90% of people in various regions of Africa (91, 92).

rAd5 was tested in humans in the STEP study, a proof-of-concept HIV vaccine clinical trial in phase IIb with the purpose of inducing cell-mediated immune responses for prevention of HIV-1 infection. The study compared rAd5 encoding HIV-1 clade B *gag*/*pol*/*nef* with placebo. The trial was stopped in 2007 due to lack of efficacy. Posthoc analyses have revealed that vaccinated individuals who were seropositive for rAd5 prior to enrollment in the study in fact had a higher HIV acquisition rate compared to the placebo group during the first 18 months after vaccination (93, 94). The mechanisms responsible for this increased risk of infection are still unclear, although it has been suggested that it was due to activation of rAd5-specific memory $CD4^+$ T cells after vaccination, which would render these cells susceptible to HIV infection (95).

To circumvent problems with pre-existing vector immunity, novel vectors have been derived from less common human adenovirus serotypes such rAd25 and rAd35, or from adenoviruses that normally infect chimpanzees, for example chimpanzee adenovirus (ChAd) serotype 63 (ChAd63) (74, 92, 96–99). These are being assessed in both preclinical and clinical studies against diseases such as HIV and malaria. In **paper I**, we use ChAd63 as a booster after a DNA replicon prime.

The rAd vectors described above are replication-deficient due to a deletion of the E1 gene. Although this increases the safety of these vectors, it also renders them unable to amplify the transgene and thereby possibly less immunogenic. As a strategy to increase immunogenicity without compromising safety, a "single cycle" rAd6 vector was generated with an intact E1 gene, but with a deletion in an Ad capsid gene (100). Upon infection, this virus is able to amplify the transgene to a higher degree than replication-deficient rAds, but unable to form infectious progeny virions. In rhesus macaques, immunization with single cycle rAd6 induced enhanced antigen-specific immune responses compared to replication-deficient rAd6 (101).

1.3.2 DNA vaccines

In the early 1990s, it was demonstrated for the first time that DNA plasmids encoding antigen derived from a pathogen could induce antigen-specific antibody and T cell responses (102–104). Since then, DNA vaccines have been tested extensively in preclinical and clinical studies for various diseases including HIV, hepatitis C and malaria (105). Three DNA vaccines have been licensed for veterinary use: a West Nile virus vaccine for horses, an infectious hematopoietic necrosis virus vaccine for salmon, and a therapeutic melanoma vaccine for dogs, thus demonstrating that DNA vaccines can elicit protective immunity, also in larger animals.

DNA vaccines have several advantages. They are relatively simple and quick to manufacture and are stable at room temperature, which makes the requirement for a cold chain less crucial than for many other vaccine platforms. Also, antivector immune responses are not induced, thus allowing DNA vaccines to be administered several times without compromising immune responses. Since DNA vaccines do not contain any infectious components, they are

associated with a high safety profile. Theoretical concerns with DNA vaccines, however, include the risk of integration into the host genome and induction of anti-DNA antibodies that could lead to autoimmune disease (106).

DNA vaccines are bacterial plasmids that carry the gene(s) encoding the antigen(s) of interest under control of a eukaryotic promoter. The modes of action of DNA vaccine-induced immune responses are not completely understood. After delivery into muscle or dermis, DNA vaccines are taken up by host cells that then produce the antigen(s), process it and present it on MHC class I molecules. In the case of intramuscular (i.m.) delivery, it is likely that myocytes as well as APCs such as DCs are transfected (107, 108). The dermis is rich in APCs including DCs and Langerhans cells, and it is likely that APCs are also directly transfected during intradermal (i.d.) delivery of DNA vaccines. In addition, APCs are constantly sampling the environment, and may through endocytosis take up antigen that has been secreted or released due to apoptosis. These antigens are then presented on MHC class II molecules, or on MHC class I molecules through cross-presentation. APCs will then travel to draining lymph nodes and activate antigen-specific B cells, $CD4^+$ and $CD8^+$ T cells.

Since the DNA plasmid used in DNA vaccines is bacterial in origin, it stimulates innate immune responses through PRRs that contribute to its ability to stimulate adaptive immune responses. The presence of CpG motifs leads to signaling through TLR9 in endosomes and induction of type I IFNs, although this signaling pathway does not appear to be crucial for induction of immune responses by DNA vaccines (109–111). Instead, the immunostimulatory properties of DNA vaccines leading to induction of type I IFNs and adaptive immunity are dependent on cytosolic DNA-sensing pathways (23, 112).

DNA vaccines have been shown to induce robust B and T cell responses in multiple preclinical studies. In humans, DNA vaccines have been demonstrated to be safe and tolerated in thousands of volunteers; however, immune responses have generally been low and disappointing. Therefore, attempts have been made in increasing the immunogenicity of DNA vaccines using a variety of approaches, such as vector optimization. For example, alphavirus replicons can be used to increase the immunostimulatory properties of the vector backbone. This approach will be described in section 1.4.

1.3.2.1 Delivery with in vivo electroporation (EP)

The development of improved delivery methods has resulted in significant improvement of the efficacy of DNA vaccines. One such approach that has received much attention within the DNA vaccine field is *in vivo* electroporation (EP). EP involves the application of short electrical pulses, the duration of which are in the milliseconds time scale, that form an electrical field within the tissue and induce transient and reversible permeabilization of the cell membrane. When EP is applied to the tissue immediately after injection of the DNA vaccine, it allows for increased cellular uptake of the DNA vaccine, which is then trapped in the cells after cells have resealed their membranes, a process that occurs in the seconds to

minutes time scale. In addition to increased transfection rates, EP causes local inflammation and recruitment of APCs to the site of injection (113, 114).

Much of the earliest work on *in vivo* EP for delivery of DNA vaccines focused on i.m. delivery; however, more recently there has been a shift towards i.d. delivery. Vaccination in the skin has several advantages. First, the skin is an immunocompetent organ, populated with various immune cells, including many Langerhans cells and dermal DCs (115). Also, i.d. EP is less invasive, as the needles only penetrate 2 mm into the skin, compared to 20 mm for i.m. EP (116, 117). I.d. EP is much less painful than i.m. EP, and any residual pain can be controlled by a simple application of topical anesthetics (118, 119). Both i.m. and i.d. EP have been shown to augment immune responses to DNA vaccines in both smaller animals such as mice as well as larger animals such as nonhuman primates (96, 120–122). The delivery of a plasmid encoding growth hormone-releasing hormone with EP has been licensed in Australia for use in swine (105). In humans, safety and tolerability of EP have been demonstrated in clinical trials (123–126).

1.3.3 Adjuvants

The term “adjuvant” stems from the latin *adjuvare*, meaning “to help”, and is defined as compounds that enhance adaptive immune responses against a co-administered antigen. The earliest developed vaccines did not require co-administration with an adjuvant, as they were based on live attenuated viruses that due to their origin possess intrinsic immune-potentiating properties. This approach is, however, associated with a too high safety risk for vaccines against some pathogens due to the risk of reversion to a pathogenic variant. Also, it may be preferable to target immune responses against specific antigens rather than the entire pathogen. In these cases, vaccination with purified subunit antigen is a more favorable approach. Protein antigens are in themselves not very immunogenic as they are highly purified and do not stimulate innate immunity. They therefore often require administration with an adjuvant to induce antigen-specific immune responses. The use of adjuvants has the additional benefit that they can decrease the antigen dose required, thereby lowering production costs.

