Chikungunya virus-like particle vaccine

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This research was conducted under the auspices of the Graduate School of
Production Ecology and Resource Conservation.

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Thesis
submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof. dr. M.J. Kropff,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Monday 24 June 2013
at 1.30 p.m. in the Aula

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Chikungunya virus-like particle vaccine
139 pages

Thesis, Wageningen University, Wageningen, NL (2013)
With references, with summaries in Dutch and English

ISBN 978-94-6173-565-2

Voor mijn ouders

Chapter

General introduction

The chikungunya virus

"It was last May, 25, in the afternoon at 5:00 when I noted while talking with two good friends of mine, a growing pain in my right hand, and the joints of the lower arm, which step by step proceeded upward to the shoulder and then continued onto all my limbs; so much so that at 9:00 that same evening I was already in my bed with a high fever… It has now been three weeks since I was stricken by the illness, and because of that had to stay home for 5 days; but even until today I have continuously pain and stiffness in the joints of both feet, with swelling of both ankles; so much so, that when I get up in the morning, or have set up for a while and start to move again, I cannot do so very well and going up and down stairs is very painful (Bylon, 1780). "

This passage from the logbook of Dr. David Bylon, a 'Staads Chirurgyn' in the City of Batavia 1779, is regarded as the first description of the viral illness nowadays called chikungunya fever (Bylon, 1780). Historically, the characteristic arthralgic disease was termed *knokkelkoorts*, *abu rokab*, *mal de genoux*, *dyenga* and *3-day fever*, most likely all caused by the chikungunya virus (CHIKV) (Carey, 1971). CHIKV is an arthropod-borne (arbo), single-stranded, positive-sense RNA virus (family *Togaviridae*, genus *Alphavirus*). CHIKV is transmitted through the bite of an infected mosquito of the *Aedes spp*, originally the yellow fever mosquito *Ae*. *aegypti*. In recent years, CHIKV switched to an alternative vector, the Asian tiger mosquito *Ae. albopictus*, a vector that has seen a dramatic global expansion in its geographic distribution in the last decade (**Fig. 1-1**). This resulted in the large epidemic on the Rèunion Island in 2005/2006, with an estimated 270 000 cases of CHIKV infection (Renault et al., 2007). In recent years (2004-2011), CHIKV produced the largest epidemic recorded for an *alphavirus*, with an estimated 1.4 to 6 million patients, and imported cases reported in nearly 40 countries including Japan and the USA (**Fig. 1-1**). The first authentic CHIKV infections in Europe (Italy in 2007 and France in 2010) were also seen during this epidemic (Gould et al., 2010).

Chikungunya fever usually develops 2-6 days post the infective mosquito bite and results in 95% of all cases in a severe and sudden onset of high fever (>38.9 °C), which poorly responds to antipyretic medication. Fever is often accompanied by myalgia, headache, fatigue, abrupt febrile illness, maculopapular rash and arthritic disease (Jaffar-Bandjee et al., 2010). The name "chikungunya" is derived from the Makonde language, meaning "that which bends up", referring to the cramped posture of infected individuals, caused by the severe arthralgia (Sourisseau et al., 2007). Although the clinical disease is usually

self-limiting, arthritic symptoms may persist from months, to several years (Borgherini et al., 2008). More severe clinical complications involve encephalopathy, heamorragic fever, neurological failure and even death in patients with underlying medical conditions (Kumar et al., 2012). Recent studies identified macrophages as being key-players in CHIKV pathogenesis. Monocyte-derived macrophages appear to be the main cellular reservoir in the persistent stage of CHIKV infection. The macrophage derived, pro-inflammatory products play important roles in the development of arthritis by CHIKV infection (Chow et al., 2011; Labadie et al., 2010; Lidbury et al., 2008)

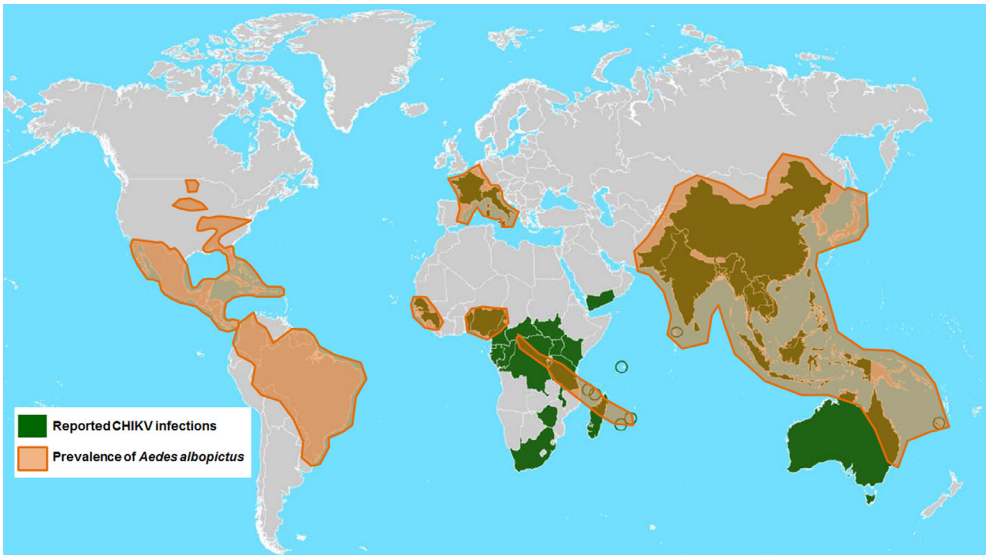

Figure 1-1. Geographical distribution of CHIKV and *Aedes albopictus*.
The green countries indicate countries in which CHIKV infections have been reported in humans. Transparent orange regions indicate regions in which *Aedes albopictus* is resident. Data according to the latest update of the Centers for Disease Control and Prevention (CDC) website (2012).

CHIKV structure and replication

CHIKV is a spherical enveloped virus of ~70 nm in diameter (**Fig. 1-2A**) and has a single-stranded, positive-sense RNA genome of approximately 11,8 kb (Khan et al., 2002b). The RNA is encapsidated in a ~40 nm wide nucleocapsid, which is enveloped by a host-derived lipid bilayer supporting 80 viral trimeric glycoprotein-spikes, which are involved in cell receptor recognition and cell entry via low pH-dependent fusion and endocytosis. The genome contains two open reading frames (ORF) encoding the non-structural polyprotein and the structural polyprotein (**Fig. 1-2B**) (Strauss and Strauss, 1994). The non-structural proteins (nsP1-4) regulate viral RNA replication and are directly translated from the 5'end of the genome. The structural proteins (Capsid or C, envelope proteins E3, E2, 6K, E1) constitute the virion proteins and are translated from a subgenomic mRNA located in the 3'-end of the genome.

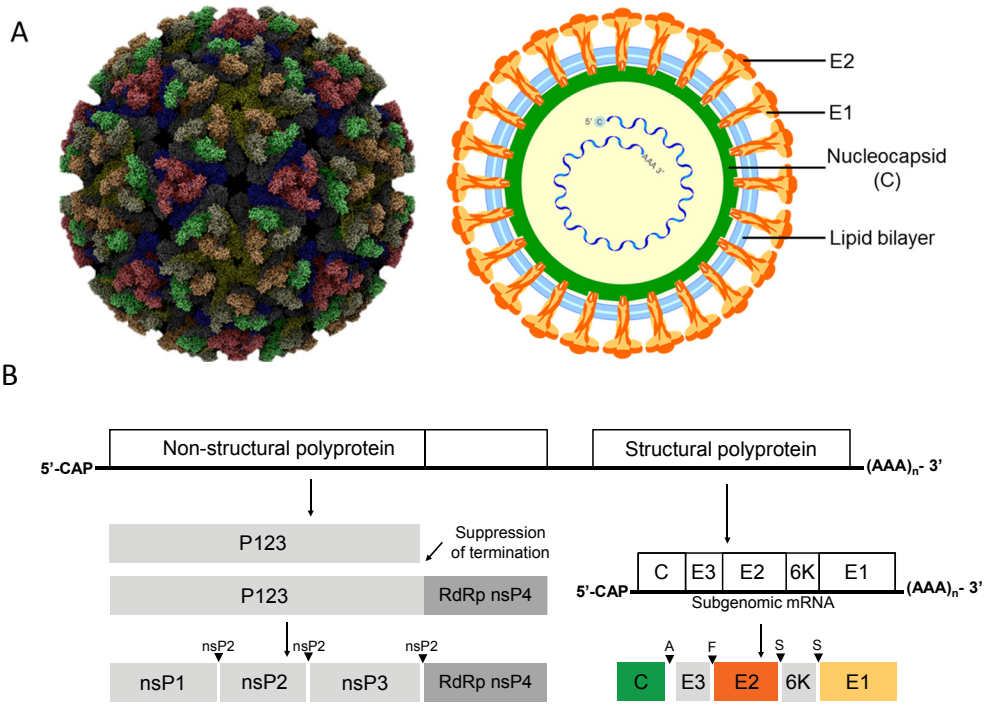

Figure 1-2. CHIKV structure and genome.
A) CHIKV virion (generated from Protein Data Bank data, based on Sindbis EM density, PDB ID: 2XFB) and schematic representation of CHIKV particle section (Metz and Pijlman, 2011). B) Schematic representation of the CHIKV genome, which encodes 2 ORFs. The non-structural polyprotein is directly translated into P123 and P1234, due to suppression of termination of nsP3. P123 and P1234 are processed by the nsP2 protease. The structural polyprotein is translated from a subgenomic mRNA. Capsid is autocatalytically cleaved off (A) from the polyprotein. Host signalases (S) process 6K and E3 is cleaved off from E3 by furin-dependent cleavage (F).

In CHIKV isolates 37997, Asian and some Indian isolates, initial translation of the non-structural region generates two polyproteins P123 and P1234 (**Fig. 2B**). P1234 is produced due to a read-through strategy (due to a leaky stop signal) at the C-terminus of nsP3 (Ding and Schlesinger, 1989). *In cis*-cleavage of P1234, yields P123 and nsP4, the viral NA-dependent RNA-polymerase (RdRp). After or in parallel to the emergence of nsP4, nsP1 is presumably cleaved off from P123, yielding nsP1, P23 and nsP4. The nsP4 functions as a polymerase for both plus- and minus-strand RNA, but minus-strand RNA is only generated in the presence of P123.

Thus nsP1, P23 and nsP4 act together as the minus-strand RNA replication complex. After further processing of P23 into nsP2 and nsP3 by in trans-cleavage by nsP2, the template preference of the replicase is altered to minus-stranded RNA, thereby promoting synthesis of plus-strand and subgenomic RNA. This shut-off mechanism of minus-strand RNA synthesis is tightly regulated by nsP2, since no viral RNA replication is observed with increased P123 processing efficiency and minus-stranded RNA accumulates with inactivated nsP2 (Lemm et al., 1994).

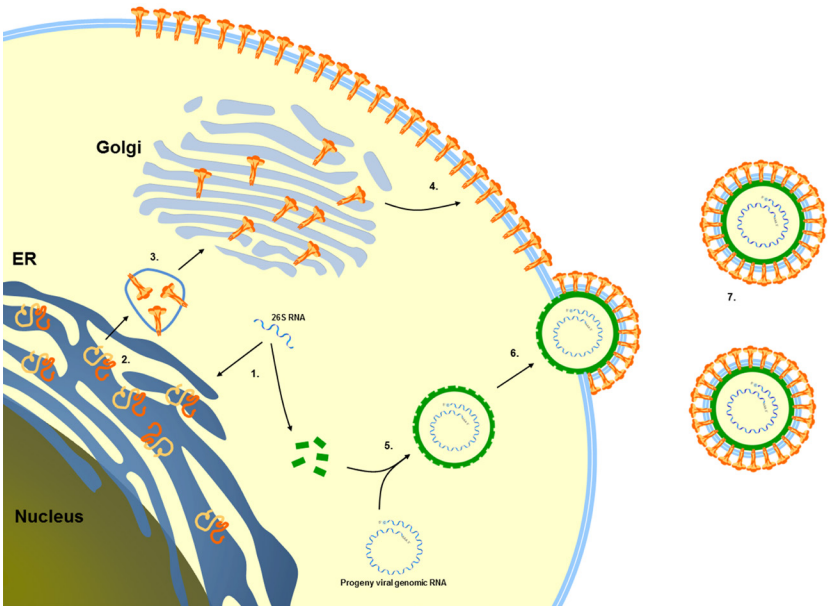

Figure 1-3. Chikungunya virus particle formation.
The viral 26S mRNA encodes the CHIKV structural proteins capsid, E3, E2, 6K and E1 (1). The envelope glycoproteins are translocated to the ER, complex into E1-PE2 heterodimers (2) and are transported to the Golgi (3), where trimeric spike formation and furin-dependent maturation takes place. Trimeric spikes are transported to the plasma membrane and are exposed on the surface of the cell (4). Progeny viral genomic RNA is encapsidated by capsid in the cytoplasm (5), after which the nucleocapsid buds from the cell (6), taking along the trimeric spikes anchored in the lipid bilayer (7).

During translation of the structural polyprotein (**Fig. 1-3**) from a subgenomic viral mRNA, C is autocatalytically cleaved off and the remaining envelope cassette is translocated to the ER by signal sequences in E3 and 6K (**Fig. 1-2B** and **1-4A**). Proteolytic processing by host signalases cleaves 6K at the N-and C-terminal end, yielding E3E2 (precursor E2 or PE2), 6K and E1 (Kuhn, 2007). Following processing, PE2 and E1 form heterodimers, which are assembled into heterotrimers in the early Golgi compartment. Subsequently, E3 is released from PE2 by furin-dependent cleavage and maturation in the trans-Golgi system receiving glycosylation. Furin cleavage is not a prerequisite for virion assembly, but incomplete processing will result in virions with impaired fusion properties (Strauss and Strauss, 1994).

The mature heterotrimers are displayed at the cell-surface as trimeric spikes (**Fig. 1-4B**) and the viral RNA is encapsidated in nucleocapsids composed of 240 copies of C (Weiss et al., 1989). Virion budding is most likely regulated upon the interaction between the intracellular domain of E2 with a single C molecule within the nucleocapsid (Garoff et al., 2004). The nucleocapsid buds out from the cell, taking along the hosts plasma membrane and a total of 80 trimeric spikes (Garoff et al., 2004). The trimeric spikes are essential for cell-receptor recognition (E2) and pH dependent endocytosis (E1). Upon receptor binding, conformational changes in E2 trigger the onset of E1 homotrimer formation, after which the mild acidic conditions in the endosome, induce membrane fusion (Gibbons et al., 2000).

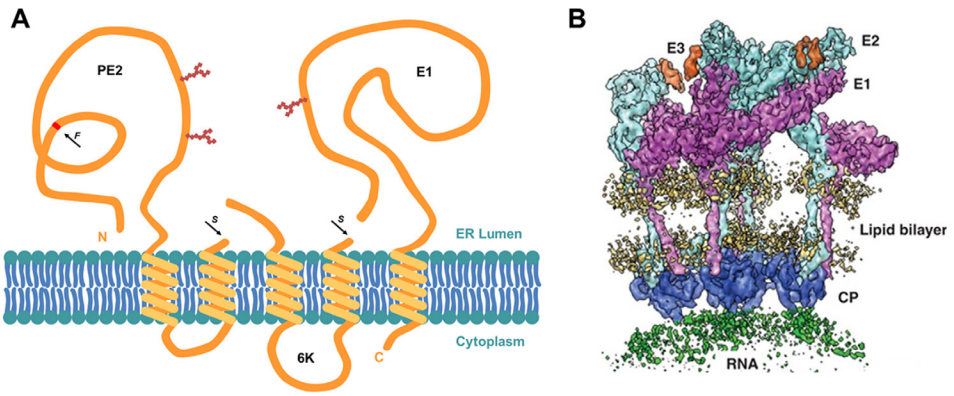

Figure 1-4. CHIKV trimeric spike structure.
A) Schematic representation of ER-membrane organisation of the CHIKV envelope cassette. N- and C-terminal end are indicated. Arrows represent cleavage sites for S (host signalases) and F (furin-like proteases). Blue chains indicate N-glycosylation sites (Metz and Pijlman, 2011). B) Cryo-EM density of an alphavirus trimeric spike, with E3 (orange) incorporated in the spike composed of E2 (cyan), E1 (magenta) and capsid (blue). Viral RNA (green) and the host-derived lipid bilayer (yellow) are also depicted (Zhang et al., 2011).

It would be detrimental for the virus if E1 assembles into homotrimers before the trimeric spikes have reached the cell-surface. To prevent this from happening, PE2 acts as a chaperone and initially forms heterodimers with the downstream encoded E1. This enables transport to the Golgi apparatus and delaying or preventing premature E1 self-aggregation (Andersson et al., 1997). E2 is not able to form dimers with E1 in the absence of E3. Then E1 is retained in the ER and no further processing will take place. Presumably, PE2 protects the immature trimeric spike from undergoing conformational changes in the acidic Golgi environment, due to interactions between E3 and the top domain of E1 (Lobigs et al., 1990). When E3 is not cleaved from PE2, due to inefficient furin processing, E1 can only exert its fusogenic activity at a pH < 5.0, which is lower than the normal intracellular pH-range of pH = 5.5-5.8. Sporadically, E3 remains associated to the trimeric spike after efficient furin processing of pE2. This however, will not impede E1 from forming homotrimers (Wu et al., 2008).

CHIKV vaccine development

The spread of CHIKV could in theory be prevented by effective vector control. However, this has proven to be difficult (Weaver et al., 2012), which makes vaccination the most effective way of protecting humans and limiting further CHIKV transmission. CHIKV infection is believed to induce life-long immunity through a protective antibody response (Tiwari et al., 2009), indicating that naïve individuals could be protected for life, if a safe and efficacious vaccine induces a similar antibody response. Currently, there are no specific treatments (antiviral) or commercial vaccines for CHIKV infections available. However, the emergence of CHIKV and the clinical manifestation of disease in humans have triggered vaccine development. The first vaccine prototype was a formalin-inactivated virus, developed by the United States Army Medical Research Institute of Infectious Diseases (USAMRIID) (Harrison et al., 1971; Tiwari et al., 2009). Although efficacious, a major drawback is that production of the virus must take place in costly, biosafety level 3 facilities. As an alternative, live-attenuated virus vaccines have been developed (Levitt et al., 1986). Live-attenuated virus vaccines have the advantage of inducing an efficient, balanced immune response, but have as drawback the risk of reversion to virulence (Noad and Roy, 2003). The live-attenuated virus CHIKV vaccine (181/clone25) was tested in phase II clinical trials and elicited a protective immune response. However, 8% of tested volunteers developed transient arthralgia (Edelman et al., 2000). Although it is an effective vaccine candidate, the US Army has stopped further development of the attenuated vaccine strain (Weaver et al., 2012). Recent studies on the attenuated 181/clone25CHIKV strain showed attenuation of the virus as a consequence of only two point mutations, which were reversed in immunized mice. This clearly highlights the genetic instability of CHIKV attenuation (Gorchakov et al., 2012). The prospect of live-(attenuated) alphavirus vaccine development remains questionable due to vaccine-induced clinical manifestations and the undeniable risk of transmission by mosquito vectors, but most of all, the possible reversion to virulence.