Adjuvants have been used in vaccines for almost a century, yet very few adjuvants are licensed for use in humans. This is largely because there has been a lack of comprehension of the mechanisms of action of adjuvants and efficacious vaccines. Currently licensed adjuvants include alum (aluminium salts), oil-in-water emulsions (MF59, AS03 and AF03), virosomes and AS04 (monophosphoryl lipid A (MPL) with alum) (127, 128). Recently, there has been an increased understanding of innate immunity and PRRs, and it has become apparent that one of the most successful licensed vaccines, the live attenuated yellow fever vaccine, stimulates DCs through multiple PRRs and induces type I IFNs (59, 129, 130). This has led to a rise in development of novel adjuvants designed to stimulate innate immunity, and in particular TLRs. Several different TLR ligands have been tested as adjuvants in humans, and recently the TLR4 ligand MPL, a non-toxic derivative of lipopolysaccharide (LPS), was licensed in Europe and the USA for use in a human papillomavirus vaccine (127).

Several chemically synthesized TLR4 agonists are being developed that are more receptor-specific with less side effects than MPL, which is manufactured by isolation from a gram-negative bacterium. For example, Glucopyranosyl Lipid Adjuvant (GLA) has been demonstrated to be more potent than MPL in stimulating innate immune responses (131). GLA promotes humoral and cellular immune responses towards co-administered protein antigen derived from influenza or HIV in preclinical models and is currently being evaluated in clinical trials (128, 132–135).

Other PRR ligands are also being assessed as potential adjuvants. For example, the bacterial molecule flagellin binds to TLR5 and NLRC4 and is being evaluated for use in an influenza vaccine (136–140). Flagellin has also been inserted into viral vectors based on alphavirus, adenovirus, paramyxovirus or vesicular stomatitis virus, resulting in enhanced cytokine expression by DCs and immune responses to co-administered antigen (141–145). Consistently, agonists of different TLRs can synergize in induction of immune responses (146–150). For example, it was demonstrated in mice that synthetic nanoparticles containing antigen together with TLR4 and TLR7 agonists induces synergistic increases in antigen-specific neutralizing antibodies compared to particles containing the antigen with only one of the TLR ligands (148).

1.3.4 Heterologous prime-boost vaccination

Multiple immunizations are often required for a vaccine to be successful. In currently licensed vaccines such as the tetanus-diphteria or hepatitis B vaccines, the same vaccine is given multiple times in homologous prime-boost vaccination. It has been demonstrated that different types of vaccines that contain the same antigen can be combined into heterologous prime-boost regimens, and that this often results in antigen-specific immune responses that are much stronger than those obtained by homologous prime-boost immunization. This approach also circumvents potential problems with antivector immunity that could build up by repeatedly immunizing with the same vector. Weakly immunogenic vaccine modalities such as DNA vaccines are often used for priming specific responses that are greatly expanded by more complex modalities such as virus vectors.

Heterologous prime-boost immunization was initially demonstrated in a malaria mouse model that showed that an influenza virus vector followed by a vaccinia virus vector boost induced protective immunity dependent on $CD8^+$ T cells (151). Reversing the order in which the modalities were given failed to induce protection. In another study, it was demonstrated that priming with DNA followed by an MVA boost induced cellular immune responses that were greater than those obtained by immunizing with only one of the vaccine modalities or reversing the order in which they were given (152). Various preclinical and clinical studies have been carried out using DNA as a prime followed by a poxvirus or adenovirus boost for vaccines against pathogens such as HIV, tuberculosis and malaria (64, 73–76, 96, 153–156).

The immunological mechanisms for the increased responses observed in heterologous prime-boost immunization are not well understood, although one explanation may be that this

approach helps to focus the immune response on the antigen (72, 157, 158). The DNA prime induces a weak yet highly specific response against the antigen of interest. When a greatly immunogenic modality such as a virus vector, encoding the same antigen, is given as a boost, the immune response to the antigen will have an advantage over induction of responses against the vector. This allows for efficient expansion of immune responses focused towards the antigen. A high replicative capacity of the booster appears to be important, as demonstrated in a recent study in which an LCMV booster with an enhanced replicative capacity induced augmented immune responses and improved protective capacity compared to an LCMV strain that is more rapidly controlled by the immune system (159).

Another prime-boost combination that is being assessed is boosting with a recombinant protein antigen after priming with a weakly immunogenic vector. Various studies have employed DNA vaccines for priming antibody responses that are boosted by protein subunit vaccines (160, 161). The most extensive HIV vaccine study using a heterologous protein boost was the phase III trial in Thailand (RV144) involving 16,000 individuals. The vaccine tested in the trial was composed of four initial administrations of ALVAC followed by two boosters with recombinant HIV Env protein and resulted in modest efficacy (77).

Since viral vectors greatly expand T cell responses, and protein antigens are optimal for boosting antibody responses, vaccination regimens containing both of these vaccine modalities may be a strategy for induction of both arms of adaptive immunity (83, 134). Another way of tailoring immune responses is by altering the interval between prime and boost. Although the ideal time interval depends on the vaccine modality, in general responses are optimized by administering the booster only after the primary response has contracted (99, 162–164).

1.4 ALPHAVIRUS REPLICONS IN VACCINATION

The *Alphavirus* genus belongs to the *Togaviridae* family and is a group of enveloped viruses containing a positive-sense ssRNA genome. Thirty different alphaviruses have been identified, including Semliki Forest virus (SFV), Sindbis virus (SIN), Venezuelan equine encephalitis virus (VEE) and Chikungunya virus (CHIKV) (165). SFV was first isolated in 1942 from mosquitoes captured from the Semliki Forest in Uganda. SFV generally does not cause disease in humans. In the rare cases where human infection has been observed, SFV caused only mild symptoms such as fever, rash and headache. In mice, however, SFV is highly virulent and causes encephalitis (166). CHIKV has recently received much attention due to its reemergence and spread in Africa, Asia, Europe and the Americas. CHIKV is mosquito-borne and causes severe arthralgic disease in humans which in rare cases can be fatal (167). Currently, no vaccine is available against CHIKV, although several vaccine candidates are in preclinical development and a few in clinical trials (82, 83, 168–171).

1.4.1 Genome and replication

The alphavirus genome has a 5’ cap structure and is polyadenylated at the 3’ terminus (**Fig. 2**). It is approximately 11.5 kb in length and contains two ORFs. The first ORF comprises the

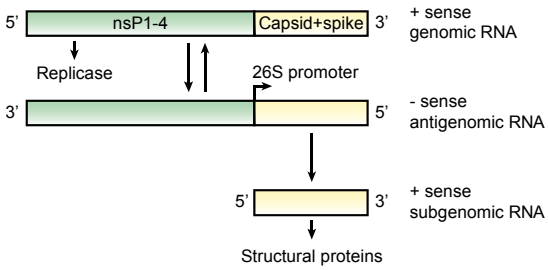

Figure 2. Replication of the alphavirus genome. The alphavirus replicase is translated from genomic RNA. The replicase then drives genome replication and transcription of subgenomic RNA, from which the structural proteins are translated.