In addition to the 181/clone25 vaccine, a broad range of vaccine strategies have been described (**Table 1-1**), including other live-attenuated virus vaccines (Levitt et al., 1986; Partidos et al., 2012; Plante et al., 2011), chimeric virus vaccines (Wang et al., 2008), DNA vaccines (Mallilankaraman et al., 2011; Muthumani et al., 2008), adenoviral vectored vaccines (Wang et al., 2011), subunit protein vaccines (Kumar et al., 2012) and virus-like particle (VLP) formulations (Akahata et al., 2010). However, these vaccination strategies have their own specific complications in manufacturing, immunogenicity, safety, recombination and large scale production (Edelman et al., 2000; Kutzler and Weiner, 2008).

Recent studies have shown promising results in the development of a CHIKV VLP-based vaccine (Akahata et al., 2010). The complete structural cassettes of different CHIKV strains were expressed via plasmid DNA transfection of 293T human kidney cells, thereby producing CHIKV VLPs. Rhesus macaques were subsequently immunized with VLPs. The

VLPs elicited a neutralizing antibody response against homologous and heterologous strains and protection against high-dose CHIKV challenge (Akahata et al., 2010). Regardless of the vaccine strategy, data obtained so far clearly showed that neutralizing antibodies play a major role in the protection against CHIKV infections (Weaver et al., 2012). During natural infections, CHIKV induces high neutralizing antibody titers. The IgG antibodies are mainly directed against an N-terminal epitope on the exposed domain of E2 and to a lesser extent to E1, making them primary targets for subunit vaccine development (Hunt et al., 2010; Kam et al., 2012b; Strauss et al., 1991; Vrati et al., 1988). The presence of the E2-directed IgG neutralizing antibodies correlates to the long-term protection and viral clearance (Kam et al., 2012b; Kam et al., 2012c).

To explore the potential of VLPs for the development of alphavirus in general, and CHIKV in particular, CHIKV-E1 and -E2 were expressed as an individual subunit or expressed together with all CHIKV structural proteins as part of a VLP. This has been shown to be a safe and effective alternative to inactivated or attenuated live-virus formulations (Akahata et al., 2010; Kumar et al., 2012) but is produced in a poorly scalable production system. The production platform of choice should be safe to use, reach high production yields and be industrially scalable. The recombinant baculovirus-insect cell expression system serves as an elegant and safe production platform and has recently received wider attention for the large scale production of VLP-based vaccine candidates against human viral disease (Vicente et al., 2011).

Table 1-1. Vaccine candidates against CHIKV infections.

Strategy	Expression system	Antigen	Developmental stage	References
Inactivated virus	GMK[1]-cells	Whole virus	Preclinical	Harrison et al., 1971
Live-attenuated	GMK-cells	Whole virus	Terminated post clinical Phase II	Levitt et al., 1986
DNA	pVax1 expression vector	C, E1, E2	Preclinical	Mallilankaraman et al., 2011; Muthumani et al., 2008
Chimeric-vector	BHK[2]-21-cells HEK[3]-293-cells	C-E3-E2-6K-E1	Preclinical	Wang et al., 2011; Wang et al., 2008
IRES	Vero-cells	Whole virus	Preclinical	Plante et al., 2011
Subunit	Baculovirus	E1, E2	Preclinical	Metz et al., 2011b
VLP subunit	293T-cells	C-E3-E2-6K-E1	Clinical Phase I	Akahata et al., 2010
VLP subunit	Baculovirus	C-E3-E2-6K-E1	Preclinical	Metz et al., 2013

[1] Green monkey kidney cells
[2] Baby hamster kidney cells
[3] Human embryonic kidney cells

The recombinant baculovirus-insect cell expression system

Baculoviruses are a large group of insect infecting DNA viruses with high host specificity. Baculovirions are rod-shaped and are characterized by having two virion phenotypes (**Fig. 1-5**). The occlusion derived virus (ODV) assembles in the nucleus of infected insect cells and is occluded in large proteinaceous occlusion bodies (OB) (van Oers, 2011). OBs are required for the horizontal virus transmission between insects. The second virion type, the budded virus (BV) is essential for systemic infection of the host and infection of cells in culture. Although phenotypically different, ODVs and BVs are genetically identical. During the very late phase of baculovirus replication, two proteins, polyhedrin and P10, are produced in very high amounts (Rohrmann, 1986; Van Oers and Vlak, 1997). Polyhedrin and P10 are essential for OB formation and OB release from infected nuclei, respectively, (Carpentier et al., 2008; Carpentier and King, 2009; van Oers et al., 1993), but are not required for BV production (Smith et al., 1983a).

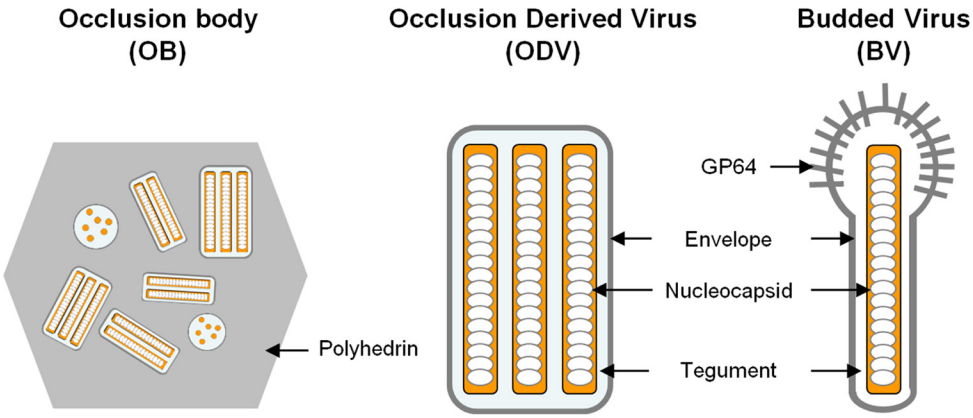

Figure 1-5. Baculovirus structure.
Enveloped occlusion derived virions (ODV) survive outside their host in the form of occlusion bodies (OB), consisting of a polyhedrin proteinaceous matrix. The second baculovirus phenotype, the budded virus (BV), is essential for systemic spread within the infected insect host. Adapted from (van Oers, 2011)

The *polh* and *p10* promoters can therefore be used to drive high-level heterologous gene expression in insect cell culture (**Fig. 1-6**). Baculoviruses have been used since the early 1980s to express transgenes in cultured insect cells (Smith et al., 1983a; Smith et al., 1983b; Summers and Smith, 1987). This now well-established technology is capable of generating high yields of heterologous protein and can be easily scaled up to large volume insect-cell bioreactors (Vicente et al., 2011). The baculovirus-insect cell expression system has resulted in the production of thousands of proteins used for scientific purposes, but also for the development of therapeutic peptides and (subunit) vaccines (Metz and Pijlman, 2011). A wide variety of

viral antigens has been expressed for this purpose by recombinant baculoviruses, ranging from relatively simple viral coat proteins to complex, secreted and/or glycosylated subunits (van Oers, 2006). Expression in insect cells is ideally suited to safely produce proteins with complex folding and post-translational processing (e.g. glycosylation) found exclusively in higher eukaryotes. Furthermore, baculoviruses do not replicate in mammals and recombinants can be constructed and produced under less stringent conditions (Metz and Pijlman, 2011). Since baculoviruses are insect viruses with optimal virus replication in the range of 25°C to 30°C, the expression system is also of high value for production of proteins derived from (viruses of) insects or other ectothermic animals that may require lower temperatures for optimal biological activity. In contrast to most other mammalian viruses, arboviruses have a very wide temperature window for viral RNA replication, ranging from approximately 10°C to 40°C. Arbovirus replication in warm-blooded or endothermic hosts, dependent on the host species, occurs around 37°C, whereas virus replication in the ectothermic, arthropod vector takes place at the ambient environmental temperatures. Recombinant baculovirus expression has resulted in a number of commercially available veterinary vaccines (van Oers,

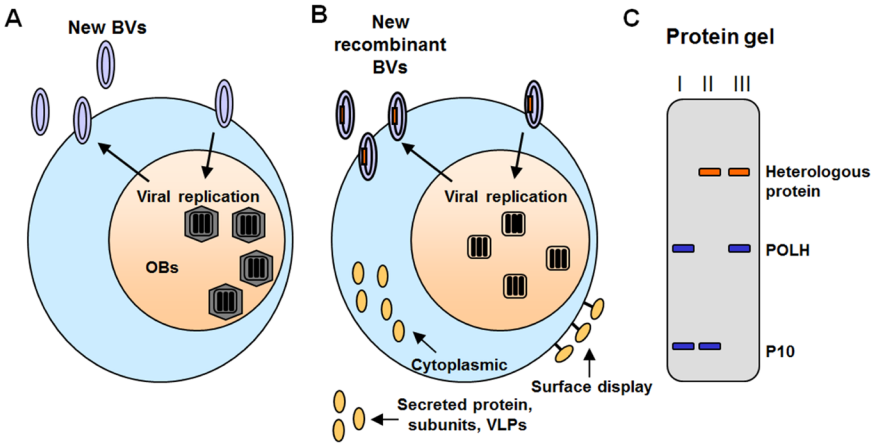

Figure 1-6. Principles of the recombinant baculovirus-insect cell expression system.
A) Infection of insect cells with wild-type (WT) BV. B) Infection of insect cells with recombinant BVs. The *polh* promoter drives heterologous protein expression, thereby disabling OB production. C) Schematic protein gel for WT-infected (I), and *polh* (II) and *P10* (III) promoter driven recombinant baculovirus-infected insect cells (van Oers, 2011).

2006), such as the classical swine fever subunit vaccine (Bouma et al., 1999) and a VLP vaccine against porcine circovirus (Circumvent® PCV, MSD). The use of baculoviruses in the prevention of human viral diseases is becoming more accepted (Roldão et al., 2010) with the first baculo-based cervical cancer VLP vaccine (Cervarix, GlaxoSmithKline) (Paavonen and Lehtinen, 2008) and a novel recombinant influenza virus vaccine (FluBlok, Protein Sciences), which has been approved by the Food and Drug Administration of the USA (Cox,

2012a; Cox, 2012b; Cox and Hollister, 2009). The first gene-therapy treatment approved in the Western world Glybera® (uniQure) uses adeno-associated virus serotype 1 (AAV1) viral vector particles that were produced using recombinant baculoviruses, for gene delivery.

Although the baculovirus expression system serves as an elegant platform for subunit vaccine development and available data are promising, the use of recombinant baculoviruses is still a fairly uncharted area and few studies have focused on the immunogenicity of CHIKV subunit or alphavirus subunits produced in insect cells. Recombinant baculoviruses expressing the full structural cassette (C-E3-E2-6K-E1) of a different alphavirus, Venezuelan equine encephalitis virus (VEEV), generated products that were antigenically indistinguishable from wild type viral proteins. Immunization of mice with lysates of infected insect cells yielded VEE neutralizing antibodies and protected mice from lethal challenge (Hodgson et al., 1999). Individual expression of VEEV E1 and E2 with their respective signal peptides 6K and E3, however, resulted in incorrect glycoprotein processing. Nevertheless, the VEEV 6KE1 construct provided full protection against challenge (Hodgson et al., 1999). This thesis provides clear evidence that the recombinant baculovirus expression is an excellent platform to produce immunogenic CHIKV subunits and VLPs and the presented date will hopefully attribute to the wider use of recombinant baculovirus expression in vaccine development.

Scope of the thesis

The continuous spread of CHIKV and increase in morbidity in man makes CHIKV one of the most important emerging arboviruses to date. A safe and effective CHIKV vaccine is clearly needed to protect humans and to stop CHIKV transmission. Several strategies have been designed to combat CHIKV infections but none of these have been licensed yet (Akahata et al., 2010; Harrison et al., 1971; Kumar et al., 2012; Levitt et al., 1986; Mallilankaraman et al., 2011; Muthumani et al., 2008; Plante et al., 2011; Wang et al., 2011; Wang et al., 2008). None of these are easy amenable to scale up. The purpose of this thesis is to design and develop a novel CHIKV vaccine, using a scalable production process.

This thesis describes the development of a recombinant CHIKV subunit and VLP vaccine using the baculovirus-insect cells expression system as a VLP production platform. This expression system for heterologous proteins is an elegant and promising manner to produce subunits and VLPs for arbovirus vaccine development (Metz and Pijlman, 2011). However, the use of recombinant baculoviruses to produce alphavirus proteins has only marginally been exploited (Hodgson et al., 1999). During natural infections of CHIKV, the alphavirus type species sindbis virus (SINV) and Ross River virus (RRV), neutralizing antibodies are primarily directed against E2, and to a lesser extent against E1 (Kam et al., 2012a; Kam et al., 2012b; Strauss et al., 1991; Vrati et al., 1988). CHIKV-E1 and -E2 glycoproteins constitute the trimeric spikes and exposed at the surface of the virion. Therefore, both E1 and E2 individually form the prime candidates for subunit vaccine development. The first experimental chapter (**Chapter 2**) describes the production and characterization of individually expressed CHIKV-E1 and E2 in *Sf*21 insect cells. Both proteins were cloned downstream of the *polh* promoter in an *Autographa californica* multicapsid nucleopolyhedrovirus (*Ac*MNPV) backbone, together with their native signal peptides 6K(E1) and E3(E2). Secreted versions of E1 (E1ΔTM) and E2 (E2ΔTM) were generated and purified to assess whether the subunits induced neutralizing antibodies upon immunization.

The next chapter (**Chapter 3**) describes the production of CHIKV VLPs using the baculovirus-insect cell expression system. The main advantage of using VLPs in vaccine development is that VLPs mimic the structure and conformation of authentic native virus, but lack the viral genome and are unable to replicate. This renders VLPs safe to use and to produce, since no infectious CHIKV is produced. The production, glycoprotein processing and CHIKV VLP morphology is investigated in this chapter.

Chapter 4 focuses on VLP expression of an unusual member of the *Togaviridae* family, the aquatic salmonid alphavirus (SAV). SAV replicates at low temperatures in fish and is the causative agent of sleeping disease and pancreas disease in Atlantic salmon and rainbow trout. SAV outbreaks cause large economic losses in aquaculture. Using recombinant baculovirus expression, SAV VLPs are obtained and purified. However, the standard 27°C incubation temperature for baculovirus expression abrogates correct SAV glycoprotein processing. A

temperature-shift regime is designed to correctly process and produce SAV VLPs, which can be used for vaccination trials.

The CHIKV subunits and the CHIKV VLPs are tested comparatively for their ability to induce protective immune responses in mice in **Chapter 5**. Immunogenicity of subunits are compared to that of the VLPs in a lethal AG129 mouse model, in terms of survival, neutralizing antibody induction and virus replication. This study clearly showed that the VLPs provide superior protection compared to that of individual protein subunits. Subsequently, the CHIKV VLPs were tested for their ability to induce complete protection in an adult wild-type immune-competent mouse model (Gardner et al., 2010) in which mice develop arthritic disease after CHIKV infection. Several doses of purified VLPs are tested and compared to an adjuvanted VLP formulation. The dominating type of immune response is determined and the mice serum is tested for its neutralizing ability (**Chapter 6**).

In **Chapter 7**, the overall results from the previous chapters are discussed in the context of other CHIKV vaccine development studies. Furthermore, the upstream and downstream processes for large-scale production of CHIKV-VLPs are discussed in which optimization strategies to further increase the VLP-yield from insect cells are highlighted.

Chapter

Functional processing and secretion of chikungunya virus E1 and E2 glycoproteins in insect cells.

Chikungunya virus (CHIKV) is a mosquito-borne, arthrogenic alphavirus that causes large epidemics in Africa, South-East Asia and India. Recently, CHIKV has been transmitted to humans in Southern Europe by invading and now established Asian tiger mosquitoes. To study the processing of envelope proteins E1 and E2 and to develop a CHIKV subunit vaccine, C-terminally his-tagged E1 and E2 envelope glycoproteins were produced at high levels in insect cells with baculovirus vectors using their native signal peptides located in CHIKV 6K and E3, respectively. Expression in the presence of either tunicamycin or furin inhibitor showed that a substantial portion of recombinant intracellular E1 and precursor E3E2 was glycosylated, but that a smaller fraction of E3E2 was processed by furin into mature E3 and E2. Deletion of the C-terminal transmembrane domains of E1 and E2 enabled secretion of furin-cleaved, fully processed E1 and E2 subunits, which could then be efficiently purified from cell culture fluid via metal affinity chromatography. Confocal laser scanning microscopy on living baculovirus-infected *Sf*21 cells revealed that full-length E1 and E2 translocated to the plasma membrane, suggesting similar posttranslational processing of E1 and E2, as in a natural CHIKV infection. Baculovirus-directed expression of E1 displayed fusogenic activity as concluded from syncytia formation.CHIKV-E2 was able to induce neutralizing antibodies in rabbits. Chikungunya virus glycoproteins could be functionally expressed at high levels in insect cells and are properly glycosylated and cleaved by furin. The ability of purifies, secreted CHIKV-E2 to induce neutralizing antibodies in rabbits underscores the potential use of E2 in a subunit vaccine to prevent CHIKV infections.

This chapter has been published as: Stefan W. Metz, Corinne Geertsema, Byron E. Martina, Paulina Andrade, Jacco G. Heldens, Monique M. van Oers, Rob W. Goldbach, Just M. Vlak and Gorben P. Pijlman (2011). *Functional processing and secretion of chikungunya virus E1 and E2 glycoproteins in insect cells. Virology Journal* ***8****;353*

Introduction

Chikungunya virus (CHIKV) is an arthropod-borne (arbo)virus that causes epidemics in Africa, India and South-East Asia (Powers and Logue, 2007). Recent outbreaks in Italy in 2007 (Enserink, 2007) and autochthonous transmission events in France in 2010 (Gould et al., 2010) exemplify the threat of continued spread of CHIKV in the Western world, which correlates with the concurrent expanding distribution of its insect vector. CHIKV is maintained in a sylvatic transmission cycle of mosquitoes, rodents and primates, with *Aedes aegyti* as the primary vector. However, the responsible vector causing the severe CHIKV epidemic on the Reunion Islands in 2005/2006 was *Ae. albopictus* (Tsetsarkin et al., 2007). This vector switch made the virus endemic in more temperate regions and resulted in the first European cases (Italy, 2007) of transmission by local populations of Ae. albopictus (Powers and Logue, 2007; Rezza et al., 2007).