5' two-thirds of the genome and encodes the four non-structural proteins (nsPs) 1-4. These proteins are translated as a polyprotein that self-cleaves into separate proteins, which assemble into a replicase complex. The replicase then directs replication and amplification of the viral genome in the infected cell cytoplasm. Later in the infection cycle, the replicase drives transcription of subgenomic RNA containing the second ORF from a 26S promoter on negative-stranded RNA formed during RNA replication. The structural proteins of the virus are encoded in the second ORF. These include the capsid protein, which interacts with the RNA genome and forms the icosahedral nucleocapsid, and the glycoproteins E3, E2, 6K and E1, which are processed into the virion spikes.

During infection, translation of host mRNA is shut off so that only viral proteins are produced by the cell. In cells infected with SFV, this is a result of the cellular stress response, leading to phosphorylation of eukaryotic translation initiation factor 2α and the transient formation of stress granules (172). Although three viral RNA species are formed during infection, only the genome is packaged into progeny virions. This is due to an RNA sequence that functions as a packaging signal and is required for packaging into virus particles. The packaging signal is located in the genes encoding nsP, and specifically in nsP2 for SFV (173). New virions are released from the host cell by budding and can infect new cells by attaching to the host cell membrane followed by receptor-mediated endocytosis.

1.4.2 Alphavirus infection and host response

Alphaviruses have a broad host range, infecting a wide variety of cell types from both insects such as mosquitoes and vertebrates including birds and mammals (165, 174). The primary mode of transmission of alphaviruses between vertebrates is by mosquito bites. The receptor that alphaviruses utilize for entry into cells is still unknown for most alphaviruses, including SFV. After subcutaneous entry, alphaviruses either infect skeletal muscle (e.g. SFV) or Langerhans cells directly (e.g. VEE), although it is not entirely clear which cells are infected. Virus is subsequently transported to draining lymph nodes, either in infected Langerhans cells or DCs, or as free virus. The virus then further replicates in the draining lymph nodes, leading to systemic infection (175, 176).

Alphaviruses are detected by the host through multiple PRRs including the endosomal receptors TLR3, TLR7 and TLR8, and the cytoplasmic receptors MDA5 and protein kinase R (177–181). The signaling through PRRs leads to induction of a strong type I IFN response,

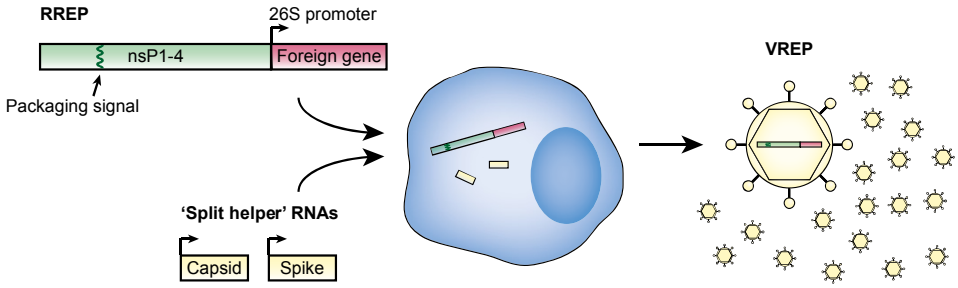

Figure 3. Packaging of VREP. Replicon RNA (RREP) and helper RNAs encoding the alphavirus structural proteins are co-electroporated into cells, resulting in the production of viral particles containing RREP (VREP). Since VREP does not contain the genes encoding the structural proteins, infection with VREP does not result in production of progeny virus.

apoptosis and thereby cross-priming of antigen epitopes on MHC class I molecules (182–185). Infection with alphaviruses results in induction of both cellular and humoral immune responses.

1.4.3 Alphavirus replicons

Expression vectors have been generated from alphaviruses by replacing the genes encoding the alphavirus structural genes by a transgene encoding a gene of interest. Due to the presence of the genes encoding the alphavirus nsPs, the resulting RNA is self-replicating and is termed a replicon (171). Since the genes encoding the structural proteins are deleted, no new virions are formed after a replicon is introduced into a host cell. Alphavirus replicons have been constructed based on SFV (186), SIN (187) and VEE (188). These can be delivered either packaged into viral particles (VREP), or as naked DNA (DREP) or RNA (RREP). In this thesis, VREP and DREP based on SFV were studied.

For packaging of VREP, the replicon RNA is transfected into a cell line together with helper vectors that supply the alphavirus structural proteins in *trans* (**Fig. 3**). To minimize the risk of recombination that could lead to the formation of replication-competent viruses, the spike and capsid genes are placed on two separate helper constructs in the so-called 'split-helper' system (189). After transfection, helper RNAs are replicated by the viral replicase from the 26S promoter. Due to the lack of packaging signal, they are not packaged into the VREP particles. Thus, VREP particles contain only the replicon RNA, but are otherwise indistinguishable from WT viral particles. The RNA in VREP particles is identical to RREP, and is produced by *in vitro* transcription of a recombinant alphavirus cDNA with an SP6 or T7 promoter (SP6 in the constructs used in this thesis). DREP is obtained by swapping this promoter with a eukaryotic promoter such as the cytomegalovirus promoter.

When the RNA of VREP or RREP enters the cytoplasm of the host cell through infection or transfection, the viral replicase is translated (**Fig. 4**). Like in natural infection with alphaviruses, the replicase drives replication and amplification of replicon RNA as well as