CHIKV (family *Togaviridae*: genus *Alphavirus*) has a single-stranded positive-sense RNA genome, which varies slightly in length between different isolates, but is approximately 11,800 nts long (Khan et al., 2002a) and encodes two open reading frames (ORF). The RNA is encapsidated in a nucleocapsid of approximately 40 nm in diameter (Strauss and Strauss, 1994). The nucleocapsid is tightly enveloped by a host-derived lipid bilayer (envelope) supporting the virus-encoded envelope proteins. Eighty glycoprotein spikes are C-terminally anchored within the viral envelope and are exposed on the surface of virions and of infected cells. The nonstructural proteins required for viral RNA replication are directly translated from the 5' two-thirds region of the viral genome. The structural polyprotein is translated from a viral subgenomic mRNA (sgRNA), located at the 3'one-third part of the genome (Schlesinger and Schlesinger, 2001; Simmons and Strauss, 1972). The five structural proteins (capsid, E3, E2, 6K, E1) are translated as a single polyprotein, from which capsid (C) is autocatalytically cleaved off to encapsidate new plus-strand RNA molecules. The envelope polyprotein precursor E3-E2-6K-E1 is then translocated to the endoplasmatic reticulum (ER). Host signalases process the polyprotein at the N- and C-terminal end of the 6K peptide, resulting in E3E2 (also known as precursor E2: PE2), 6K and E1 (Kuhn, 2007), all anchored in the ER membrane. After this proteolytic cleavage, E3E2 and E1 will eventually form heterotrimers in the early Golgi compartment. Subsequently, E3E2 undergoes a furin-dependent maturation cleavage in the trans-Golgi system at the consensus cleavage signal R-X-(K/R)-R. This furin cleavage is not a prerequisite for virion assembly (Zhang et al., 2003). The hetero-trimeric spikes consisting of E2 and E1 facilitate cell receptor recognition, cell entry via pH-dependent endocytosis and support viral budding (Schlesinger and Schlesinger, 2001). The major clinical symptoms of a CHIKV infection are febrile illness and severe joint pains (Solignat et al., 2009). Recently, macrophages were identified as being key players in CHIKV infection, persistance and pathogenesis. Macrophage-derived pro-

inflammatory products are strongly involved in the muscle and joint immunopathological findings after alphavirus infection (Labadie et al., 2010; Lidbury et al., 2008). Currently, there are no specific treatments for CHIKV infections and no licensed vaccine for any alphavirus is available for human use. During an infection with the alphavirus type species sindbis virus (SINV) neutralizing antibodies are generally directed against E2 and to a lesser extent to E1. This holds true for other alphaviruses as well, suggesting that E1 and E2 are conserved among alphaviruses as epitope donors (Strauss et al., 1991; Vrati et al., 1988). Therefore glycoproteins E1 and E2 serve as the major targets in the development of a (subunit) vaccine against CHIKV infections. A recently developed, experimental CHIKV vaccine based on virus-like particles (VLPs) containing both E1 and E2 induced a protective immune response in non-human primates (Akahata et al., 2010). While this VLP approach may be a way forward in the development of a CHIKV vaccine, the described method of transfecting large DNA plasmids into mammalian cells remains challenging in terms of upscaling. Therefore, a subunit approach may be better compatible with industrial operations (Bouma et al., 1999; Cox and Hollister, 2009). In this study, the baculovirus-insect cell expression system was used to study recombinant CHIKV glycoprotein subunit formation. This production system is a safe and efficient way of expressing heterologous proteins on a large scale in eukaryotic cells (Kost et al., 2005) and the proven technology has resulted in several commercially available veterinary vaccines (van Oers, 2006), including a veterinary subunit vaccine targeting Classical Swine Fever (Bouma et al., 1999) and a human vaccine against cervical cancer (Cervarix, GlaxoSmithKline) (Paavonen and Lehtinen, 2008). A recombinant human vaccine against influenza virus (FluBlok) has recently been approved by the Food and Drug Administration in the USA (Cox and Hollister, 2009). Recombinant protein expression by baculoviruses is based on the use of the strong *polyhedrin* promoter and the exchange of the polyhedrin gene for the heterologous gene of interest. This late phase baculoviral protein is expressed in large amounts in infected cells, but is not essential for baculovirus replication (Smith et al., 1983b). Furthermore, protein expression in insect cells allows post-translational modifications, accurate folding and efficient secretion (van Oers, 2006; Vialard et al., 1995). *Drosophila* cells have recently been used to efficiently express and process CHIKV-glycoprotein complexes (Voss et al., 2010), yet there are few reports on successful expression of alphavirus structural polyproteins with recombinant baculoviruses (Hodgson et al., 1999; Oker-Blom and Summers, 1989), and there are no studies that describe the expression of individual full-length alphavirus E1 and/or E2 glycoproteins. Here, we investigated the expression of individual CHIKV E1 and E2 glycoproteins in insect cells and analyzed the routing and post-translation modifications of these glycoproteins as well as the fusogenic properties of recombinant E1. In addition, purification methods were developed for secreted forms of these glycoproteins, of which the ability to generate a neutralizing antibody response was investigated.

Results

Expression of CHIKV-E2 and -E1 glycoproteins in Sf21 insect cells by recombinant baculoviruses.

Four recombinant baculoviruses were generated to express membrane attached and secreted versions of CHIKV glycoproteins E1 and E2 (*Ac*-6KE1, *Ac*-6KE1ΔTM, *Ac*-E3E2 and *Ac*-E3E2ΔTM) (**Fig. 2-1A**). The glycoprotein coding sequences were cloned downstream of the polyhedrin promoter in an *Autographa californica* multicapsid nucleopolyhedrovirus (*Ac*MNPV) bacmid from which the promoters and open reading frames of the p10, cathepsin and chitinase genes were deleted (See Materials and Methods). All four glycoprotein constructs were equipped with a C-terminal 6xhis tag. *Sf*21 insect cells were infected with the respective recombinant baculoviruses at a MOI of 10 $TCID_{50}$ units per cell and cells were harvested 72 h post infection (hpi), which appeared to be the optimal time point for harvesting (data not shown). Recombinant protein content in the cell fraction was analyzed by Coomassie brilliant blue (CBB)-staining and western analysis using α-His monoclonal antibodies (mabs) and α-E1 and α-E2 polyclonal antisera (**Fig. 2-1B**).

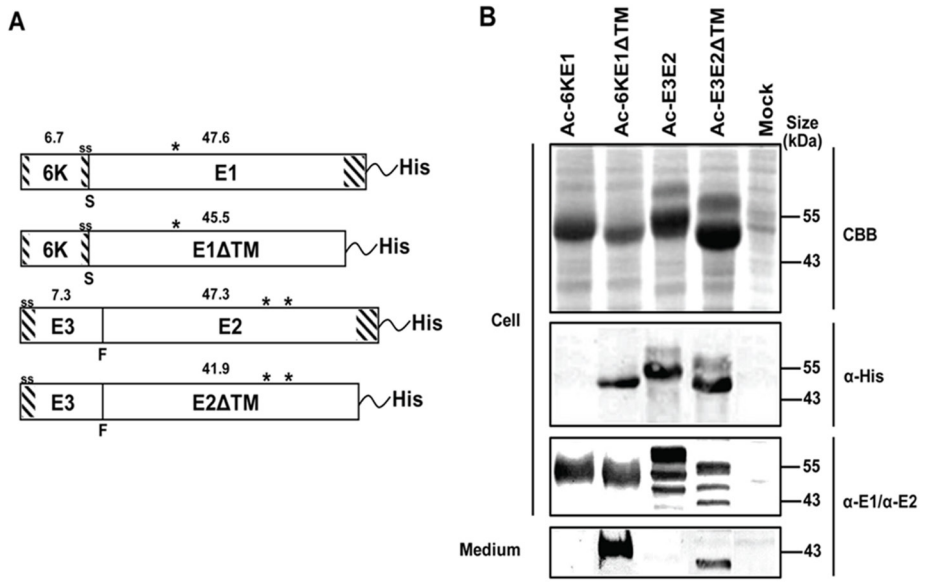

Figure 2-1. CHIKV E1 and E2 expression using recombinant baculoviruses.
A) Schematic representation of four CHIKV glycoprotein constructs expressed from recombinant baculoviruses. The shaded areas represent transmembrane domains or signal peptides (ss), asterisks indicate predicted N-glycosylation sites, S and F indicate signalase and furin cleavage sites, respectively. The predicted molecular mass of the glycoproteins is indicated. All constructs were equipped with a C-terminal 6xHis-tail. B) Gene expression in the cell and in medium fractions of infected *Sf*21 cells was analyzed by Coomassie brilliant blue-staining and western blotting using α-His mabs and rabbit α-E1 and rabbit α-E2 polyclonal antisera.

CBB staining of *Sf*21 cell lysates showed that CHIKV-E2 and -E1 were expressed at very high levels. The size of the proteins observed after infection with *Ac*-6KE1 and *Ac*-6KE1ΔTM (**Fig. 2-1B, lanes 1 and 2**) closely matched the predicted molecular mass of 6KE1 and 6KE1ΔTM of 54.2 kDa and 52.1 kDa, respectively. CHIKV 6KE1 was not detected by western analysis using α-His mabs, but was recognized well by α-E1 polyclonal antiserum. This is most likely caused by the proteolysis of the C-terminal his-tag of CHIKV 6KE1. Expression analysis of insect cells infected with *Ac*-E3E2 and *Ac*-E3E2ΔTM (**Fig. 2-1B, lane 3 and 4**) resulted in two polypeptides for each construct. The smallest ones correspond to the predicted molecular mass of unprocessed E3E2 (54.6 kDa) and E3E2ΔTM (49.2 kDa), respectively. The larger ones, estimated to be 3-4 kDa larger in size, are possibly glycosylated forms of E3E2 and E3E2ΔTM, respectively. When the cell fractions were analyzed with the α-E2 polyclonal antiserum, a third, smaller protein was seen, which most likely represents furin-processed, glycosylated E2 and E2ΔTM, respectively. To allow secretion of E1 and E2 into the cell culture fluid, the C-terminal transmembrane domains of E1 and E2 were deleted (constructs *Ac*-6KE1ΔTM and *Ac*-E3E2ΔTM). Medium fraction analysis shows that the removal of the C-terminal TM of E1 and E2 indeed resulted in secretion of E1 and E2 (**Fig. 2-1B, bottom, lanes 2 and 4**).

Glycosylation status of baculovirus expressed CHIKV-E1 and -E2.

To analyze whether the size difference observed between the upper and lower bands of E3E2 or E3E2ΔTM in CBB and western blots (**Fig. 2-1B**) can be attributed to glycosylation of E2 a tunicamycin assay was performed to analyze the N-glycosylation status of both glycoproteins. It is common knowledge that alphavirus E1 and E2 are N-linked glycosylated, but the number of glycosylation sites can vary among species (Burke and Keegstra, 1979; Knight et al., 2009; Rice and Strauss, 1981; Simizu et al., 1984). CHIKV-E1 is predicted to be glycosylated at N141 and CHIKV-E2 at N263 and N273 (Blom et al., 2004). *Sf*21 cells were infected with the 4 recombinant baculoviruses, respectively, (**Fig. 2-1A**) and incubated in the presence or absence of tunicamycin, harvested at 72 hpi and separated from the medium fraction. Whole cell lysates were analyzed by western blotting (**Fig. 2-2A**) and by Periodic Acid Schiff staining (PAS) (**Fig. 2-2B**), which stains glycosyl groups on proteins (Kapitany and Zebrowski, 1973).

Expression of 6KE1 and 6KE1ΔTM in tunicamycin treated *Sf*21 cells resulted in a band with lower molecular mass (**Fig. 2-2A, left**). This suggests that E1 is at least partially glycosylated. PAS-staining of 6KE1 and 6KE1ΔTM resulted in protein bands with a size similar to the proteins detected by western blotting and confirmed that CHIKV-E1 was glycosylated. Western blot detection using α-E2 polyclonal antiserum showed that infection with *Ac*-E3E2 and *Ac*-E3E2ΔTM resulted in the triple-band pattern previously observed (**Fig. 2-1B**). The minor fourth upper-band that was detected is only observed occasionally and

may represent E2 aggregates. Upon tunicamycin treatment of *Ac*-E3E2 and *Ac*-E3E2ΔTM-infected cells, the E2-specific polypeptides with the highest molecular mass (~58 and ~55 kDa, respectively) disappeared, suggesting that these are the N-glycosylated forms of E3E2 and E3E2ΔTM (**Fig. 2-2A**). PAS-staining, which is absent in the tunicamyin treated lanes, confirmed this result. Thus it can be concluded that the bands of ~55 kDa and ~49 kDa in infections with *Ac*-E3E2 and *Ac*-E3E2ΔTM, respectively, represent non-glycosylated E3E2 and E3E2ΔTM proteins. The smaller polypeptides of ~50kDa and ~44kDa, correspond in size to glycosylated, furin-cleaved E2 and E2ΔTM, respectively. PAS-staining on the Ac-E3E2ΔTM infected cell fraction also stains this additional protein of ~44 kDa (**Fig. 2-2B**), suggesting that glycosylated, furin-cleaved E2ΔTM is indeed produced, albeit in relatively low abundance. Immunodetection of the baculovirus GP64 envelope protein was used as a positive control (**Fig. 2-2C**). GP64 is essential for baculovirus budding and cell entry, and is known to be heavily glycosylated (Monsma et al., 1996; Pijlman et al., 2006) at 4 of the 5 predicted positions (Jarvis et al., 1998). As expected, a significant decrease (~6 kDa) in molecular mass was observed as a result of tunicamycin treatment. GP64 is best detected on highly concentrated budded virus preparations and more difficult in insect cells. PAS staining is not sensitive enough to detect glycans on minor amounts of GP64, compared to overexpressed CHIKV glycoproteins, which explains the lack of PAS staining in cells infected the *Ac*-GFP control virus (**Fig. 2-2B, right**). These results show that a substantial fraction of CHIKV E1 and E2 proteins expressed by recombinant baculoviruses in *Sf*21 insect cells is N-glycosylated and that only a minor fraction of the glycosylated E3E2 precursor is processed by furin into mature, N-glycosylated E2.

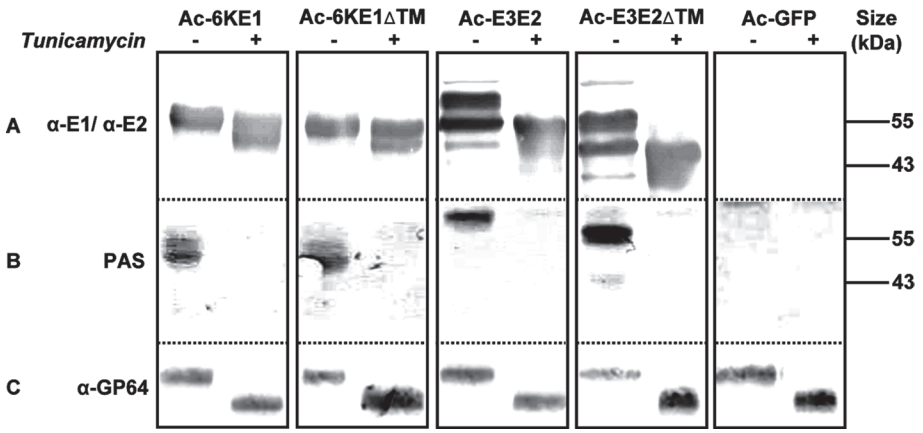

Figure 2-2. N-linked glycosylation of CHIKV E1 and E2 synthesized with baculovirus vectors.
A) Infected *Sf*21 cells were treated with tunicamycin and cell fractions were analyzed by western blotting using α-E1 and α-E2 polyclonal antisera. B) Glycosylation of recombinant CHIKV E1 and E2 was confirmed by PAS-staining. C) Detection of baculovirus glycoprotein GP64 verified tunicamycin activity.

Furin processing of recombinant CHIKV-E3E2 and -E3E2ΔTM

During natural alphavirus glycoprotein maturation, E3 is released from E3E2 via cleavage by a furin-like protease (Zhang et al., 2003). However, the triple-band patterns found during infection with *Ac*-E3E2 and *Ac*-E3E2ΔTM (**Fig. 2-2A, right**) suggest that the bulk of intracellular E3E2 is incompletely processed by furin into the individual E3 and E2 proteins. To investigate the level of furin-processing and to confirm the nature of the smallest polypeptide in the triple-band pattern, *Sf*21 cells were infected with *Ac*-E3E2 and *Ac*-E3E2ΔTM in the presence or absence of furin inhibitor. Western blot analysis of *Ac*-E3E2 infected cells shows that the smallest polypeptide (~44 kDa) detected by anti-E2 antiserum disappeared in the presence of furin inhibitor (**Fig. 2-3A, left**) indicating that this represents the mature E2, which according to the previous experiment was also N-glycosylated (**Fig. 2**). As expected, all E2-specific polypeptides were retained in the cell fraction (**Fig. 3B, left**) unless the TM was deleted (**Fig 2-3B, right**) in accordance with the experiments showing that E2 is only secreted when the C-terminal TM-domain was deleted (**Fig. 2-1B**). It should be noted that furin-processed E2ΔTM could hardly be observed in the cell fraction, which might suggest that the protein is secreted shortly after it is cleaved by furin in the trans-Golgi complex. Interestingly, when furin cleavage was inhibited in *Ac*-E3E2ΔTM-infected cells, the N-glycosylated fraction of E3E2ΔTM was still secreted into the culture fluid (**Fig. 2-3B, right**). It can therefore be concluded that secretion of E2ΔTM is not dependent on furin processing.

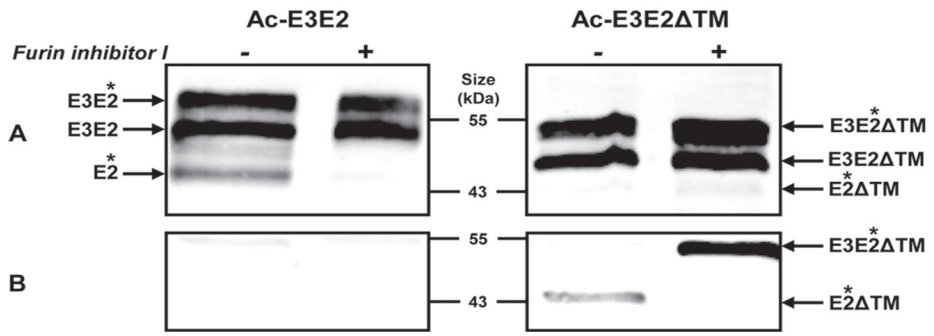

Figure 2-3. Furin inhibiton assay on *Ac*-E3E2 and *Ac*-E3E2ΔTM infected *Sf*21 cells.
*Sf*21 cells were infected with *Ac*-E3E2 and *Ac*-E3E2ΔTM in the absence (-) or presence (+) of furin inhibitor. The cell fraction (A) and the concentrated medium fraction (B) were analyzed by western blotting using α-E2 polyclonal antiserum. Glycosylated proteins are indicated with an asterisk.