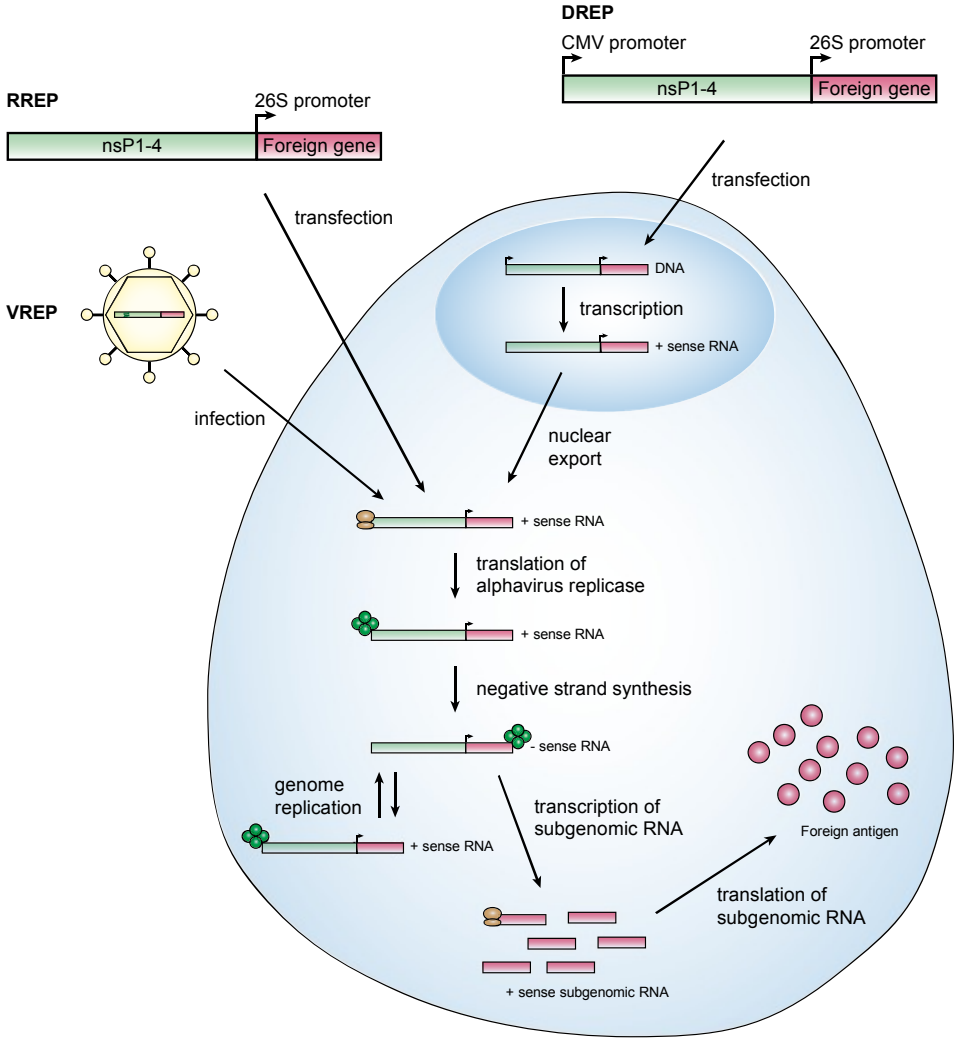

Figure 4. Cellular processing of alphavirus replicons. Alphavirus replicons can be delivered packaged into viral particles (VREP), or as naked RNA (RREP) or DNA (DREP). After transfection, DREP is transcribed into RNA that is identical to RREP and the RNA of VREP. From this RNA, the alphavirus replicase is translated, which then drives amplification of replicon RNA as well as transcription of subgenomic RNA encoding an antigen of interest. From this RNA, large amounts of the foreign antigen is produced. CMV, cytomegalovirus.

transcription of subgenomic RNA, from which the antigen of interest can be translated. When cells are transfected with DREP, the DNA is first transported into the cell nucleus, where it is transcribed into replicon RNA that is then transported to the cytoplasm. The replicon RNA transcribed from DREP is identical to the RNA of VREP and RREP, and thus the amplification cycles are identical for the three platforms once RNA has been transcribed from DREP and has been transported to the cytoplasm.

1.4.3.1 Translational enhancer

The 5' end of the capsid gene of SFV and SIN, but not VEE and CHIKV, contains a translational enhancer (190–192). In SFV, the enhancer is present in the RNA encoding the first 34 amino acids of the capsid. Placing these residues upstream of the transgene encoding the antigen of interest in the replicon RNA increases transgene expression eight-fold (192). In order to obtain translation of antigen that is not attached to this capsid fragment, a 17 amino acid sequence of the 2A autoprotease of foot-and-mouth disease virus is inserted in frame directly after the capsid translational enhancer. During translation, the 2A nascent peptide modifies the activity of the ribosome to carry out ribosomal 'skipping' (193, 194). In this process, the ester linkage between tRNA and the C-terminal amino acid of 2A is hydrolyzed, resulting in release of the nascent capsid-2A peptide from the ribosome. The ribosome then continues translating the downstream sequence, resulting in production of a peptide that is not attached to the capsid-2A residues.

1.4.4 Applications in vaccines

The intrinsic immunostimulatory properties of alphavirus replicons as well as their ability to stimulate cellular and humoral immune responses make them ideal for use as vaccines. The amplification cycle of replicon RNA in host cells mimics viral infection with production of ssRNA and dsRNA intermediates that stimulate multiple PRRs as described above, leading to induction of type I IFNs, apoptosis and thereby promotion of cross-priming (182–185). DREP, being a DNA vaccine, additionally stimulates TLR9 and cytosolic DNA sensors (23, 109–112). The resulting type I IFN response has a dual effect, since type I IFNs on the one hand promote immune responses, but on the other hand can suppress expression of the transgene and thereby actually limit antigen-specific responses (96, 179).

Additional features that make alphavirus replicons attractive vaccine platforms include their broad host range and high levels of transgene expression. Also, antivector immunity is not prevalent, and although antibodies directed at the vector are induced after immunization, they do not interfere with the immune response to the vectored immunogen in cases where the replicon is administered multiple times (188, 195–197). Induction of apoptosis by the replicon reduces the theoretical risk of integration of the viral genome into the host genome. Since the RNA of RREP and VREP replicates in the cytoplasm, this further eliminates the risk of integration. Replicons are associated with a high safety profile with a minimal risk of causing disease. Moreover, the replicons used in this thesis were based on SFV, which is associated with only mild human disease.

1.4.4.1 Viral replicon (VREP)

The VREP vector has been shown to induce robust antigen-specific immune responses of both the cellular and humoral arms and has been tested in both preclinical and clinical studies (171). The majority of vaccine studies using alphavirus replicons have focused on the VREP platform, and thus most data on alphavirus replicon vaccines stems from these studies. VREP has been tested in multiple animal models including mice and non-human primates for

diseases such as HIV, influenza and dengue fever (98, 195, 197–199). In **paper II**, we characterize the kinetics and phenotype of the antigen-specific $CD8^+$ T cell responses induced by the VREP vector. VREP has also been tested together with other vectors in heterologous prime-boost studies. For example, VREP was shown to prime HIV-specific T cells that are efficiently boosted using an MVA vector (200). In humans, VREP has been demonstrated to be safe, but immunological data is still limited (171).

Due to its strong stimulation of innate immunity, VREP can also be used as an adjuvant. When co-administered with protein antigen, VREP potentiates antigen-specific cellular and humoral immune responses (201–203). The adjuvant effect is dependent on type I IFN signaling (143, 201). In **paper IV**, we show that the adjuvant effect of VREP can be further enhanced by incorporation of the TLR5 and NLRC4 agonist flagellin.

1.4.4.2 DNA replicon (DREP)

As described above, the development of DNA vaccines has been hampered by low immunogenicity in humans and thus the requirement of high doses (105, 119). We and others have demonstrated the use of the DREP vector as a strategy to increase the immunogenicity of DNA vaccines (96, 179, 204–208). This vector carries intrinsic immunostimulatory properties, as described above, and thereby requires lower doses while still inducing stronger antigen-specific cellular immune responses compared to conventional plasmid DNA vectors. The use of EP for delivery of DREP further increases antigen-specific immune responses and allows for an additional dose-sparing effect (96).

DREP can also be given as a prime prior to a heterologous boost that further expands T cell responses. For example, we show in **paper I** in mice and rhesus macaques that DREP is superior to conventional plasmid DNA in priming HIV-specific T cells that are boosted by an MVA or ChAd63 vector encoding the same immunogen (96). In **paper III** we further explore the prime-boost regimen by boosting with both MVA and protein antigen in GLA adjuvant.

DREP is furthermore being evaluated as a vaccine candidate for CHIKV. In this case, DREP contains the whole CHIKV genome but lacks the capsid-encoding sequences. It thus only expresses the envelope membrane proteins of CHIKV and is unable to form infectious virions upon transfection into cells (83). CHIKV-specific T cell and antibody responses primed by DREP are boosted to high levels by protein and MVA boosts, and confer protection against CHIKV in a murine model.

1.4.4.3 RNA replicon (RREP)

Alphavirus replicons can also be delivered as a naked RNA vector, or RREP. This approach has the advantage that the theoretical risk of genome integration is eliminated. Also, gene expression is transient and does not persist. Therapy with RNA is not classified as gene therapy by regulatory authorities, allowing for a more rapid progress of RNA vaccines into clinical trials. The naked RNA vaccine platform has not been studied as extensively as viral

vectors or DNA vaccines due to initial concerns about instability of RNA and difficulties with large-scale manufacturing. With recent development in manufacturing technology and delivery methods, these are no longer viewed as hurdles, which has given the RNA vaccine platform more attention as an alternative to other approaches. Also, RREP appears to be rather resistant to degradation, possibly due to its secondary structure.