Secretion of recombinant CHIKV-E1ΔTM and –E3E2ΔTM from insect cells.

The recombinant baculovirus expression analysis and the furin inhibition assay showed that CHIKV E1ΔTM and E2ΔTM are secreted from *Sf*21 cells when they are expressed with their native signal peptide 6K and E3, respectively (**Figs. 2-1B and 2-3B**). To investigate whether other heterologous signal peptides might have a stronger effect on protein secretion, 6K and E3 were both exchanged for the honey bee melittin signal peptide (HBM), which has been shown to improve both expression and secretion of heterologous proteins in baculovirus infected cells (Tessier et al., 1991). In other constructs, the first 41 amino acids from 6K were deleted as they are not part of the signal peptide necessary for ER translocation of E1. The remaining 6K signal sequence was cloned upstream of E1ΔTM (*Ac*-6K^{-40}E1ΔTM) and of E2ΔTM (*Ac*-6K^{-40}E2ΔTM). The level of intracellular or secreted E1 and E2 was compared by western blotting. A control lane of CHIKV-infected *Ap*61 mosquito cells was loaded to indicate the size of native E1 and E2 proteins.

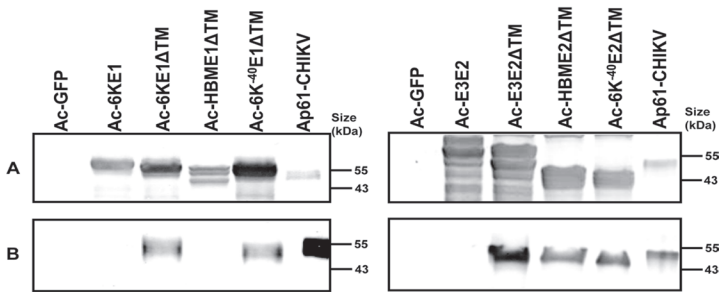

Figure 2-4. CHIKV E1ΔTM and E2 protein secretion.
Both CHIKV E1ΔTM and E2ΔTM were expressed with E3, HBM or 6K^{-40} as signal sequence. The cell fraction A) and the medium fraction B) were analyzed by western blot using α-E1 and α-E2 polyclonal antibodies. CHIKV infected *Ap*61 mosquito cell-lysate was used as a control.

The replacement of 6K for HBM upstream of E1ΔTM resulted in lower intracellular E1 levels (**Fig. 2-4A**), while the secretion level was also dramatically decreased (**Fig 2-4B**). Deletion of the first 41 amino acids from 6K resulted in equal secretion levels compared to the native complete signal peptide 6K. It could be concluded that the signal sequence in 6K is sufficient for efficient secretion of E1ΔTM. When the same signal peptides were exchanged with E3 to test whether E2ΔTM secretion could be enhanced, all constructs were expressed successfully (**Fig. 2-4A**), but medium fraction analysis showed that neither the HBM nor the 6K^{-40} signal sequence could improve E2ΔTM protein secretion. This indicates that the CHIKV-6K and E3 signal peptides direct E1ΔTM and E2ΔTM secretion better than other well studied signal peptides like the HBM signal sequence. These results suggest that the CHIKV signal peptides could potentially serve as strong signal peptides for secretion of other heterologous proteins.

Purification of secreted recombinant CHIKV-E1ΔTM and -E2ΔTM

Both *Ac*-6KE1ΔTM and *Ac*-E3E2ΔTM constructs were tagged with a C-terminal polyhistidine-tail for detection and purification purposes. Medium fraction analyses showed that E1ΔTM and E2ΔTM were effectively secreted from the cell, which enables protein purification by Co^{2+}-histidine interaction. Total medium fractions were loaded onto Talon® spin columns and bound proteins were eluted with 150 mM imidazol. CBB analyses of the three elution fractions (**Fig.2-5A**) showed a dramatic increase in concentration of both E1ΔTM and E2ΔTM compared to the non-purified fractions, indicating that both recombinant proteins were efficiently purified from the medium fraction. Western analyses using the polyclonal antisera (**Fig. 2-5B**) confirmed that the purified fractions indeed contained E1ΔTM and E2ΔTM and that multiple elution steps are required to completely elute the recombinant CHIKV glycoproteins from the column. Two bands were detected in the elution fractions of *Ac*-E3E2ΔTM (**Fig. 2-5B, right**). The protein band with the highest molecular mass most likely represents glycosylated E3E2ΔTM, which complies with the finding that secretion of E2ΔTM is not dependent on furin cleavage (**Fig. 2-3B**). Protein concentrations in the elution fractions were determined by Bradford assay and resulted in a total yield of 38 and 30 mg/l secreted protein for CHIKV-E1ΔTM and CHIKV (E3)E2ΔTM, respectively, at a cell concentration of $6.7x10^5$ *Sf*21 cells/ml. Metal affinity purification of secreted CHIKV-E1ΔTM and –E2ΔTM resulted in a protein recovery of ~60% and >95%, respectively.

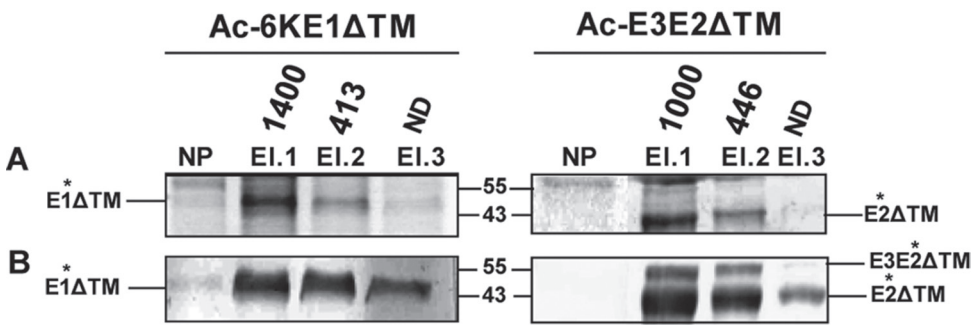

Figure 2-5. Purification of secreted, recombinant CHIKV E1ΔTM and E2ΔTM proteins.
*Sf*21 cells were infected with *Ac*-6KE1ΔTM and *Ac*-E3E2ΔTM and the respective serum-free medium fractions were loaded onto Talon® spin columns. Bound proteins were eluted 3 times with 150 mM imidazol. The elution fractions were analyzed with CBB (A) and western blotting, using α-E1 and α-E2 polyclonal antisera (B). NP represents non-purified fractions and El.1-3 indicate the subsequent elution fractions. Total protein concentration of the elution fractions (1 ml each) are noted above in µg/ml.

Surface expression of CHIKV-E1 and -E2 in Sf21 cells.

Alphavirus glycoproteins are, during a natural infection, expressed at the surface of the host cell to allow budding of progeny virus (Strauss and Strauss, 1994). From the foregoing experiments it became clear that baculovirus expressed CHIKV E1 and E2 are (at least partially) glycosylated and secreted from *Sf*21 cells when their C-terminal TM domain is deleted. It was also observed that E3E2 is (at least partially) processed by furin, suggesting that the posttranslational processing of CHIKV glycoproteins in baculovirus-infected cells may resemble the processing during a natural CHIKV infection (Kuhn, 2007). If this is the case, E1 and E2 are expected to be transported to the cell membrane during baculovirus infection and be exposed at the cell surface.

Therefore, non-permeable, living *Sf*21 cells infected with *Ac*-6KE1 or *Ac*-E3E2 were subjected to immunofluorescence using polyclonal E1 and E2 antisera (Kaba et al., 2003). Positive staining indicated that the glycoproteins are exposed at the surface of the cell (**Fig. 2-6**). From the ring-like structure observed by confocal microscopy (**Fig. 2-6, top and middle**) it could be concluded that both E1 and E2 are translocated to the plasma membrane when expressed in insect cells. This ring-like structure was not observed in mock infected cells (**Fig. 2-6, bottom**).

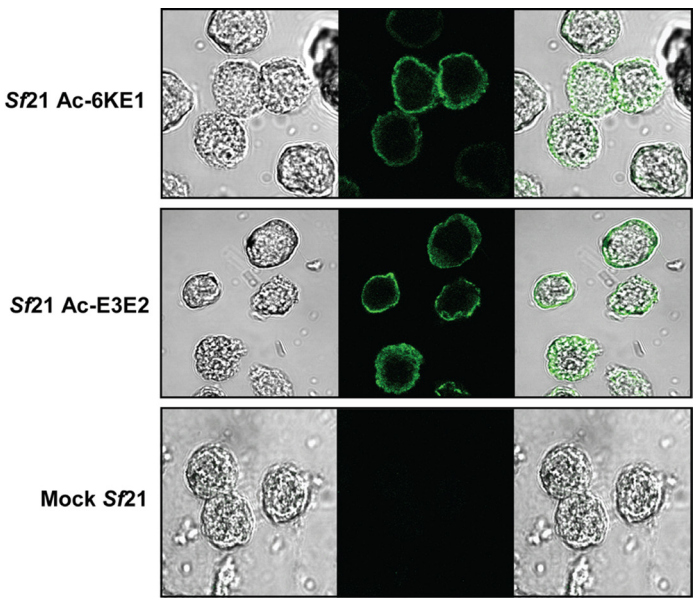

Figure 2-6. Surface expression of CHIKV E1 and E2 on the plasma membrane.
*Sf*21 cells were infected with recombinant baculoviruses and living cells were immunostained against E1 and E2. Confocal microscopy reveals the presence of CHIKV-E1 and-E2 at the surface of infected *Sf*21 cells.

Fusogenic activity of CHIKV-E1 expressed in Sf21 cells

This study has shown that production of CHIKV E1 and E2 insect cells using baculovirus vectors leads to glycosylation, (partial) furin processing and plasma membrane translocation and or secretion of E1 and E2. The individually expressed glycoproteins appear to follow similar processing steps as compared to wildtype CHIKV infections (Kuhn, 2007), which might imply that the recombinant glycoproteins retained their functionality. The following experiment was designed to test the fusogenic activity of baculovirus expressed E1, which in CHIKV infection regulates fusion during endocytosis in a pH-dependent manner, with a pH-optimum of 5.5 (Wu et al., 2008). Since alphavrus fusion is cholesterol dependent (Kielian

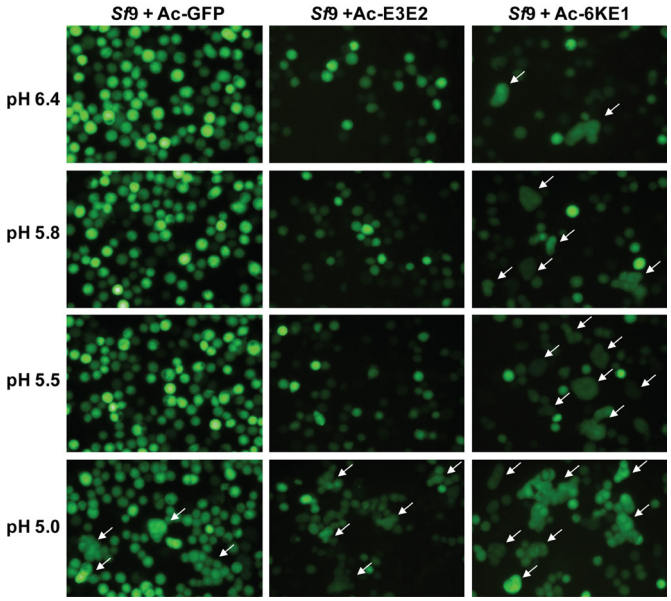

Figure 2-7. Syncytia formation after surface expression of CHIKV-E1 in insect cells.
*Sf*9-ET cells in cholesterol supplemented Sf900 II medium (pH=6.4) were infected with *Ac*-6KE1, *Ac*-E3E2 and *Ac*-GFP. Cells were incubated 72 h post infection with medium of pH=5.8, pH=5.5 and pH=5.0 for 2 min. Pictures were taken 4 h post induction. Syncytia are indicated with arrows

and Helenius, 1984) and insect are cholesterol auxotrophs, *Sf*9-ET cells were cultured in cholesterol-supplemented *Sf*900 II insect medium (pH=6.4) and were infected with *Ac*-6KE1, *Ac*-E3E2 and *Ac*-GFP. *Sf*9-ET is a transgenic cell line expresing eGFP under the control of the polyhedrin promoter, thereby only allowing eGFP expression during baculovirus replication (Hopkins and Esposito, 2009). In this way, baculovirus infected cells could be easily visualized. Therefore, *Sf*9-ET cells infected with Ac-GFP express higher amounts of GFP, compared to *Ac*-6KE1 and *Ac*-E3E2 infected *Sf*9-ET cells.

Cells were treated with acidified medium of pH=5.8, pH=5.5 and pH=5.0. Cells were screened for syncytia formation 4 h post treatment. Syncytia formation after induction with

medium of pH6.4, pH=5.8 or pH=5.5 was only observed in cells producing CHIKV-E1 at 72 hpi (**Fig. 2-7, right**). In contrast, cells infected with *Ac*-E3E2 (**Fig. 2-7, middle**) or *Ac*-GFP (**Fig. 2-7, left**) did not form syncytia, unless they were induced with acidified medium of pH=5.0 (**Fig. 2-7, bottom**). At this low pH, baculovirus GP64 induces syncytia formation (Volkman, 1986). Thus, expression of E1 is correlated with syncytium formation and E1 is functionally active as viral fusion protein in a defined pH range.

The fusogenicity of CHIKV E1 is in the same order of magnitude to what has been found for the major envelope fusion protein F of baculoviruses with this particular assay suggesting that E1 is highly fusogenic (Long et al., 2007). These data concerning pH-dependent syncytia formation exclude the possibility that the syncytia formation in this assay at pH values of 5.8 and 5.5 was caused by the baculovirus infection itself. These findings are in line surface expression of CHIKV-E1 (**Fig. 2-6**) and indicate that E1 retains its fusogenic activity when expressed in *Sf*-cells.

Virus neutralization test

Purified CHIKV-E1ΔTM and -E2ΔTM (**Fig. 2-5**) were used to generate polyclonal antisera in rabbits. To analyse the neutralizing activity of the polyclonal α-E1 and α-E2, the antisera were incubated with 100 $TCID_{50}$/ml CHIKV and subsequently incubated for 4 days with BHK-21 cells. Total suppression of cytopathic effect was used as a microscopic marker for virus neutralization. Rabbits vaccinated with CHIKV-E2ΔTM developed neutralizing antibody titers against CHIKV, up to a dilution of 1:40. No neutralizing antibodies were measured in sera of the animals vaccinated with CHIKV-E1ΔTM. The sera of CHIKV infected mice was used as a control and neutralized CHIKV up to 1:320 dilution. These results show that purified CHIKV-E2ΔTM, expressed by recombinant baculoviruses, is able to induce neutralizing antibodies in rabbits.

Discussion

The continued spread of CHIKV worldwide, the associated threat for invasion of Europe and other Western countries, and the economic impact of a CHIKV epidemic highlights the demand for an effective antiviral therapy and/or vaccine. Several attempts have been undertaken to design such a vaccine and include the development of a life attenuated CHIKV strain and, more recently, a VLP-based approach (Akahata et al., 2010). VLPs have safety profiles very similar to those of subunits and immunogenic properties comparable to killed vaccines. It has been shown that there is great potential for the baculovirus expression system to generate effective (subunit) vaccines (van Oers, 2006) and several vaccines produced in this system are on the market or in the process of registration. Furthermore, CHIKV is transmitted by mosquitoes and replicates to high viral titers in mosquitoes cells, thus it might be beneficial to express CHIKV proteins in insect cells (Metz and Pijlman, 2011). In this research, vaccine candidates were generated consisting of individual CHIKV glycoproteins expressed in insect cells using recombinant baculoviruses.

CHIKV glycoprotein genes were cloned downstream of the polyhedrin promoter of baculovirus *Ac*MNPV to analyze individual expression of E1 and E2 with their native signal peptides and in the presence or absence of their respective C-terminal transmembrane domains. Results of SDS-PAGE and western blot analyses indicated that all proteins were expressed at high levels in *Sf*21 cells and that a substantial fraction of these proteins was processed in a similar fashion as during a natural CHIKV infection. The glycosylation patterns and resulting size changes found for CHIKV E1 and E2 appear to correspond with the postulated number of glycosylation sites in E1 and E2 (1 and 2, respectively). Our findings are in agreement with results obtained in glycoprotein expression studies for other alphaviruses and for the related rubellavirus, that used recombinant baculoviruses (Cho et al., 2008; Hodgson et al., 1999; Oker-Blom et al., 1995; Oker-Blom and Summers, 1989).

We have shown that the glycoproteins travel through the ER, which is very sensitive to any homeostatic alterations and disturbances. Such ER stress is induced by protein misfolding, considerable protein overproduction and inhibition of N-linked glycosylation (Kaufman, 1999). Generally, two baculovirus proteins cathepsin and chitinase are abundantly expressed and the latter tends to accumulate in the ER (Saville et al., 2004), thereby clogging up the ER and competing with recombinant proteins. Even though chitinase is deleted from the recombinant baculovirus backbone, it appears that the CHIKV glycoproteins are expressed in such massive amounts, that the unfolded protein response (UPR), which is normally induced in response to ER-stress (Rutkowski and Kaufman, 2004), is not sufficient to mitigate ER stress, as concluded from the fact that a fraction of the 6KE1, 6KE1ΔTM, E3E2 and E3E2ΔTM appear in their unprocessed forms. Incomplete glycosylation and retention in the ER of intracellular viral glycoproteins expressed at very high levels in baculovirus-infected

cells is common (van Oers et al., 2001), but this did not compromise membrane localization and subunit secretion.

While glycosylation of recombinant CHIKV-E1 and -E2 was relatively efficient, expression analysis with the baculoviruses *Ac*-E3E2 and -E3E2ΔTM indicated that only a small fraction was cleaved by cellular furin. This, however, did not prevent secretion of the uncleaved, glycosylated E3E2 precursor as observed in the furin inhibition experiment and during metal-affinity chromatographay purification of secreted E2 subunits. We can therefore conclude that not furin activity, but rather the accumulation and retention of non-glycosylated E3E2 precursors in the ER, is limiting subunit secretion. This phenomenon has been well described for high level expression in insect cells of CSFV-E2, a similar glycoprotein from a different virus (van Oers et al., 2001).