I.m. injection with RREP expressing influenza antigens into mice results in strong humoral and cellular immune responses that are protective against challenge (209, 210). These responses are further augmented by improved delivery methods such as EP or formulation in lipid nanoparticles (117, 211–213). RREP formulated in lipid nanoparticles was recently demonstrated to induce potent HIV-specific cellular and humoral immune responses in rhesus macaques (213).

2 AIMS OF THIS THESIS

Detailed understanding of immune responses induced by different vaccine platforms is important for rational vaccine design. The aim of this thesis was to evaluate the use of alphavirus replicons as a vaccine platform with the intent to use this knowledge to optimize this platform for future use in the clinic. Specifically, the aims were defined as follows:

- To further enhance the immunogenicity of the alphavirus replicon by improved delivery methods or incorporation of additional PRR stimulants.
- To evaluate the use of DREP as a prime in heterologous prime-boost regimens prior to a viral vector or protein boost.
- To characterize memory T cell populations induced by alphavirus replicons, and assess how these are affected by varying factors such as timing and dose.

3 RESULTS AND DISCUSSION

In this thesis, we studied antigen-specific $CD8^+$ T cell and antibody responses induced by alphavirus replicons using a murine model, with the purpose of evaluating this platform for use in future vaccines. VREP and DREP constructs used in this thesis were based on SFV. We used HIV antigens that are being assessed in clinical trials in **papers I** and **III** and model antigens in **papers II** and **IV**. Specifically, in **paper I** we used HIVconsv, an HIV immunogen designed to encode 14 of the most conserved regions of the proteome of the four major HIV clades A, B, C and D. We assessed T cell responses against a single immunodominant epitope inserted for the purpose of preclinical evaluation. In **paper III**, we used Env and a Gag-Pol-Nef fusion protein from HIV clade C. Chicken ovalbumin was used as a model antigen in **paper II**, as it has a well-characterized and strong $CD8^+$ T cell epitope. The model antigen β-galactosidase was used in **paper IV**.

Antigen-specific $CD8^+$ T cell responses were enumerated by IFN-γ enzyme-linked immunosorbent spot (ELISpot), a sensitive assay that detects cells that secrete IFN-γ in response to *ex vivo* peptide stimulation. To assess functionality of $CD8^+$ T cells, cells were stained with the intracellular cytokine staining (ICS) method to detect production of IFN-γ, IL-2 and TNF-α in response to peptide stimulation. In **paper III**, we additionally assessed cytotoxic function by detecting mobilization of degranulation marker CD107a in the ICS assay. In **paper II**, we characterized $CD8^+$ T cell responses with pentamer staining, a method that utilizes a complex of five MHC-peptide molecules to detect $CD8^+$ T cells with complementary T cell receptors. In the pentamer assay, we stained for surface markers including CD127, CD62L, CD43 and CD27 to characterize the phenotypes of memory T cells.

Antigen-specific antibody levels were assessed with enzyme-linked immunosorbent assay (ELISA), an assay that measures the amount of antibodies that can bind to the antigen, but not their ability to neutralize. Total antigen-specific IgG responses were assessed, as well as IgG1 and IgG2a isotype responses, as a measure for Th2- and Th1-type responses, respectively.

3.1 IMMUNE RESPONSES INDUCED BY ALPHAVIRUS REPLICONS

3.1.1 $CD8^+$ T cell responses

3.1.1.1 Dose-response

Delivery of DNA vaccines by *in vivo* EP greatly enhances antigen-specific immune responses (116, 119–122). This delivery method had prior to this thesis, however, not been evaluated for delivery of DREP. DREP induces multiple inflammatory signals, and therefore it was not obvious whether this would overshadow the inflammation and enhancement of immune responses potentiated by EP. We tested this in **paper I** by immunizing mice with various doses of DREP or conventional plasmid DNA, delivered using either the i.m., i.d. or i.d. EP

immunization routes. Using IFN-γ ELISpot, we compared the magnitudes of antigen-specific $CD8^+$ T cell responses.

With all immunization routes, substantially lower doses of DREP were required to induce responses equivalent in magnitude as conventional DNA. Delivering DREP with EP resulted in significantly increased responses compared to immunization via the i.m. or i.d. routes without EP. Responses induced by delivery of DREP with EP reached a higher level compared to the responses obtained without EP and were dose-dependent up to 2 μg, where the dose-response curve reached a plateau. In addition to obtaining stronger responses, the use of EP allowed for dose sparing. With the EP delivery route, the dose of DREP could be reduced 125 times to obtain the same magnitude of response induced without EP. Furthermore, a dose of DREP that was 625-fold lower than conventional DNA induced equivalent responses. Doses typically used in DNA vaccine studies in mice range from 25-100 μg. For example, an optimized non-replicon DNA vaccine given with i.m. EP required 35 μg for induction of optimal T cell responses (214). Thus, the results from **paper I** suggest that the dose can be substantially reduced by the use of DREP rather than conventional DNA.

We also showed in **papers I** and **III** that the responses induced by a single immunization with an optimal or suboptimal dose of DREP are boosted to a higher level by two homologous boosts. Similarly, a study in rhesus macaques showed that multiple immunizations with a conventional DNA vaccine results in increased responses, whereas the responses induced by rAd5 were not increased after homologous boosts, probably due to induction of antivector immunity (215). T cells induced by the DNA vaccine were characterized by greater replicative capacity and increased polyfunctionality compared to responses induced by rAd5. In **paper III**, we showed that the proportions of multifunctional cells induced by DREP are greater than those induced by conventional DNA.

3.1.1.2 Kinetics

In **paper II**, we characterized the kinetics of antigen-specific $CD8^+$ T cell responses induced by the VREP and DREP vectors. This knowledge is important for understanding memory T cell formation and can be used to optimize the time interval in prime-boost vaccination. We immunized mice with VREP or DREP constructs and followed the development of antigen-specific $CD8^+$ T cell responses after a single immunization using IFN-γ ELISpot. We observed that responses induced by VREP or DREP had a sharp peak, followed by a rapid contraction.

For mice immunized with VREP, the responses peaked on days 7-8 and had contracted by day 21. Following immunization with DREP, responses peaked after 10 days and had contracted substantially 2 weeks after immunization. The pattern of the $CD8^+$ T cell responses induced by VREP and DREP are similar to those induced by the live attenuated yellow fever virus and smallpox vaccines, two highly successful human vaccines. With these vaccines, responses peak rapidly within 2 weeks and have contracted by 4 weeks after vaccination (216, 217). In contrast, T cell responses induced by rAd5, which was used in the

STEP trial, have a later peak, between days 15 and 25, and do not rapidly contract but rather remain at a high level (85, 92, 99, 218).

When a second homologous replicon immunization was given after 3 weeks, responses peaked to higher levels and more rapidly than after the primary immunization. Responses peaked already 6 days after a second immunization with VREP and 8 days after immunization with DREP. After contraction, the number of $CD8^+$ T cells was maintained at a higher level than after the primary response, in accordance with previous knowledge that contraction after an anamnestic response is less pronounced than after the primary response (219–222).