Although E3 plays a major role during CHIKV replication in the formation of E1 and E2 heterodimers (Kuhn, 2007), and the presence of uncleaved E3E2 in progeny alphavirus particles induces defects in virus production (Ozden et al., 2008), it is not known if the presence of E3 has negative effects on the stability, functionality or antigenicity of E2 subunits. Considering that alphavirus virions with incorporated uncleaved E3E2 are able to bind efficiently to the cell surface, which is modulated by E2 (Lobigs et al., 1990), we expect that E3E2 is still sufficiently immunogenic. In fact, a recent report has shown that E3 also harbours protective epitopes (Parker et al., 2010). Future studies might further explicate the effect of glycosylation on subunit antigenicity and epitope presentation.

Confocal microscopy showed that E1 and E2 are present at the surface of *Sf*21 cells. Since cells are able to secrete uncleaved, but glycosylated E3E2ΔTM, it is likely that cells expressing E3E2 display the protein in its glycosylated configuration on the surface of the cell. The reason that displayed or secreted proteins are all glycosylated, might be explained simply by the fact that glycosylation occurs prior to entering of the secretory pathway. Cells infected with *Ac*-6KE1 were able to form syncytia, indicating that E1 protein displayed at the surface was able to induce membrane fusion. Subjecting *Ac*-6KE1, *Ac*-E3E2 and *Ac*-GFP infected *Sf*9-ET cells to a range of acidified *Sf*900 II medium (pH=5.0, 5.5, 5.8 and 6.4) excluded the possibility that syncytia formation was induced at pH 5.8 by the baculovirus fusion protein GP64, which is also expressed at the surface of infected cells (Volkman, 1986). The pH of *Sf*900 II medium was in the same range as it would be during endocytosis (pH=5.5-pH=5.8). Surprisingly, fusogenic activity was also shown at a pH=6.4, which is expected to be slightly out of the fusogenic range of E1. This phenomenon was most likely caused by the addition of cholesterol to the culture medium. Cholesterol is known to be an important factor in alphavirus fusion (Kielian and Helenius, 1984), and a supplement activates CHIKV-E1 to be fusogenic. Whether its fusogenicity means that E1 is present in the form of homotrimers on the surface of the cell needs experimental confirmation.

The virus neutralization test shows that the rabbit polyclonal antiserum elicited against purified CHIKV-E2ΔTM is able to neutralize CHIKV. This is the first time that a secreted form of CHIKV-E2, expressed by the recombinant baculovirus-insect cell expression system elicits neutralizing antibodies in rabbits. No neutralizing antibodies were detected in the serum of E1ΔTM vaccinated rabbits, which is not entirely unexpected since E1 is partially covered by E2 in mature virions (Li et al., 2010). This is clear proof for antigenicity of the E2 subunit vaccine candidate, which will now be further studied in vaccination trials in an animal model. The fact that expression of CHIKV-glycoproteins in insect cells results in correct processing similar to the processing found during wildtype infections, proposes it to be a very useful and promising expression system in the generation and development of alphavirus subunit vaccines. In addition, proteins appear to retain their original function, paving the way for recombinant baculoviruses to be used in functionality studies in insect cells.

Conclusions

In conclusion, this study has shown that expression of full-length CHIKV 6KE1 and E3E2 glycoproteins using baculovirus vectors in insect cells leads to glycosylation, furin processing, plasma membrane translocation of E1 and E2, and CHIKV-E1 retains its functional activity as a membrane fusion protein. In addition, the deletion of the C-terminal transmembrane domain enables secretion of glycoprotein E1 and E2 independent of furin cleavage. The purified, glycosylated and secreted CHIKV E2 subunit induced neutralizing antibodies in rabbits and can now be tested for its ability to provide protection against CHIKV challenge in an animal model.

Acknowledgements

Han van den Bosch (Nobilon International BV) and Albert Osterhaus (EMC) are acknowledged for initiating the project, their continued interest and advice. Dowty Movita constructed the *Ac*MNPV deletion bacmid and Leon Oosterik helped with cloning of the HBM constructs. We would like to thank Lizzy Buijs for generating polyclonal antibodies against CHIKV-E1 and -E2. This work was performed within the framework of Dutch Top Institute Pharma, project “Development of recombinant live and subunit vaccines against chikungunya virus infections“ (project nr. T4-301).

Methods

Cells and viruses

Spodoptera frugiperda (*Sf*21) insect cells (Invitrogen) were maintained in tissue culture flasks (Greiner) as a monolayer culture in Grace's insect medium (Invitrogen), supplemented with 10% fetal bovine serum (FBS, Hyclone). *Sf*9-easy titer (ET) cells (Hopkins and Esposito, 2009) were maintained as a monolayer culture in *Sf*900 II (Invitrogen) serum free medium. The Bac-to-Bac baculovirus expression system (Invitrogen) based upon *Autographa californica* multicapsid nucleopolyhedrovirus (*Ac*MNPV) was used to generate recombinant baculoviruses. The bacmid backbone AcΔcc contained a deletion of the promoters and large parts of the ORFs of cathepsin and chitinase, thereby leaving flanking essential genes intact (Kaba et al., 2004). Furthermore, the *p10* promoter and ORF were deleted from the *Ac*Δcc backbone by replacing these elements with a zeocin resistance marker by Lambda Red recombination (Datsenko and Wanner, 2000; Pijlman et al., 2002). This zeocin resistance marker was flanked by modified loxP sequences as described by (Suzuki et al., 2005) for bacterial genome modifications. This allowed the subsequent removal of the marker gene with Cre recombinase. Baculovirus titers were determined by end point dilution assay and expressed as tissue culture infectious dose 50 ($TCID_{50}$) per ml. Viral RNA of chikungunya virus isolate S27 (Tanzania, 1953) provided by Erasmus Medical Center (EMC), Rotterdam, was used as a source for cDNA synthesis. CHIKV-S27 virus stocks were produced in BHK-21 cells in a BSL3 laboratory. Input virus was removed by extensive washing, and supernatant containing infectious virus was harvested four days post infection (pi) and filtered. Virus titration assays were performed on BHK-21 cell monolayers in 96 wells plate and titer was calculated using the Karber method. Virus stock titers were expressed as $TCID_{50}$ per ml. CHIKV stocks containing 10^7 $TCID_{50}$ / ml were aliquoted and stored at -80°C.

Construction of recombinant baculoviruses encoding CHIKV glycoproteins.

Amplicons of CHIKV 6KE1 and E3E2 were generated using SuperScript One-Step RT-PCR with Platinum Taq (Invitrogen), cloned in pGEM-Teasy vectors (Promega) and sequenced (Eurofins Operon, Germany). CHIKV cDNA sequences encoding 6KE1, 6KE1ΔTM, E3E2 and E3E2ΔTM (**Fig. 1-1**) were PCR amplified from these plasmids using extended primers (6K-F; ggggacaagtttgtacaaaaaagcaggcttaggatccaccatggccacataccaagaggctgc, E3-F; ggggacaagtttgtacaaaaaagcaggcttaggatccaccatgagtcttgccatcccagttatg, E1-R; ggggaccactttgtacaagaaagctgggtaaagcttctaatgatgatgatgatgatgcatgtgcctgctgaacgacacgc, E1ΔTM-R; ggggaccactttgtacaagaaagctgggtaaagcttctaatgatgatgatgatgatgcatccatgacatcgccgtagcgg, E2-R; ggggaccactttgtacaagaaagctgggtaaagcttctaatgatgatgatgatgctgcagcgctttagctgttctgatgc and E2ΔTM-R;

ggggaccactttgtacaagaaagctgggtaaagcttctaatgatgatgatgatgctgcagcagctcataataatacagaa) that introduce AttB recombination sites (underlined) to enable Gateway® cloning (Invitrogen). Resulting PCR products were cloned into pDONR207 donor plasmid (Invitrogen) and subsequently transferred to the pFastBac1 derivative pDEST8 (Invitrogen). Recombinant baculoviruses encoding the CHIKV protein constructs were generated using the Bac-to-Bac baculovirus expression system (Invitrogen), resulting in *Ac*-6KE1, *Ac*-6KE1ΔTM, *Ac*-E3E2 and *Ac*-E3E2ΔTM.

Protein analysis.

For analysis of protein expression, $6x10^6$ *Sf*21 cells were seeded into 75 cm^2 culture flasks. Cells were infected with recombinant virus at a multiplicity of infection (MOI) of 10 $TCID_{50}$ units per cell. Cells were harvested 72 hpi and washed twice in 1 ml phosphate buffered saline (PBS). Finally, cells were resuspended in 500µl PBS and stored at -20°C. Whole cell lysates were analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie brilliant blue staining. A lysate of CHIKV-infected Ap61 mosquito cells was used as positive control. Proteins were transferred to an immobilon transfer membrane (Millipore) for Western blot (WB) analyses. Membranes were blocked in PBS + 0.1% Tween-60 (PBST) containing 3% skim milk for 1 h at 37°C. Next, the membranes were washed with PBST and incubated with rabbit polyclonal antiserum (supplied by Nobilon International BV) at a dilution of 1:15,000 for 1 h at 37°C. After incubation, membranes were washed and treated with 1:3,000 diluted Alkaline Phosphatase (AP) conjugated goat anti-rabbit IgG antibodies (Sigma) for 45 min at 37°C. Proteins were detected by NBT/BCIP staining (Roche).

Tunicamycin assay.

A tunicamycin assay was performed to analyze the glycosylation status of recombinant CHIKV E1 and E2. *Sf*21 cells were infected with recombinant baculoviruses at an MOI of 10 $TCID_{50}$ units per cell. Cells were incubated at 27°C in the presence of 10 µg/ml tunicamycin (Sigma) and harvested 72 hpi. Protein glycosylation was visualized using Western blotting and Periodic acid Schiff staining. Proteins were blotted on an immobilon transfer membrane (Millipore) and soaked in PAS solution (1% perjodium acid in 3% acetic acid) for 15 min at room temperature (RT). The membrane was washed several times in water and was incubated in the dark for 15 minutes at RT with Schiff's reagent. Finally, the membrane was washed for 5 min in 50% Na_2CO_3.

Furin inhibition assay.

*Sf*21 cells were infected with *Ac*-E3E2 and *Ac*-E3E2ΔTM at an MOI of 10 in Grace's insect medium (Invitrogen) without FBS. Cells were washed twice with medium and incubated with medium containing 50 μM of Furin Inhibitor I (Calbiochem). Cells were harvested at 72 hpi and the medium fraction was separated from the cell fraction by centrifugation. Protein processing was analyzed by Western blotting.

Immunostaining of baculovirus infected Sf21 cells.

*Sf*21 cells were infected with *Ac*-E3E2 and *Ac*-6KE1 at a MOI of 10 to determine surface expression of CHIKV-E1 and -E2. Cells were harvested 48 hpi and washed with PBS. Next, the cells were incubated for 1 h at RT with PBS+5%FBS containing 1:5,000 diluted rabbit α-CHIKV-E1 and rabbit α-CHIKV-E2 polyclonal antibodies. Cells were washed 3 times and incubated for 1 h at RT with PBS+5%FBS containing 1:1,000 diluted goat-anti-rabbit-Alexa Fluor 488 (Invitrogen). Cells were analyzed using laser scanning confocal microscopy on a Zeiss LSM 510 Meta, Axiovert 100m (Zeiss) with Argon laser (488 nm) and images were analyzed with Zeiss LSM image browser.

Syncytium formation assay

The syncytium formation assay was performed by infecting *Sf*9-easy titer (ET) cells (Hopkins and Esposito, 2009) with *Ac*-E3E2, *Ac*-6KE1 at an MOI of 10 in *Sf*900 II medium (pH=6.4), supplemented with cholesterol (0.2 mg/ml, Sigma). A recombinant *Ac*MNPV expressing GFP (*Ac*-GFP) was used as a negative control. Syncytium formation was induced 72 hpi, by subjecting infected cells for 2 minutes to acidified medium with pH=5.8, pH=5.5 and pH=5.0. Syncytium formation was scored 4 h post induction using fluorescence microscopy.

Purification of secreted CHIKV-E1 and –E2 subunits from insect cells.

*Sf*21 cells were infected with *Ac*-6KE1ΔTM and *Ac*-E3E2ΔTM at an MOI of 10 in Grace's insect medium without FBS and were incubated for 72 h at 27°C. The recombinant proteins were purified from the medium fraction using Talon® spin columns (Clontech) according to the manufacturer's protocol. Bound protein fractions were eluted with 150 mM imidazol and total protein content was determined via a Bradford protein assay (Biorad) according to the manufacturer's procedure.

Virus neutralization test.

Virus neutralizing antibody titers in sera of rabbits vaccinated with purified CHIKV-E1ΔTM and E2ΔTM, were determined as follows. Serial two-fold dilutions of heat-inactivated rabbit sera were prepared in triplicate in 96-wells plate and 100 $TCID_{50}$ of CHIKV suspension was added to each well. After one hour of incubation at 37°C, 1 x 10^4 BHK-21 cells were added to each well and plates were incubated for another four days. Neutralizing titers were determined microscopically and expressed as the reciprocal of the highest serum dilution still giving 100% suppression of cytopathic effect.

Chapter

Chikungunya virus-like particle production in insect cells.

The emerging arthritogenic, mosquito-borne chikungunya virus (CHIKV) causes severe disease in humans and represents a serious public health threat in countries where *Aedes spp* mosquitoes are present. This study describes for the first time the successful production of CHIKV virus-like particles (VLPs) in insect cells using recombinant baculoviruses. This well-established expression system is rapidly scalable to volumes required for epidemic responses and proved well suited for processing of CHIKV glycoproteins and production of enveloped VLPs. Expression of the CHIKV structural cassette (C, E3, E2, 6K, E1) resulted in correctly glycosylated, furin-processed and fusogenically active glycoproteins (E1 and E2), which are exposed as trimeric spikes on the surface of infected cells. The VLPs were isolated from the medium fraction and appeared morphologically indistinguishable to wild type virus. This study underscores the potential use of CHIKV VLPs produced in insect cells, in CHIKV vaccine development.

This chapter has been published together with chapter 6 as: Stefan W. Metz, Joy Gardner, Corinne Geertsema, Thuy T Le, Lucas Goh, Just M. Vlak, Andreas Suhrbier, Gorben P. Pijlman (2013). *Effective chikungunya virus-like particle vaccine produced in insect cells. PLoS NTD, 7(3);e2124*

Introduction

Chikungunya virus (CHIKV) is a mosquito-borne, single-stranded, positive-sense RNA virus (genus *alphavirus*) that has caused sporadic outbreaks every 2-50 years of predominantly rheumatic disease, primarily in Africa and Asia. CHIKV recently (2004-2011) produced the largest epidemic recorded for an alphavirus with an estimated 1.4 to 6 million patients, and imported cases reported in nearly 40 countries including Europe, Japan and the USA. The first autochthonous CHIKV infections in Europe (Italy in 2007 and France in 2010) were also seen during this epidemic. Although *Aedes aegypti* is the traditional vector for CHIKV, the recent outbreak was associated with the emergence of a new clade of CHIKV viruses, which were efficiently transmitted by *Aedes albopictus* mosquitoes, a vector that has seen a dramatic global expansion in its geographic distribution (Lambrechts et al., 2010; Suhrbier et al., 2012). CHIKV is a biosafety level 3 (BSL3) pathogen and has been declared a Category C Priority Pathogen by the National Institute of Allergy and Infectious Disease (NIAID) in the United States. The US Army has long recognized that CHIKV could be used as a biological weapon (Treble, 2002). The word "chikungunya" is derived from the Makonde language (Tanzania) and means "that which bends up" referring to the severe joint pain-induced posture of afflicted individuals. CHIKV disease is characterized by acute and chronic polyarthritis/polyarthralgia, which is usually symmetrical and often incapacitating, with other symptoms such a fever, rash, myalgia and/or fatigue often also present during the acute phase. Arthropathy usually progressively resolves over weeks to months, usually without long-term sequelae; however, CHIKV infections can sometimes cause severe disease manifestations and mortality (Schwartz and Albert, 2010; Suhrbier et al., 2012).

CHIKV is an enveloped virus of ~70 nm and has an RNA genome of ~11,800 bp (Khan et al., 2002b). Alphaviral RNA encodes two polyproteins; the non-structural polyprotein and the structural polyprotein. The structural polyprotein is translated from a 26S subgenomic mRNA and is processed into the 5 structural proteins; capsid (C), E3, E2, 6K and E1 (Schlesinger and Schlesinger, 2001). The viral RNA is encapsidated in a ~40 nm nucleocapsid, which is tightly enclosed by a host-derived lipid bilayer envelope displaying the viral envelope glycoproteins E1 and E2. The glycoproteins are arranged in 80 trimeric spikes composed of three assembled E1-E2 heterodimers. The trimeric spikes are essential for budding of new virus particles, host receptor recognition and attachment (via E2), and cell entry via pH-dependent endocytosis (via E1). Upon translation of the structural polyprotein, the capsid protein C is autocatalytically cleaved off from the structural polyprotein and encapsidates cytoplasmic viral genomic RNA. The remaining envelope polyprotein (E3E26KE1) is further processed in the endoplasmic reticulum (ER). The resulting membrane bound E3E2 (also known as precursor E2 or PE2) and E1 form heterodimers, with three of these heterodimers assembling to form the trimeric spikes. Prior to surface exposure of the trimeric spikes, PE2

undergoes furin-dependent cleavage to release E3 from the trimeric spike (Kuhn, 2007; Li et al., 2010; Mulvey and Brown, 1996).

At present, no licensed vaccine or particularly effective drug is available for human use for any alphavirus. The use of virus-like particles (VLP) in vaccine development is very promising. VLPs are structurally identical to wild type virus and previous studies have shown that VLP-based vaccines induce protective immune responses in a large variety of viruses (Roldão et al., 2010). Here, we demonstrate successful CHIKV VLP production in insect cells, using recombinant baculovirus expression. We show that CHIKV glycoproteins and VLPs are efficiently processed and that the trimeric spikes retain fusion activity. In addition, the production of VLPs was followed over time and VLP morphology was investigated.

Results

Expression analysis of the CHIKV structural polyprotein, produced in insect cells by recombinant baculoviruses

A recombinant baculovirus (*Ac*-S27) was generated to produce CHIKV VLPs by expressing the complete CHIKV-S27 structural polyprotein (C, E3, E2, 6K, E1) (**Fig. 3-1A**). The coding sequence of the structural polyprotein was cloned downstream the polyhedrin promoter in an *Ac*MNPV backbone, after which *Sf*21 cells were infected with a MOI = 10 $TCID_{50}$/ml. Glycoprotein expression in the cell fraction as well as in the medium fraction was analyzed by WB using α-E1 (**Fig. 3-1B**) and α-E2 (**Fig. 3-1C**) polyclonal antibodies. A recombinant baculovirus expressing GFP (*Ac*-GFP) was used a negative control. Western analysis of the cell fraction yielded protein bands of ~50 kDa for α-E1 (**Fig. 3-1B, lane 2**) and two bands of ~50 kDa and ~57 kDa for α-E2 (**Fig. 3-1C, lane 2**). These sizes correspond to predicted molecular masses of E1 (47.5 kDa), E2 (47.3 kDa) and its precursor E3E2 (54.6 kDa), respectively. CHIKV glycoproteins were also detected in the medium fractions after PEG-precipitation (**Fig. 3-1B and 3-1C, lane 4**). The molecular mass of observed protein bands corresponds to mature E1 and E2. The PEG-precipitated medium fraction containing the VLPs was subjected to discontinuous sucrose gradient purification. The 70% - 40% intermediate phase was isolated and analyzed on WB (**Fig. 3-1B, lane 5**). This resulted in a further concentration of CHIKV- E1 and E2. In addition, a ~30kDa protein band, corresponding to the predicted molecular weight of CHIKV-C, was readily observed on the coomassie-stained SDS-PAGE gel (not shown), suggesting that the VLPs contain a nucleocapsid. To analyze the glycosylation status of the glycoproteins E1 and E2, the infected cell fraction and the purified VLPs were treated with PNGase F, which enzymatically removes glycan residues from N-glycosylated proteins fractions. CHIKV-E1 is predicted to be N-glycosylated at N141, whereas E2 is predicted to be N-glycosylated at N263 and N273 (**Fig. 3-1A**) (Blom et al., 2004). PNGase F treatment resulted in an expected reduction in

molecular mass of CHIKV-E1 in both the cell fraction and purified VLPs (**Fig. 3-1C, lane 2-5**), indicating that E1 was efficiently N-glycosylated by the insect cells. The size difference between non-treated and treated samples was significantly larger for E2 than that of E1, which may suggest that E2 is N-glycosylated at the two predicted loci (**Fig. 3-1C, lane 2-4**). In addition, both protein bands (presumably E2 and E3E2, based upon earlier observations (Metz et al., 2011b)) in the double-band pattern that were found using α-E2 detection (**Fig. 3-1B and 3-1C**), appeared to be fully glycosylated.

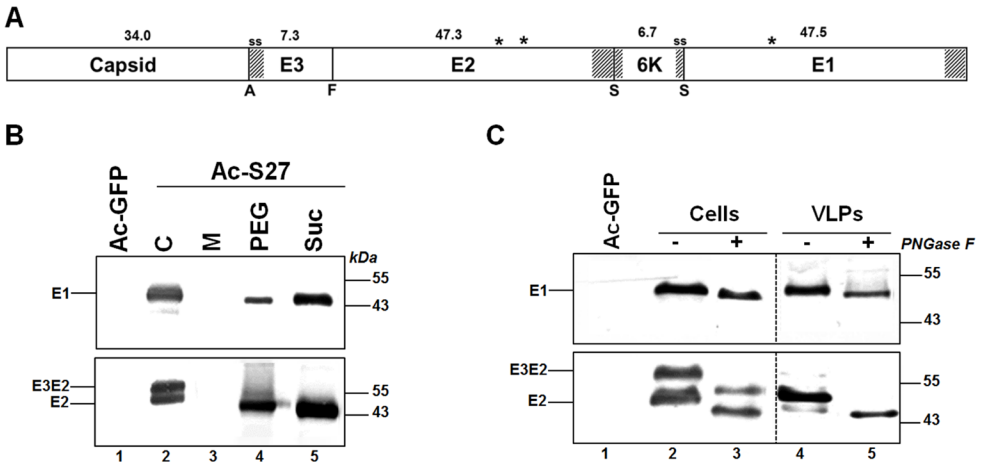

Figure 3-1. CHIKV structural cassette expression and VLP production, using recombinant baculoviruses. A) Schematic representation of the CHIKV structural cassette, as it was expressed in insect cells. Shaded areas represent transmembrane domain and ss signifies signal sequences. A, F and S indicate autocatalytic, furin and signalase cleavage sites, respectively. Asterisks indicate N-glycosylation sites and the molecular mass (in kDa) of the proteins is depicted. B) CHIKV E1 and E2 expression in the *Sf*21 cell (C) and medium (M) fraction was analysed by WB using α-E1 and α-E2 polyclonal antibodies. VLPs were precipitated using PEG-6000 (PEG) and subsequently purified using discontinuous sucrose gradient centrifugation (Suc). C) The glycosylation status of CHIKV E1 and E2 was analysed by WB after PNGase F treatment. Ac-GFP was used as a negative control.

CHIKV glycoproteins E1 and E2 localize to the surface of Sf21-cells.

During natural infections, CHIKV-E1 and -E2 are assembled into trimeric spikes, which are expressed at the surface of the host cell, to enable budding of new virus particles. Expression analysis has made clear that E1 and E2 are both glycosylated and that a fraction of PE2 is processed by furin. To analyze whether the glycoproteins were subsequently translocated to the cell plasma membrane, non-permeable *Sf*21 cells infected with *Ac*-S27 were treated with α-E1 and α-E2 to enable immunofluorescence analysis. Treated cells displayed ring-like structures (**Fig. 3-2, middle and bottom**), indicating that the glycoproteins are indeed exposed at the surface of the infected cells. The non-infected mock cells did not reveal these ring-like structures (**Fig. 3-2, top**).

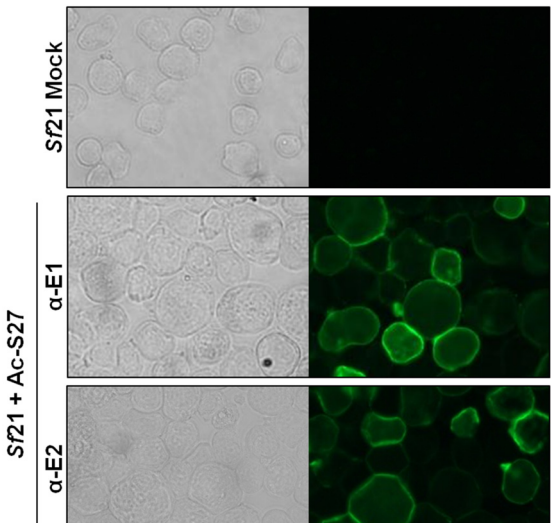

Figure 3-2. CHIKV-E1 and –E2 detection on the surface of Ac-S27 infected *Sf*21 cells.
*Sf*21 cells were infected with *Ac*-S27 and subjected to immunostaining using α-E1 and α-E2 antibodies. Cells were analysed by fluorescent microscopy where positive staining indicates E1 or E2 surface exposure.

CHIKV E1 retains fusogenic activity when expressed by recombinant baculoviruses.

So far, experiments have shown that CHIKV E1 and E2 are expressed, processed correctly and exposed at the surface of the infected host cell. This suggests that maturation of the recombinant CHIKV structural proteins appears to correspond to what happens during natural virus infection. To test whether CHIKV E1 retains its functionality as a fusion protein, a pH-dependent syncytia formation assay was performed. *Sf*9-ET cells were infected with *Ac*-S27, *Ac*-6KE1 and *Ac*-GFP (negative control). *Ac*-6KE1 is a recombinant baculovirus that expresses individual fusogenic E1 (Metz et al., 2011b) and was used as a positive control. Infected cells were treated for 2 min with acidified medium (pH = 5.8, pH = 5.5 and pH = 5.0) and screened for syncytia formation 4 h post treatment.

Syncytia formation at pH =6.4, pH = 5.8, pH = 5.5 was readily observed in cells expressing CHIKV structural proteins (*Ac*-S27) (**Fig. 3-3, right**) and in the positive control (*Ac*-6KE1) (**Fig. 3-3, middle**) but not in the negative control (*Ac*-GFP) (**Fig. 3-3, left**). Syncytia observed in Ac-S27 infected cells were slightly more abundant and also larger in size, suggesting that E1 in its native conformation, i.e. in trimeric spikes closely associated with E2, displays increased fusogenic activity as compared to individual E1 expression in *Ac*-6KE1 infected cells. In contrast to *Ac*-S27 and *Ac*-6KE1, *Ac*-GFP infected cells were only able to fuse when treated with acidified medium of pH = 5.0 (**Fig. 3-3, left**). This was due to the pH-dependent activity of the baculovirus fusion protein GP64, which becomes active only at values below pH= 5 (Volkman, 1986). The formation of syncytia correlates with the presence of E1 on the surface, individually expressed or expressed as a part of the CHIKV structural polyprotein. Therefore, it can be concluded that E1 retains its fusogenic properties when expressed in *Sf*-cells.

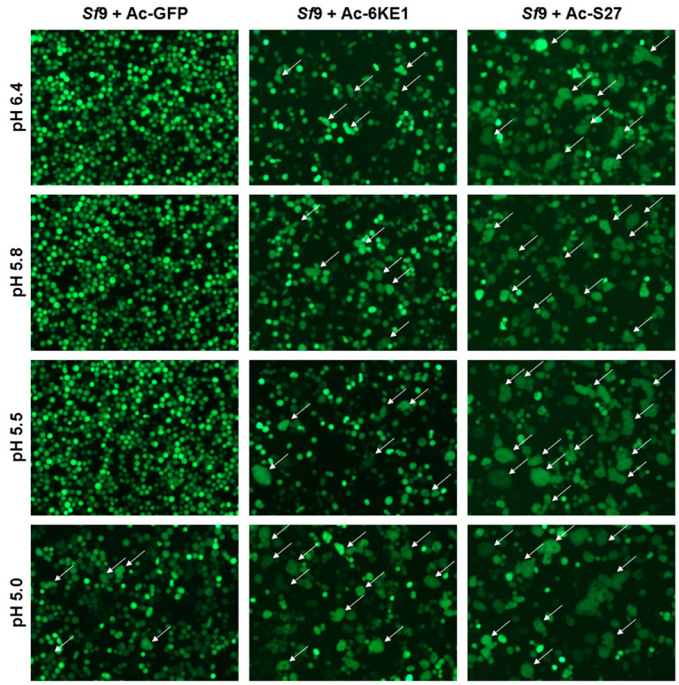

Figure 3-3. Syncytia formation assay on *Ac*-S27 infected insect cells.
*Sf*9 ET- cells were infected with *Ac*-S27 and treated 72 hpi with acidified culture medium of pH = 6.4, 5.8, 5.5 and 5.0 for 2 min. Cells were analysed 4 h post induction and syncytia are indicated with white arrows.

Production of CHIKV virus-like particles in insect cells

Baculovirus expression of the complete CHIKV structural cassette in insect cells leads to correct processing of the CHIKV glycoproteins E1 and E2, which both are exposed on the surface of the host cell. Western analysis of the medium fraction indicated that both E1 and E2 are present in the medium and can be concentrated by discontinuous sucrose gradient purification. These findings strongly suggest that VLPs were formed and were secreted in the medium. To analyze VLP production in time, a time-course expression assay was performed and CHIKV-VLPs were detected by a sandwich-ELISA, using α-E2 antibodies (**Fig. 3-4A**). *Sf*21-cells were infected in duplo with *Ac*-S27 and medium samples were taken within 7 to 9 h intervals. VLP production in *Sf*21-cells initiated at ~28 hpi, typical for polyhedrin expression. Production peaked at ~ 61 hpi and longer production periods, yielded lower amounts of VLPs probably due to cell death.

For the final verification of CHIKV-VLP production, insect cells were infected with *Ac*-S27 and the medium fraction was subjected to discontinuous sucrose gradient purification. The isolated VLP fraction was analyzed by transmission electron microscopy (TEM) (**Fig. 3-4B**). Spherical, enveloped particles of ~65-70 nm, were detected in large numbers and were absent in control infections with *Ac*-GFP. The isolated VLPs varied in size, within a range of ~55 -80 nm. The diameters of 200 VLPs were measured to determine a relative size distribution (**Fig. 3-4C**). The CHIKV VLPs fitted a size of 68 ± 14 nm, which is consistent with the reported size (65-70 nm) of alphavirus virions. The specific E2 protein content of the isolated VLPs was determined at 40 mg/l.

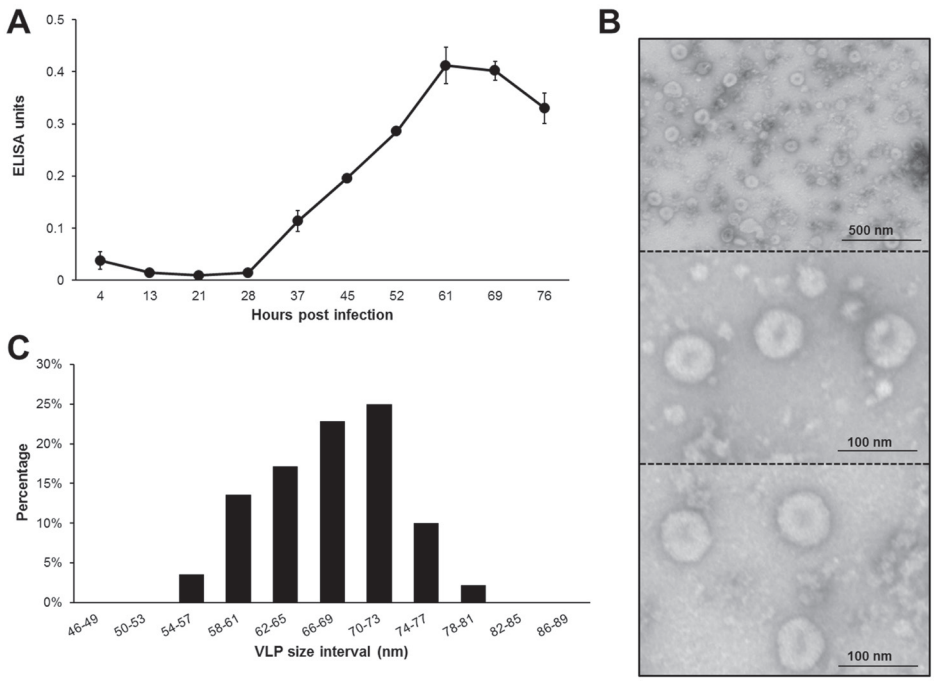

Figure 3-4. CHIKV VLP production in insect cells.
*Sf*21 cells were infected with *Ac*-S27 in duplo and VLP production was followed over time. Medium fraction samples were taken at the indicated time points and analysed on E2 content by sandwich ELISA. B) Electron micrographs of CHIKV-VLPs at a 12,000:1 (top) and 40,000:1 (middle and bottom) magnification. The medium fraction of infected *Sf*21 cells was analysed by transmission electron microscopy (TEM) for the presence of VLPs. C) The size of 200 VLPs were determined using TEM to determine the relative VLP size range.

Discussion

In this study, recombinant baculoviruses were used to produce CHIKV VLPs in *Sf*21 insect cells. The VLP proteins were correctly processed and the VLPs provided complete protection against CHIKV viraemia and arthritic disease in a mouse model after a single dose of CHIKV VLPs.

The complete CHIKV structural cassette was cloned downstream the strong polyhedrin promoter of the AcMNPV baculovirus (*Ac*-S27). Western analysis on cell and medium fractions of *Sf*21-cells that were infected with *Ac*-S27 indicated that glycoprotein processing (glycosylation, furin cleavage and surface localization) is efficient and that the functionality of E1 as fusion protein is retained. Western analysis on the precipitated medium fraction and purified VLPs shows that only fully matured and glycosylated E2 was incorporated into the VLPs, which suggest that the recombinant VLPs are homogenous. This contrasts with previous findings that uncleaved E3E2 is present in progeny alphavirus particles (Ozden et al., 2008; Zhang et al., 2011). However, both immature (presumably E3E2) and mature, furin-cleaved E2 fractions were found intracellularly, most likely a result of the very high expression levels of the CHIKV proteins. Alphaviral processing intermediates are commonly found in many different expression systems, including recombinant baculoviruses (Cho et al., 2008; Hodgson et al., 1999; Oker-Blom et al., 1995; Oker-Blom and Summers, 1989). The triple-banded E2 pattern previously observed upon individual expression of E3E2 (Metz et al., 2011b) was not found after expression using *Ac*-S27, indicating that in this case all glycoproteins were efficiently glycosylated. The postulated number of N-glycosylation sites of E1 (n=1) and E2 (n=2) correspond to the protein size shifts after PNGase F treatment. These results demonstrate that the processing efficiency of E3E2 in insect cells increases when CHIKV glycoproteins E1 and E3E2 are co-expressed as part of a polyprotein, when compared with expression of E3E2 by itself (Metz et al., 2011b). To obtain more insight in the exact number and type of glycan-moieties on both E1 and E2, in-depth studies are required (e.g. mass spectrometry), which can precisely indicate to what extent the glycoprotein intermediates are processed.

In addition to correct processing, CHIKV-E1 and E2 were found exposed on the surface of the infected insect cells. The green fluorescent rings found after immunostaining *Ac*-S27 infected insect cells, mark the final stage of processing and translocation of the glycoproteins within the host cell, just prior to the budding of the VLPs. In addition, the retained fusogenic function of E1 was shown by treatment of *Ac*-6KE1, *Ac*-GFP and *Ac*-S27-infected *Sf*9-ET cells with acidified culture medium (pH = 5.8, 5.5 and 5.0) resulting in increased syncytia formation. This was not a consequence of the baculovirus GP64 fusion protein, as the control baculovirus Ac-GFP only formed syncytia at pH= 5.0 (Volkman, 1986).

The VLPs were efficiently isolated using discontinuous sucrose gradient purification. Transmission electron microscopy (TEM) analysis revealed that the VLPs were morphologically similar to CHIKV and other alphavirus VLPs and that they had a similar diameter of 68 ± 14 nm (Akahata et al., 2010; Metz et al., 2011a). The overall baculovirus CHIKV VLP yield (40 mg/L) appeared to be higher than in another study, in which VLPs were produced by DNA-transfection of 293F cells (10-20 mg/L) (Akahata et al., 2010). This underscores one of the major advantages of the baculovirus-insect cell system for production of recombinant proteins. A further gain in VLP yield is expected in an optimized large-scale insect-cell bioreactor configuration (Vicente et al., 2011).

This study provides clear evidence that CHIKV VLPs can be efficiently produced in insect cells using the recombinant baculovirus-insect cell expression system. The immunogenicity of the CHIKV VLPs and their potential use in a VLP-based CHIKV vaccine will be investigated in a follow up study involving vaccination trials in mice (Gardner et al., 2010).