We also tested different time intervals between prime and boost and observed that it was necessary to wait until the $CD8^+$ T cell response had contracted before administering a booster. Administering a booster during the peak response resulted in a memory response of lower magnitude compared to that obtained when the booster was given 3 weeks or longer after the prime. This was likely due to the necessity of formation of a sufficient memory cell frequency before the response can be boosted. Accordingly, we observed that less than half of the antigen-specific $CD8^+$ T cells had developed a memory phenotype during the peak response, whereas this frequency was close to 90% after contraction. Previous studies have also shown that waiting a longer time between prime and boost enhances the secondary effector response (47, 99, 162, 164). The interval observed with alphavirus replicons was, however, shorter than for rAd vectors, for which an interval of 8 or more weeks between prime and boost is optimal (99, 162). In an influenza vaccine trial, boosting with an inactivated influenza vaccine gave an optimal response when waiting at least 12 weeks after a DNA prime (164).

3.1.1.3 Memory phenotype

In **paper II**, we characterized the memory phenotype of $CD8^+$ T cell responses induced by VREP and DREP. We immunized mice and characterized antigen-specific $CD8^+$ T cell responses with tetramer staining as well as memory marker staining. Tem and Tcm subpopulations were identified based on CD127/CD62L staining (46–49, 223). In addition, CD27/CD43 staining was used to identify $CD27^+$ $CD43^-$ memory T cells known to have a high recall capacity (56–58).

A single immunization with either VREP or DREP induced a memory $CD8^+$ T cell population characterized by both Tem and Tcm. The memory population that developed after a homologous boost consisted of a higher Tem:Tcm ratio, in accordance with previous observations that repeated antigen exposure promotes generation and maintenance of Tem, and that secondary memory T cells are slower to acquire a Tcm phenotype (222, 224). Immunization with increasing doses of VREP resulted in an enhancement of Tem formation, whereas the proportions of Tcm decreased with higher doses. These observations were in line with a model proposing that increased signal strength favors formation of Tem rather than Tcm (46). Vectors based on other nonpersistent viruses such as vaccinia virus also induce

both Tcm and Tem (82). In contrast, low-level persisting viruses such as rAd and CMV induce predominantly Tem and are poor inducers of Tcm (85, 218, 225).

Analyzing the CD27/CD43 phenotype, we saw that the majority of antigen-specific $CD8^+$ T cells had a $CD27^+$ $CD43^+$ phenotype during the acute response after a single immunization with DREP or VREP. Over time, the proportion of this population decreased, while the $CD27^+$ $CD43^-$ cell subpopulation increased, in accordance with previous observations with a Sendai virus model (56). After a second immunization, the proportion of $CD27^+$ $CD43^+$ cells further decreased. In contrast to what we observed with the Tem and Tcm phenotypes, the proportions of $CD27^+$ $CD43^+$ and $CD27^+$ $CD43^-$ cells did not differ by varying the dose of VREP.

As described in the introduction, the type of memory T cells that are optimal for pathogen control will depend on the specific pathogen. Tem are important for control of infections that replicate in the periphery including HIV (38, 55), malaria (226, 227) and vaccinia virus (52), whereas Tcm play an important role against systemic LCMV infection, which replicates in lymphoid organs (52–54), and against a P18 tumor challenge (90). Memory T cells with a $CD27^+$ $CD43^-$ phenotype have been implicated as important for control of Sendai virus, hepatitis C virus and LCMV infection, although this phenotype has not been as extensively studied as the Tem and Tcm subpopulations (56–58). The characterization of memory T cell subpopulations induced by replicons, and knowledge of how these can be altered by varying factors such as dose, can be used to tailor vaccination regimens to induce responses that are optimal for control and/or clearance of a specific pathogen and are therefore important for design of future clinical trials.

3.1.2 Antibody responses

The rationale for the development of DNA vaccines was to induce cellular immunity, and 'first-generation' DNA vaccines were generally poor inducers of antibody responses. More recently, however, optimized DNA vaccines have demonstrated the ability to induce antibody responses after multiple immunizations (105, 214). DREP has also been shown to induce modest levels of antibodies (204, 208). In **paper III**, we assessed antibody responses induced by DREP delivered by i.d. EP. We immunized mice with one, two or three homologous administrations of either a high (10 μg) or low (0.2 μg) dose of DREP. Three weeks after the last immunization, we analyzed total antigen-specific IgG responses. A low but clearly detectable response was induced in all groups except in the group given the low dose once. A single immunization of 10 μg CHIKV DREP delivered by i.d. EP was also recently shown to induce antibodies that protected 4 of 5 mice from a CHIKV challenge (83). Administering CHIKV DREP twice resulted in antibodies that were boosted to a higher level and protected all mice from CHIKV challenge.

3.2 ALPHAVIRUS REPLICONS IN HETEROLOGOUS PRIME-BOOST REGIMENS

DNA vaccines have been shown to be excellent priming agents in heterologous prime-boost vaccine regimens (72–76, 105, 153–155). Results from clinical trials have, however, shown that repeated immunization with high doses of DNA is required for induction of long-lived immune responses. Thus, more work is needed for the DNA vaccine platform to be viable for use in human vaccines. Since we showed in **paper I** that electroporated DREP is superior to conventional DNA vaccines in inducing antigen-specific immune responses, we assessed the outcome of administering DREP by i.d. EP as a prime prior to a heterologous boost. We compared DREP with conventional DNA and investigated the effect of varying dose and number of DREP primes.

3.2.1 DREP compared to conventional DNA vaccine as a prime

In **paper I**, we compared the ability of DREP and conventional DNA to prime antigen-specific $CD8^+$ T cell responses prior to a heterologous virus vector boost. We immunized mice once with DREP or conventional DNA followed by a boost with either MVA or ChAd63. We selected two priming doses for both DREP and conventional DNA: one dose that in the dose-titration experiment described above induced a maximal response (2.5 μg of DREP and 25 μg of conventional DNA), and one dose that induced intermediate T cell frequencies (0.05 μg of DREP and 2.5 μg of conventional DNA). Responses primed by DREP were superior in magnitude to those primed by conventional DNA. This was evident at both 1 and 5 weeks post-boost. MVA and ChAd63 appeared similar as boosting agents, both in terms of frequencies of antigen-specific $CD8^+$ T cells and expression of cytokines. The DREP-MVA regimen was also assessed in a CHIKV vaccine study. Similar to this study, CHIKV DREP or MVA by themselves induced only modest T cell responses, but by administering MVA after a single prime with DREP, T cells were expanded to extremely high levels (83). The MVA used in the CHIKV study had an enhanced immunogenicity due to deletion of immunomodulatory genes (82). In **paper II** we show that the MVA boost mainly favored expansion of Tem cells when given after a CHIKV DREP prime, similarly to previous observations with MVA by itself of given after a conventional DNA vaccine prime (82, 228–230). rAd also induces T cell responses predominantly of the Tem phenotype (85, 218, 225).