Acknowledgements

We thank Roy Hall for supplying monoclonal antibodies and Hannelie Semmelink for initial studies on CHIKV VLP expression. We acknowledge Jacco Heldens and Han van den Bosch (Nobilon International BV) and Ab Osterhaus and Byron Martina (Erasmus Medical Centre, Rotterdam) for their support and continued interest. This work was performed within the framework of Dutch Top Institute Pharma, project “Development of recombinant live and subunit vaccines against chikungunya virus infections” (project nr. T4-301).

Material and Methods

Cells and viruses.

Adherent *Spodoptera frugiperda* (*Sf*21)-cells (Invitrogen) were maintained as a monolayer tissue culture in Grace's insect cell medium (Invitrogen), supplemented with 10% foetal bovine serum (FBS, Gibco). *Sf*9-easy titration (ET) cells (Hopkins and Esposito, 2009) were maintained as a monolayer cell culture using *Sf*900II (Invitrogen) serum-free medium (SFM), supplemented with 5% FBS and 200µg/ml Geneticin (Gibco). Recombinant baculoviruses were generated according to the Bac-to-Bac baculovirus expression system, using an adapted Autographa californica nucleopolyhedrovirus (*Ac*MNPVΔp10Δcc) backbone (Kaba et al., 2004; Metz et al., 2011b). The cloning fragment of the complete CHIKV-S27 structural polyprotein (Genbank accession # AF369024) was synthetically generated (GeneArt®) and equipped with AttB recombination sites to enable Gateway® cloning (Invitrogen). The 3842 bp CHIKV fragment was cloned into pDONR207 (Invitrogen) donor plasmid and subsequently transferred to the pFastBacI analogue pDEST8 (Invitrogen). The CHIKV-S27 structural cassette was then recombined into the *Ac*MNPVΔp10Δcc, resulting in *Ac*-S27. Recombinant baculovirus titers were determined by end point dilution assays using *Sf*9-ET cells and expressed in tissue culture infectious dose 50 ($TCID_{50}$)/ml.

CHIKV VLP production and purification.

To produce CHIKV VLPs, 8×10^6 *Sf*21-cells were seeded in a 75 cm^2 culture flask and infected with *Ac*-S27 at a multiplicity of infection (MOI) of 10 $TCID_{50}$ units per cell. Infections were performed under serum free conditions on a shaking platform at 27°C and cells were incubated at 27°C for 72 h. Next, cells were separated from the medium fraction by low speed centrifugation. The cell fraction was washed in phosphate buffered saline (PBS) and finally stored in 200 µl PBS at -20°C. Secreted protein fractions were precipitated from the medium with 7% (w/v) polyethylene glycol (PEG)-6000 and 0.5M NaCl for 2 h at room temperature (RT). Pellets were resuspended in 1 ml GTNE buffer (200nM Glycine, 50mM Tris/HCl, 100mM NaCl, 1mM EDTA, pH 7.3) and loaded on a discontinuous 70% (w/v), 40% (w/v) sucrose in GTNE gradient. Sucrose gradients were centrifuged at 27,000 rpm (SW55 rotor, Beckman) for 2 h at 4°C. The 70%-40% interphase band was isolated and resuspended in 5 ml GTNE buffer. The VLPs were pelleted by centrifugation with 30000 rpm, for 30 min at 4°C. The pellet was resuspended in 50 µl GTNE, checked for integrity by transmission electron microscopy and stored at -80°C. VLPs were quantified based on specific E2 protein content, which was determined by Western analysis and via Bradford protein assay (Biorad) and calculated using purified E2 subunit (Metz et al., 2011b) as a reference.

Protein analysis.

Protein expression and processing of infected *Sf*21-cell fractions and purified CHIKV VLP fractions were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie Brilliant Blue (CBB) staining. The purified VLPs and cell fractions were denatured in a gel loading buffer containing SDS and β-mercaptoethanol, incubated for 10 min at 95°C and clarified by centrifugation for 1 min at 14,000 rpm. After electrophoresis, denatured proteins were transferred to an Immobilon membrane (Millipore) for analysis by Western blot (WB). Membranes were blocked in 3% skimmed milk in PBS-0.1% Tween-60 (PBST) for 1 h at RT or overnight (ON) at 4°C. Blocked membranes were washed 3 x 5 min with PBST and subsequently incubated for 1 h at RT with rabbit polyclonal anti-E1 and anti-E2 (Metz et al., 2011b), 1:15,000 and 1:20,000 diluted in PBST, respectively. Membranes were washed and treated with alkaline phosphatase (AP) conjugated, goat anti-rabbit IgG monoclonal antibodies (Sigma), 1:3000 times diluted in PBST, for 45 min at RT. Membranes were washed 2 x 5 min with PBST and 1 x 10 min with AP-buffer (100mM NaCl, 5mM $MgCl_2$, 100mM Tris-HCl, 0.1% Tween 20, pH=9.5). Proteins were detected by NBT/BCIP staining (Roche).

PNGase F treatment.

Infected cell- and medium-fractions were treated with PNGase F (New England Biolabs) to determine the glycosylation status of the CHIKV glycoproteins E1 and E2. Protein samples were treated with 1 µl denaturing buffer in 9 µl MilliQ for 10 min at 95°C. The denatured proteins were subsequently incubated with 2 µl G7 reaction buffer, 2 µl 10% NP40 buffer, 0.5 µl PNGase F in 4.5 µl MilliQ for 1 h at 37°C. Treated and non-treated protein samples were analyzed by SDS-PAGE and WB.

Syncytia induction assay.

*Sf*9-ET cells (Hopkins and Esposito, 2009) were infected with *Ac*-S27 and *Ac*-GFP with a MOI of 10 $TCID_{50}$/ml in *Sf*900-II SFM medium. A recombinant *Ac*MNPV expressing CHIKV 6KE1 (*Ac*-6KE1) (Metz et al., 2011b) was used as a positive control. The medium (pH = 6.4) was supplemented with 0.2 mg/ml cholesterol (Sigma) as described previously (Metz et al., 2011b). Cell fusion was induced 72 hpi, by treating the cells for 2 min with acidified medium with pH = 5.8, pH = 5.5 and pH = 5.0, respectively. Syncytia formation was analyzed 4 h post treatment by fluorescence light microscopy.

Immunofluorescence assay.

To determine surface expression of CHIKV-E1 and -E2, *Sf*21-cells were infected with *Ac*-S27, with a MOI of 10 $TCID_{50}$/ml in Grace's insect SFM (Invitrogen). Cells were harvested 72 hpi and washed with PBS. Next, cells were incubated with PBS containing 1:5000 diluted rabbit α-E1 and rabbit α-E2 polyclonal antibodies, for 1 h at RT. Cells were washed 3 x 5 min with PBS and treated with 1:1000 diluted goat-anti-rabbit Alexa fluor 488 (Invitrogen) for 1 h at RT. Finally, cells were washed and treated with 1:100 diluted Hoechst stain for 5 min at RT. Cells were analyzed using fluorescence light microscopy.

Time course expression assay and VLP- ELISA.

To analyze CHIKV-VLP production in time, *Sf*21 cells were infected with *Ac*-S27 and samples were taken at intermediate time points from 4 h to 69 hpi. The medium fraction was analyzed for the amount of VLPs in triplo by enzyme linked immunosorbent assay (ELISA). ELISA plates (Greiner Bio-One) were coated with 2.5 µg/ml rabbit α-E2 polyclonal antibodies (Metz et al., 2011b) in coating buffer for 2 h at RT. Plates were washed three times in PBST, and medium samples were loaded for 2 h at 37°C. The plates were washed three times and treated with 1:500 diluted α-E2 monoclonal antibodies (52B2, provided by Lucas Goh) for 2 h at 37°C. Plates were washed and incubated for 2 h at 37°C with 1:500 diluted, AP-conjugated, goat-anti-mouse IgG monoclonal antibodies (Sigma). Finally, plates were washed three times and treated with 1 mg/ml phosphatase substrate (Sigma) in substrate buffer for 45 min at 37°C. Absorbance was measured at 405 nm using a FLUOstar Optima (BMG Labtech).

Electron Microscopy.

Copper 400 square mesh grids (Veco) were treated by Argon gas discharge and loaded with 10 µl sample for 2 min at RT. Excess liquid was removed and the grids were washed five times with MilliQ. Finally, grids were treated with 2% uranyl acetate for 15 s, excess uranyl acetate was carefully removed using filter paper. The grids were air dried and analyzed with a JEOL JEM 1011 transmission electron microscope.

Chapter

Low temperature-dependent salmonid alphavirus glycoprotein processing and recombinant virus-like particle formation.

Pancreas disease (PD) and sleeping disease (SD) are important viral scourges in aquaculture of Atlantic salmon and rainbow trout. The etiological agent of PD and SD is salmonid alphavirus (SAV), an unusual member of the *Togaviridae* family (genus *Alphavirus*). SAV replicates at lower temperatures in fish. Outbreaks of SAV are associated with large economic losses of ~17 to 50 million $/year. Current control strategies rely on vaccination with inactivated virus formulations that are cumbersome to obtain and have intrinsic safety risks. In this research we were able to obtain non-infectious virus-like particles (VLPs) of SAV via expression of recombinant baculoviruses encoding SAV capsid protein and two major immunodominant viral glycoproteins, E1 and E2 in *Spodoptera frugiperda Sf*9 insect cells. However, this was only achieved when a temperature shift from 27°C to lower temperatures was applied. At 27°C, precursor E2 (PE2) was misfolded and not processed by host furin into mature E2. Hence, E2 was detected neither on the surface of infected cells nor as VLPs in the culture fluid. However, when temperatures during protein expression were lowered, PE2 was processed into mature E2 in a temperature-dependent manner and VLPs were abundantly produced. So, temperature shift-down during synthesis is a prerequisite for correct SAV glycoprotein processing and and recombinant VLP production.

This chapter has been published as: Stefan W. Metz, Femke Feenstra, Stephane Villoing, Mariëlle C. van Hulten, Jan W. van Lent, Joseph Koumans, Just M. Vlak, Gorben P. Pijlman (2011. *Low temperature-dependent salmonid alphavirus glycoprotein processing and recombinant virus-like particle formation. PLoS one 6;10;e25816*

Introduction

Global fish-aquaculture has grown extensively over the past 50 years, producing yearly up to 52.5 million tons worth of an estimated US$98.5 billion. Approximately 50% of the world's fish supply is derived from fish farming, of which salmon and trout represent high value cultivates with an output of over 1.5 million tons worth US$7.2 billion per year (Bostock et al., 2010). Unlike other reared and farmed animals, fish are often cultured in open systems, exposing them to a wide variety of naturally occurring pathogens, in particular fish infecting viruses (Snow, 2011).

Salmonid alphavirus (SAV) is a serious pathogen for European farmed Atlantic salmon, *Salmo salar* and rainbow trout, *Oncorhynchus mykiss* and is the etiological agent of pancreas disease (PD) and sleeping disease (SD), respectively. PD is associated with lack of appetite, lethargy, pancreatic and heart lesions and increased mortality up to 48% of infected fish (McLoughlin and Graham, 2007). SD is characteristically presented by fish lying on their side on the bottom of the culture tank due to severe skeletal muscle necrosis (Boucher and Laurencin, 1996). SAV is an unusual alphavirus and differs from other alphaviruses like sindbis virus (SINV), Semliki forest virus (SFV) or chikungunya virus (CHIKV) as it solely replicates in fish (mainly salmonids) and has no known arthropod vector. Although SAV has successfully been detected in parasitic salmon louse species, the supposition of lice being a vector for SAV transmission remains to be proven, since active SAV replication within lice has not yet been confirmed (La Linn et al., 2001; Petterson et al., 2009). In addition, it has been shown that SAV can be transmitted from fish to fish in cohabitation experiments in the absence of an arthropod vector (Boucher et al., 1995) (McLoughlin et al., 1996).

SAV belongs to the genus *Alphavirus* within the *Togaviridae* family and represents at least six closely related subtypes, of which three are of special interest: salmon pancreas disease virus (SPDV or SAV1) from Ireland, sleeping disease virus (SDV or SAV2) from France and the Norwegian salmonid alphavirus (NSAV or SAV3) (McLoughlin and Graham, 2007). SAV virions are enveloped spherical (~65 nm) particles and contain a positive sense, single-stranded RNA genome of approximately 12kb (Nelson et al., 1995). The viral RNA encodes two open reading frames (ORF); the non-structural ORF, which is directly translated from the genomic RNA, and the structural ORF, which is encoded by a 26S sub-genomic mRNA and is processed into 5 structural proteins – capsid, E3, E2, 6K, and E1. SAV transmembrane glycoproteins E1 and E2 are exposed on the virion surface as trimeric spikes, facilitating cell receptor recognition (presumably E2), cell entry via pH-dependent endocytosis (presumably E1) and support budding. E2 also serves an important role in regulating the fusion activity of E1 so that this does not occur before endocytosis (White and Helenius, 1980). The acidic endosomal environment dissociates the trimeric spike and causes E1 to initiate fusion with the endosomal membrane (Gibbons et al., 2000; Wahlberg et al., 1989), thereby releasing the

nucleocapsid and subsequently the viral RNA into the cytoplasm of the cell.

During translation of the structural polyprotein, the capsid protein is autocatalytically cleaved off from the structural polyprotein to encapsidate newly synthesized genomic RNA. The remaining envelope cassette (E3-E2-6K-E1) is subsequently translocated to the endoplasmatic reticulum (ER) and is processed by host signalases at the N-terminal and C-terminal end of 6K, yielding E3E2 (precursor E2; PE2), 6K and E1(Kuhn, 2007). The membrane anchored PE2 and E1 form heterodimers and three PE2-E1 dimers will eventually assemble into heterotrimers in the rough ER of infected cells (Li et al., 2010; Mulvey and Brown, 1996; Strauss and Strauss, 1994; Ziemiecki et al., 1980). The presence of E3 within the heterotrimers offers resistance against the acidic environment of the Golgi apparatus, to avoid premature trimer activation by homotrimerization of E1 (Kuhn, 2007). In the trans-Golgi system, PE2 undergoes furin-dependent maturation, thereby releasing E3 from E2. However, E3 may remain associated with the E1-E2 trimers in the acidic compartments of the cell and dissociates once the trimers reach the cell surface to prime the spikes for acidic activation (Sjoberg et al., 2011b).

SAV infection in farmed salmonids can be minimized by reducing stress and good hygienic culture methods, such as sea-lice control and proper boat and transporter cleaning and disinfection. Next to these control measures, vaccination in fish has shown to be effective in protecting farmed salmon and trout from SAV infections. Few studies have focused on SAV-specific immune responses in fish, but it has been shown that infected salmonids generate a short-lasting (~9 months) protective immune response against subsequent SAV infections (Houghton, 1994) and that, similar to other alphaviruses, immune cells are involved in dissemination of infection in the host (Houghton, 1995; Lidbury et al., 2008). Although the immune response against SAV is not fully understood, it has been demonstrated in multiple studies that salmonids can be protected against SAV challenge by immunization with inactivated virus formulations and that immunized fish elicit neutralizing antibodies, resulting in viral clearance (Desvignes et al., 2002; McLoughlin et al., 1996). Several vaccination strategies have been developed and tested, such as formalin-inactivated viral vaccines, recombinant and attenuated live vaccines (Benmansour and de Kinkelin, 1997; Lopez-Doriga et al., 2001; McLoughlin and Graham, 2007; Moriette et al., 2006; Sommerset et al., 2005). Although these vaccines provide cross-protection to all SAV-subtypes, safety issues such as incomplete inactivation of active virus remain problematic. The use of a subunit vaccine may serve as an elegant alternative for inactivated or live-attenuated vaccines, in particular the use of virus-like particles (VLPs). VLPs are non-pathogenic virus look-alikes, since they are morphologically similar to virions but are non-infectious as they do not contain viral RNA. Recent studies have shown that the expression of the CHIKV-structural polyprotein in culture cells results in the formation of VLPs, which induced a protective immune response in non-human primates (Akahata et al., 2010). However, the low

temperature replication and assembly of SAV are potentially major hurdles to the production of SAV VLPs.

This study focuses on the genesis of SAV VLPs using the recombinant baculovirus-insect cell expression system. This expression system has proven to be an efficient and safe way to express heterologous proteins on large scale in insect cells, which resulted in the production of several commercially available human and veterinary viral vaccines (Bouma et al., 1999; Cox and Hollister, 2009; Kost et al., 2005; Paavonen and Lehtinen, 2008; van Oers, 2006). The recombinant insect cell expression system is based upon the exchange of the baculovirus polyhedrin gene for the heterologous gene of interest. Polyhedrin expression is controlled by the strong *polyhedrin* promoter, thereby enabling high heterologous protein expression. Next to high level protein expression, insect cells enable post-translational protein modification and accurate folding (van Oers, 2006; Vialard et al., 1995) and several studies have shown efficient alphavirus protein expression, using recombinant baculoviruses (Hodgson et al., 1999; Metz et al., 2011b; Oker-Blom and Summers, 1989). In addition, the baculovirus-insect cell expression has been shown to be an elegant method for VLP production of enveloped and non-enveloped arboviruses (Metz and Pijlman, 2011). In this study, we show that the formation of SAV VLPs in insect cells does not take place under standard conditions, but that it is dependent on the level of processing of the envelope glycoprotein E2. In addition, we show that E2 processing is a function of temperature and is only complete at low temperatures. Finally, we present an optimized production process involving a temperature-shift regime to allow the efficient secretion of SAV VLPs in the culture fluid of baculovirus-infected insect cells.

Results

Expression of SAV3 structural cassette in Sf9 insect cells by recombinant baculoviruses.

A recombinant baculovirus was generated (*Ac*-SAV3) to express the complete SAV3 structural cassette C-E3-E2-6K-E1 (**Fig. 4-1A**). The complete coding sequence of the structural cassette was cloned downstream of the polyhedrin promoter of an adapted AcMNPV backbone. Insect cells were infected with a MOI of 10 $TCID_{50}$ / cell and were incubated for 72 h. Protein expression in the cell fraction was analyzed by CBB and WB using α-E1 and α-E2 mabs. Total protein-staining by CBB showed high levels of expression based upon the abundant band of approximately 35 kDa, which closely matches the predicted size of the SAV capsid protein (**Fig. 4-1B, left**). Western analysis using α-E1 and α-E2 mabs yielded bands of ~50 kDa and ~55 kDa, respectively (**Fig. 4-1B, center**). These sizes correspond to the predicted molecular mass of E1 (49.2 kDa) and E3E2 (54.8 kDa), suggesting that expression of the SAV3 structural cassette results in an E3E2 intermediate that is not further processed by host furin.