We also compared DREP and conventional DNA in their abilities to prime T cell responses in rhesus macaques. We primed animals three times with either 400 μg of DREP or 4 mg of conventional DNA; i.e. we used a 10-fold lower mass, equivalent to a 20-fold lower molar dose, of DREP than conventional DNA. Animals were boosted sequentially with MVA and ChAd63 vaccines. Despite the lower dose of DREP given, frequencies of antigen-specific $CD8^+$ T cells induced by DREP or conventional plasmid DNA were equivalent, as determined by IFN-γ ELISpot using peptide pools as stimuli. Analyzing the responses induced by the different peptide pools, we observed that the breadth of responses were also similar. Macaques primed with DREP consistently displayed higher frequencies of

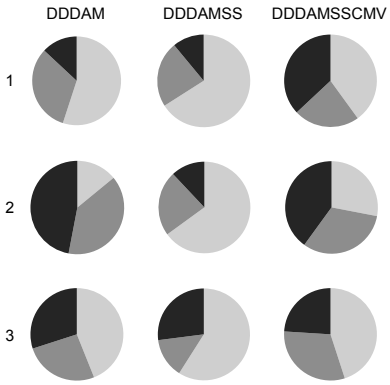

Figure 5. Functional profiles of HIVconsv-specific CD8$^+$ T cells. Three rhesus macaques were immunized with the regimen stated above pie charts. Monkey number id is shown to the left of charts. All vaccine constructs encoded the HIVconsv immunogen. Cells were assayed with ICS for production of IFN-γ, IL-2 and TNF-α. The proportion of antigen-specific CD8$^+$ T cells producing 3 (black), 2 (dark gray) or 1 of these cytokines is illustrated in the pie charts. D, DNA; A, rAd5; M, MVA; S, synthetic long peptides; C, ChAd63; V, VREP. Figure modified from (98).

multifunctional CD4$^+$ and CD8$^+$ T cells than animals primed with conventional DNA, although these differences were not statistically significant.

Multifunctional T cells play a central role for protection against viral disease (43, 44). In another study in rhesus macaques, we assessed the potential of using alphavirus replicons as a boost. We boosted rhesus macaques with VREP encoding the HIVconsv immunogen following immunization with DNA, synthetic long peptides, MVA and ChAd63 (98). The results from this study indicated that polyfunctionality of antigen-specific T cells increased after administration of VREP (**Fig. 5**). Other studies have also indicated increased polyfunctionality with the use of mixed modality vaccine regimens. For example, in a hepatitis C vaccine study, immunizing mice with a DNA-MVA vaccine regimen resulted in broader and more polyfunctional T cell responses compared to an MVA-MVA regimen (228). It would be relevant to address broadness of responses induced by DREP more extensively in future studies, as immune responses against subdominant epitopes will be important for future vaccines against highly variable pathogens such as HIV and hepatitis C virus.

3.2.2 Effect of varying the number of primes with DREP

Preclinical and clinical prime-boost studies most often utilize multiple administrations of DNA due to its weak immunogenicity. As we had demonstrated higher immunogenicity of DREP than conventional DNA vaccines in **paper I**, and in particular when delivered by i.d. EP, we asked in **paper III** what the effect would be of varying the number of DREP primes prior to a heterologous boost, and whether there would be a difference by priming multiple times with different doses. We therefore primed mice either one or three times with either 10 μg or 0.2 μg of DREP encoding antigens from HIV clade C. Mice were then boosted with one of the following: 1) MVA encoding the same antigens, 2) gp140 protein in GLA aqueous formulation (GLA-AF) adjuvant, 3) MVA followed by a second boost with protein/adjuvant, or 4) MVA and protein/adjuvant simultaneously. The rationale was that MVA would boost T cells while protein/adjuvant would boost antibodies. As a shortened vaccine regimen would

be favorable in a clinical setting since it may increase compliance, we tested whether we could administer the two boosts simultaneously without compromising immune responses.

A single prime with a low dose of DREP was sufficient for priming of antigen-specific $CD8^+$ T cells that were expanded by an MVA boost and antibody responses that were boosted by protein antigen. The strongest responses were observed when multiple DREP primes were given. The two doses were, however, equally efficient as priming components. Whereas immunizing with gp140 without a prime induced a Th2-biased response characterized by IgG1 antibodies, mice that were primed with DREP displayed a more balanced Th1/Th2 response with induction of both IgG1 and IgG2a antibodies. Thus, DREP promotes formation of Th1 type responses, in accordance with observations with conventional DNA vaccines and CHIKV DREP (83, 105, 200).

When both MVA and protein were given as boosts, both T cells and antibodies directed at the antigen were induced. Accordingly, a CHIKV DREP prime followed by protein and MVA boost resulted in decreased disease progression compared to mice boosted with only one of the components, although a boost with only protein or MVA appeared to be sufficient for protection against viremia (83). No major differences were seen in **paper III** between the sequential and simultaneous MVA and protein/adjuvant boosts, both on the T cell and antibody responses. Another recent study used the same MVA and protein boosts, but instead after a non-replicon DNA prime. In that study, humoral responses were also similar when MVA and protein were given sequentially or simultaneously (134). T cell responses were, however, greater in magnitude when administering MVA and protein simultaneously. The increased response observed in that study may have been due to the higher dose of GLA-AF used, which could exert immunostimulatory effects that enhanced responses against MVA-encoded immunogens.

Another aspect that could be considered is whether there would be a difference in reversing the order of the sequential MVA-protein regimen. Here we administered MVA followed by protein, which in part mimics the RV144 HIV vaccine trial in which an ALVAC prime was given followed by a protein boost (77). One study showed that priming with gp140 protein formulated in IC31 adjuvant followed by a NYVAC boost induced Env-specific $CD4^+$ and $CD8^+$ T cell responses that were superior to those induced by the reversed order of protein and NYVAC administration (231). In **paper III**, the majority of observed antigen-specific T cells were directed at epitopes within Pol, with only weak responses against Env. This was in part a result of immunodominance that occurred when Env and Gag-Pol-Nef immunogens were given simultaneously encoded by DREP or MVA modalities. Thus, it is plausible that Env-specific responses could be increased by priming with Env, either as protein or encoded by DREP, without the presence of Gag-Pol-Nef, in order to focus immune responses towards Env.

3.3 ENHANCEMENT OF THE REPLICON BY INCORPORATION OF BACTERIAL FLAGELLIN

Ligands of different PRRs may act in synergy to induce greatly augmented immune responses (146–150). In addition, it has been demonstrated that one of the most effective vaccines developed, the live attenuated yellow fever vaccine, stimulates multiple innate signaling pathways (59, 129, 130). We therefore asked in **paper IV** whether we could further enhance the immunogenicity of the replicon with additional PRR stimuli. We hypothesized that this could be achieved by incorporating flagellin, which stimulates cell surface-bound TLR5 and cytosolic NLRC4. Since VREP and flagellin stimulate different PRRs with different downstream pathways, they might act in synergy when co-administered and augment immune responses towards antigens expressed by VREP or co-immunized protein antigen. We therefore constructed VREP encoding FliC flagellin from *Salmonella* Typhimurium (VREP-FliC).

3.3.1 Responses to replicon-encoded antigen

Antigens expressed from VREP have previously been shown to induce antigen-specific antibody responses (201, 232, 233). To evaluate whether these responses could be augmented by the presence of flagellin, we immunized mice with VREP encoding the antigen mixed with either 1) VREP-FliC, 2) soluble FliC and control VREP encoding an irrelevant antigen, or 3) VREP encoding an irrelevant antigen. Three weeks after immunization, serum was assayed for antigen-specific IgG responses. No significant differences were observed between the groups. We additionally assessed antigen-specific $CD8^+$ T cells with IFN-γ ELISpot on day 7 after immunization and observed no differences.

Flagellin has been shown to act as an adjuvant when fused to the protein antigen, and an influenza vaccine based on a flagellin-antigen fusion protein is currently being evaluated in clinical trials (136–140). We therefore investigated whether flagellin might act as an adjuvant if expressed from VREP as a protein fused with the antigen. For this purpose, we produced VREP encoding a FliC-antigen fusion protein and immunized mice with this construct. No enhancement of antibody or $CD8^+$ T cell responses were observed compared to immunizing with VREP encoding the antigen without flagellin. We then considered that it might be necessary for the fusion protein to be secreted from cells in order to obtain an adjuvant effect of flagellin, so we added a signal sequence for secretion on the fusion protein; however, this also had no effect on antigen-specific antibody responses.

3.3.2 Responses to co-administered protein antigen

It has previously been shown that VREP acts as an adjuvant for antigen-specific antibody responses when co-delivered with protein antigen (201, 202). We asked in **paper IV** whether this adjuvant effect could be further enhanced by incorporation of the flagellin gene into VREP. We therefore immunized mice with protein antigen mixed with either a control VREP encoding an irrelevant antigen, or with VREP-FliC. Three weeks after immunization, serum was assayed for antigen-specific IgG responses. Mice immunized with the VREP-FliC

adjuvant displayed a stronger IgG response than mice immunized with VREP not encoding flagellin. Whereas control VREP promoted IgG2a antibodies, VREP-FliC enhanced both IgG1 and IgG2a antibodies, indicating that VREP-FliC has an adjuvant effect on both Th1 and Th2 type responses. Thus, we demonstrated that VREP expressing intracellular flagellin is a more potent adjuvant than VREP alone.

We then asked whether the adjuvant effect of VREP-FliC was dependent on intracellular expression of FliC from the replicon, or whether administering soluble FliC together with VREP not expressing FliC would have a similar adjuvant effect. We immunized mice with protein antigen mixed with soluble FliC and VREP and did not observe an increased adjuvant effect on antigen-specific IgG responses compared to immunizing with soluble FliC protein or VREP on their own.

We also investigated which innate signaling pathways are involved in the adjuvant effect of VREP-FliC. Type I IFN signaling has previously been shown to be crucial for the adjuvant effect of VREP (201). We showed that for VREP-FliC, IgG responses against co-immunized protein antigen were diminished but not abolished in mice lacking type I IFN signaling. This was likely due to the absence of contribution from VREP on inducing innate immunity. Similarly, we observed that the adjuvant effect of VREP-FliC in mice lacking TLR5 was diminished although not abrogated. In these mice, the VREP-FliC adjuvant did exert a stronger IgG response than the VREP adjuvant by itself, indicating that other pathways are also involved in the adjuvant effect of intracellularly expressed flagellin.

For soluble flagellin, it has been reported that the adjuvant activity is codependent on TLR5 and NLRC4, although TLR5 alone was necessary for the adjuvant effect of flagellin administered as a fusion protein with the antigen (234, 235). In addition, flagellin expressed from a vesicular stomatitis virus vector stimulates NLRC4 (144). Thus, it is likely that flagellin expressed intracellularly from VREP signals through NLRC4. Cells infected with VREP-FliC will eventually die from apoptosis due to VREP infection or pyroptosis due to NLRC4 signaling, which would release FliC into extracellular space, where it would be able to access TLR5 on cell surfaces.

The difference in the adjuvant effect exerted by VREP expressing intracellular flagellin compared to VREP and soluble flagellin could be explained by timing and location. Infection of cells with VREP and amplification of replicon RNA is a process that takes several hours. Thus, for VREP encoding flagellin, stimulation of PRRs such as TLR3, TLR7, TLR8 and MDA5 by replicon RNA and TLR5 and NLRC4 by flagellin can occur at the same time and in the same cell. When VREP is instead administered with soluble flagellin, the flagellin is available for PRR stimulation immediately, and thus the effect of VREP and flagellin will not occur simultaneously.

4 CONCLUSIONS AND FUTURE PERSPECTIVES

In this thesis, we characterized immune responses elicited by alphavirus replicons by immunizing mice or rhesus macaques and characterizing antigen-specific T cell and antibody responses. We demonstrated in **paper I** that T cell responses induced by DREP can be further elevated by delivery by i.d. EP. Responses were superior to those induced by a conventional DNA vaccine. We also established that a dose of 2 μg of DREP induced a maximal response. In **paper II** we showed that the kinetics of T cell responses induced by VREP or DREP exhibited a sharp peak followed by rapid contraction. To obtain an optimal effect of a booster, it was necessary to wait 3 weeks after prime until immune responses had contracted before administering a booster. Memory T cells were comprised of both Tem and Tcm subpopulations, and the Tem:Tcm ratio correlated with the dose given. We used the knowledge of optimal doses and timing obtained from **papers I** and **II** to assess DREP as a prime prior to a heterologous boost with MVA and/or protein antigen in **paper III**. DREP potently primed T cells that were expanded by an MVA boost and antibody responses that were boosted by protein antigen. These results in part confirmed our results in **paper I**, where we showed that DREP was superior to conventional plasmid DNA in priming T cells prior to a boost with MVA or ChAd63. Lastly, we took a different approach in **paper IV**, where we used VREP as an adjuvant for potentiating antibody responses against co-immunized protein antigen. We showed that this adjuvant effect could be further enhanced by incorporation of bacterial flagellin, which stimulates innate immune pathways different from those induced by VREP.

Alphavirus replicons can be delivered as VREP, DREP or RREP. Which of these platforms is most attractive for future clinical use? The VREP vector is the alphavirus replicon platform that has been evaluated most extensively; however, several factors make the DREP and RREP approaches perhaps more favorable in a clinical setting. First, the production of VREP involves the use of cell lines, which is a complicated and expensive procedure that may hinder large-scale production. VREP based on SFV for preclinical use is produced in baby hamster kidney cell lines, which are not approved for GMP production. For clinical purposes, VREP would be prepared in Vero or 293 cell lines, which yield lower virus titers. Also, infectivity with viral particles may be decreased by antivector immunity in the host. Preexisting antivector immunity against SFV is not prevalent, and any antivector immune responses induced during immunization does not hinder the response to a homologous booster (188, 195–197). However, for safety reasons it is desirable to avoid induction of any irrelevant immune responses in a vaccine.

We showed in this thesis that DREP is an excellent prime for T cell and antibody responses prior to a heterologous boost. However, RREP has certain benefits over DREP that may make it a more appealing vaccine platform such as the elimination of risk of integration into the host genome. The advances in delivery methods including EP and lipid nanoparticle formulation have shown to work well with RREP, inducing potent immune responses in mice and nonhuman primates (117, 211–213). The RREP platform has only recently gained

increased interest, and therefore data on its immunogenicity and use in prime-boost regimens is still limited. Further testing of RREP in heterologous prime-boost regimens in comparison to DREP would be valuable.

Also, a systematic comparison of immune responses induced by different vaccine modalities including magnitude, breadth, memory phenotype and longevity will be valuable for design of vaccine regimens. Thus far, studies have combined vaccine vectors largely based on availability, and immunogenicity data has been difficult to compare due to differences in dosage, timing and antigens. A head-to-head comparison would be of great value for rational design of vaccine regimens.

In conclusion, we have demonstrated that alphavirus replicons induce strong antigen-specific immune responses of both arms of adaptive immunity. In addition, we have characterized the kinetics and phenotypes of these responses allowing for tailoring of vaccination regimens. The results obtained in this thesis in addition to the past 20 years of preclinical testing of alphavirus replicons strongly support further development of these vectors in clinical trials.

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