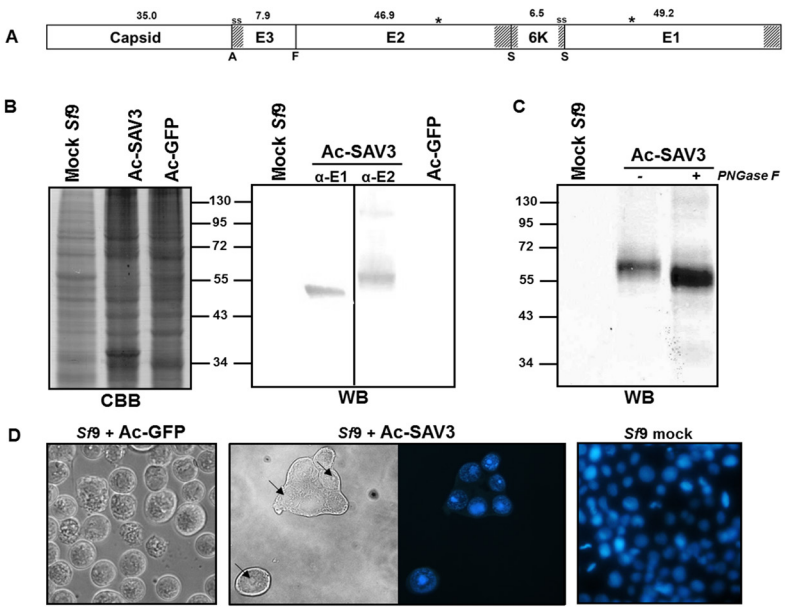

Figure 4-1. SAV3 structural cassette expression, using recombinant baculoviruses.
A) Schematic representation of the SAV3 structural cassette as it is expressed by recombinant baculoviruses. The molecular mass of the proteins are indicated and shaded areas represent transmembrane domains or signal sequences (ss). Autocatalytic (A), furin (F) and signalase (S) cleavage sites are indicated, asterisks represent N-linked glycosylation sites. B) Protein expression in *Sf*9 cells was analyzed by CBB and WB using SAV α-E1 and SAV α-E2 mabs. C) Whole cell lysates were treated with/without PNGase F and analyzed with SAV α-E2 mabs. D) Cells infected with *Ac*-GFP and *Ac*-SAV3 and stained with Hoechst. CPE was evaluated by brightfield and fluorescence microscopy. Arrows indicate dense nuclear bodies in *Ac*-SAV3-infected insect cells.

To analyze if SAV glycoproteins were glycosylated (**Fig. 4-1C**), expression products were treated with PNGase F, which enzymatically removes carbohydrate residues from proteins. SAV3-E2 is predicted to be N-glycosylated at N318 (**Fig. 4-1A**) (Blom et al., 2004; Strauss and Strauss, 1994). As expected, PNGase F treatment reduced the size of E3E2 with a few kDa (**Fig. 4-1C**). The shift in molecular mass of the SAV3-E3E2 fraction after PNGase F treatment suggests that SAV3-E2 is indeed N-glycosylated, when expressed in *Sf*9 insect cell by recombinant baculoviruses. The absence of non-glycosylated protein fraction in the untreated sample indicates that SAV3-E2 is very efficiently glycosylated in insect cells, despite the fact that E3 is not released from E3E2 by furin cleavage. Infected cells displayed baculovirus-specific cytopathic effect (CPE) including decreased cell growth, enlarged nuclei and cell monolayer detachment. However, *Sf*9 cells infected with *Ac*-SAV3 showed additional CPE. Cell membranes fused between closely neighboring infected cells resulting in polykaryons or syncytia (**Fig. 4-1D**).

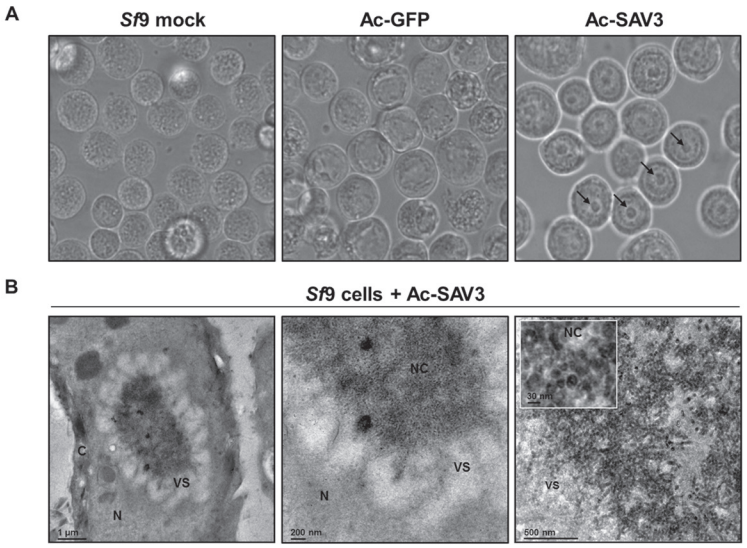

Figure 4-2. Nuclear localization and assembly of SAV3-nucleocapsids.
*Sf*9 infection with *Ac*-SAV3 and *Ac*-GFP was analyzed by A) light-microscopy at 2 d.p.i. B) *Ac*-SAV3-infected cells were fixed and embedded in gelatin and ultrathin coupes were analyzed by TEM, with C, N, VS and NC indicating cytoplasm, nucleus, virogene stroma and SAV nucleocapsids, respectively.

This syncytia formation was most likely induced by the fusogenic activity of SAV-E1, since alphavirus E1 regulates fusion during endocytosis in wildtype infections (Strauss and Strauss, 1994). In addition to the enlarged nuclei and syncytia, infected *Sf*9 cells contained dense nuclear bodies that were only detectable in cells expressing the SAV structural cassette (**Fig. 4-1D and Fig. 4-2A**, indicated with arrows) Nuclear staining using Hoechst suggested that the nuclear bodies contained nucleic acids (**Fig. 4-1D**).

To further investigate the nature of these nuclear bodies, infected cells were fixed and embedded in gelatin after which the cells were analyzed by transmission electron microscopy (TEM) (**Fig. 4-2B**). All infected cells developed the dense structures which appeared to consist of aggregated, smaller spherical structures, surrounded by a halo of viral stroma. Based on morphology and size (~30 nm) and the fact that SAV3-capsid contains a nuclear localization signal (Karlsen et al., 2010), the spherical structures corresponds most likely to assembled SAV nucleocapsids.

Temperature-dependent processing of SAV3 structural proteins.

The analysis of the SAV3 structural cassette, expressed by recombinant baculoviruses at 27°C, showed that SAV3-E2 was efficiently glycosylated, but was not released from its E3E2 precursor by host furin-like proteases. Since SAV is an alphavirus of cold-water fish, the lack of furin-processing in E3E2 might be caused by the significant difference in environmental temperature between wildtype SAV replication (12-15°C) (McLoughlin and Graham, 2007) and baculovirus expression (27°C). To investigate this putative temperature-effect on furin-dependent processing, *Sf*9-cells were infected with *Ac*-SAV3 and incubated at 12°C, 15°C, 18°C and 27°C for 14, 10, 7 and 3 days, respectively. Whole cell lysates were treated with PNGase F and analyzed using WB. Expression at 27°C (**Fig. 4-3A, lane 2**) resulted in similar protein patterns as seen before (**Fig. 4-1B**). The 55 kDa protein band, corresponding to glycosylated but unprocessed SAV-E3E2, was also detected at all lower expression temperatures (**Fig. 4-3A, lane 3, 4, 5**).

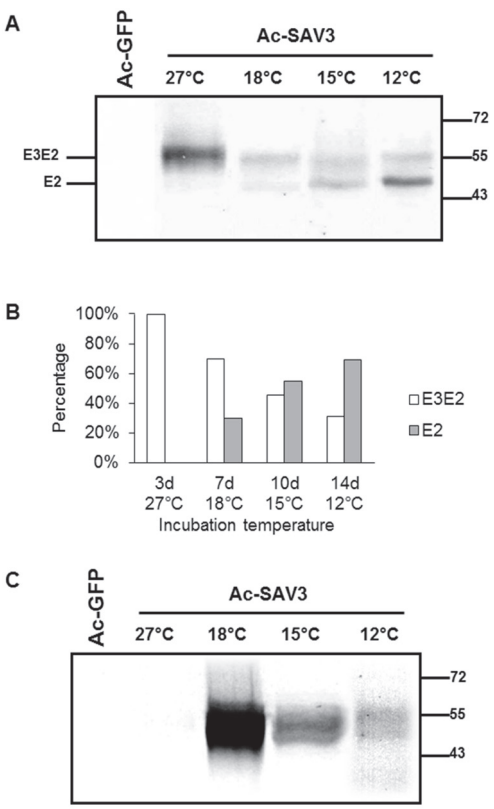

Figure 4-3. Temperature-dependent processing and secretion of SAV-E2.
A) *Sf*9 cells were infected with *Ac*-SAV3 with an MOI of 10 at 27°C, 18°C, 15°C and 12°C. SAV-E2 expression in the cell-fraction was analyzed by WB using SAV α-E2 mab (17H23). B) Relative percentages of SAV-E3E2 and E2, indicating more efficient processing of E3E2, with decreasing temperatures. C) Secretion of E2 as a function of the temperature. The medium fraction of infected *Sf*9-cell cultures was PEG-precipitated and analyzed by WB using SAV α-E2 mab.

However, in addition to E3E2, a second protein band of lower molecular mass was found. The smaller polypeptide (48 kDa) matches the predicted molecular mass of processed SAV3-E2 (46.9 kDa). This suggests that furin cleavage of E3E2 is rescued at 18°C, 15°C and 12°C. At all temperatures, PNGase I treatment resulted in an equal downward shift of both bands, suggesting that recombinant E3E2 and E2 were N-glycosylated (data not shown). From the relative intensities of the bands on the WB, it could be concluded that the intensity of the E2 fraction increased at lower temperatures. In conclusion, by decreasing the expression temperature from 27°C to 18°C, 15°C or 12°C, the ratio between E3E2 and fully processed E2 shifts towards the processed fraction (**Fig. 4-3B**). These results suggest that furin processing of the SAV3 E3E2 precursor is temperature dependent. Medium fractions of *Ac*-SAV3 infected *Sf*9 cells were PEG-precipitated to concentrate protein content and western analysis using α-E2 mabs showed that E2 can be detected in the medium fraction (**Fig. 4-3C**), but only when proteins were expressed at 12°C, 15°C or 18°C, but not at 27°C.

Surface expression of baculovirus expressed SAV3-E2 in Sf9 cells.

To generate progeny viral particles through budding, alphavirus glycoproteins assemble into heterodimers, three of which congregate into trimers, the so-called trimeric spikes. The spikes are formed in the ER and pass through the Golgi apparatus. At the end of the processing pathway, the trimeric spikes are anchored in the plasma membrane by the C-terminal transmembrane domains of E1 and E2 and are exposed on the surface of infected cells (Strauss and Strauss, 1994). So far, it has been shown that expression of the SAV3

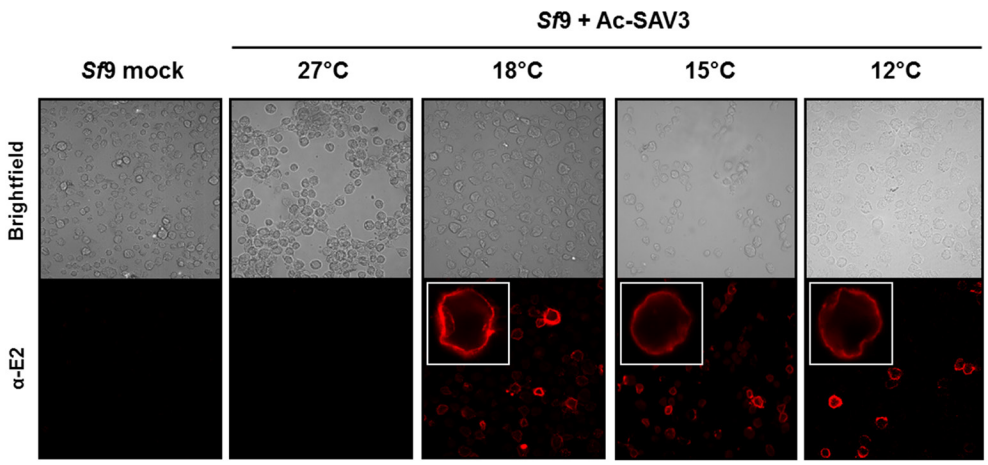

Figure 4-4. SAV3-E2 detection on the surface of *Sf*9 cells after recombinant baculovirus expression.
Cells were infected with *Ac*-SAV3 at 12°C, 15°C, 18°C and 27°C. Cells were fixed with 4% paraformaldehyde and subjected to immunostaining with α-E2 mabs. Cells were analyzed by confocal microscopy and positive staining indicates the presence of E2 at the surface of infected cells.

structural cassette by recombinant baculoviruses in insect cells results in the formation of glycosylated E3E2, which, at lower temperatures, is processed to glycosylated E2. This suggests that processing in insect cells resembles the processing as it takes place during wildtype infections in fish cells. In this case, glycoprotein spikes are exposed at the insect cell surface (Kuhn, 2007). To analyze whether or not this is true, *Sf*9 cells were infected with *Ac*-SAV3 at different temperatures. Next, non-permeable cells were subjected to immunofluorescence using α-E2 mabs (**Fig. 4-4**). Positive staining indicates that SAV3-E2 is exposed at the surface of the cells. Confocal microscopy revealed that the ring-like structures indicating surface expression could only be observed when *Ac*-SAV3 was expressed at 12°C, 15°C and 18°C. (**Fig. 4-4**). In sharp contrast, surface expression of E2 was not detected at 27°C (**Fig.4- 4**), while western analysis showed that E3E2 was expressed at high levels (**Fig. 4-3A**). No staining was observed in the mock cells (**Fig. 4-4, left**). These data comply with the previous results and demonstrate that the processing of SAV3-E2 is temperature dependent and that E2 can only be detected at the surface of infected cells at lower temperatures.

SAV3 virus-like particle formation in Sf9 cells expressing the SAV3 structural cassette.

Processing of the SAV3 structural cassette, expressed by recombinant baculoviruses in insect cells is temperature dependent. Mature E2 is produced at temperatures ranging between 12°C and 18°C, where it is then translocated to the surface of *Ac*-SAV3 infected cells and subsequently can be detected in the medium fraction. In addition, it was shown that the capsid protein is successfully auto-cleaved from the structural polyprotein (**Fig. 4-1B, left**) and E1 is successfully released from the envelope cassette (**Fig. 4-1B, middle**). When the structural cassette was expressed at 27°C, E2 retained in the cell fraction in its E3E2 precursor form (**Fig. 4-3A, lane 2**). The effect of temperature on SAV maturation is clear, but to assess if temperature has an influence on the antigenicity of recombinant SAV-E2, which carries the major neutralizing epitopes, an immune-capture antigenic mass ELISA using the SAV neutralizing mab (17H23) was performed on total cell culture lysates and medium fraction of *Sf*9 cells infected with *Ac*-SAV3 at 27°C, 18°C, 15°C and 12°C (**Fig. 4-5A**). The highest antigenic mass was detected in the combined sonicated culture fraction and medium fraction of cells incubated at 15°C. Slightly lower antigenic mass levels were detected at cells incubated at 18°C and 12°C, whereas no antigenic mass was detected at 27°C. Detection of high antigenic mass in the medium fraction of infected cells incubated at 15°C or lower, suggested the formation of SAV VLPs.

To investigate whether or not SAV VLPs could be detected, the 15°C medium fraction was evaluated by transmission electron microscopy (TEM) (**Fig. 4-5B**). VLP structures – spherical, sometimes donut-shaped, particles of 65-70 nm – that were morphologically similar to SAV3 virus particles (**Fig. 4-5B, left**) were found (**Fig. 4-5B, middle**), but these were absent in the control medium of *Ac*-GFP infection (**Fig. 4-5B, right**). This shows that SAV

VLPs can be produced by expressing the SAV3 structural cassette, using the recombinant baculovirus-insect cell expression system. Moreover, these VLPs can be detected by a SAV-neutralizing mab, suggesting that these VLPs morphologically and antigenically resemble authentic SAV virions.

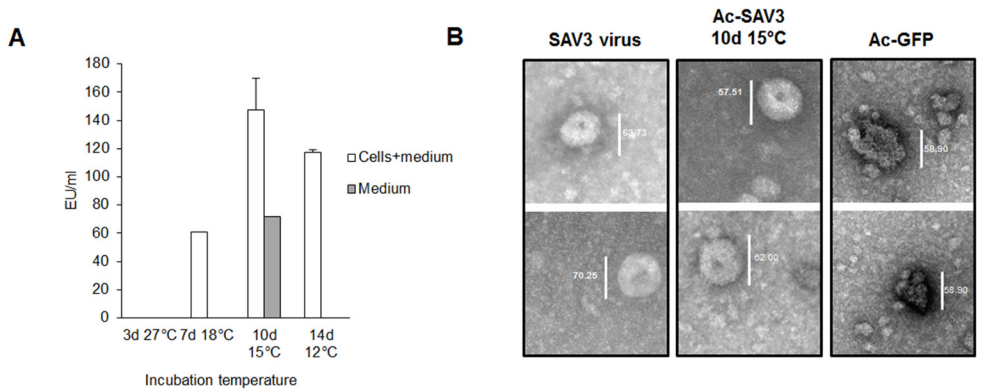

Figure 4-5. SAV-E2 antigenic mass determination and VLP production.
A) SAV-E2 antigenic mass was determined using the SAV-neutralizing mab 17H23 on cell and medium fractions of infected *Sf*9-cells, incubated at 27°C, 18°C, 15°C and 12°C. B) The medium fraction of cells infected with *Ac*-SAV3 at 15°C were analyzed by TEM to analyze SAV VLP production. Medium fractions of *Ac*-GFP infected *Sf*9 cells and SAV3 infected Chinook salmon embryo cells were used as control samples.

SAV3 structural cassette expression and VLP formation by temperature-shift in Sf9 insect cells.

The results so far have shown that SAV glycoprotein processing and secretion and VLP formation is dependent on the expression temperature. Although SAV-E2 processing appears to be most efficient at 12°C (**Fig. 4-3A**), expression at this temperature remains inefficient, due to the low metabolic rate of *Sf*9 cells and the extensive incubation time of 14 days. To optimize insect cell infection and SAV3 structural polyprotein processing, a temperature-shift experiment was performed. In this experiment, *Sf*9 cells were first infected with Ac-SAV3 at 27°C for 2 days to allow efficient baculovirus replication, after which the cells were transferred to 12°C for 3 days, to allow expression of properly processed SAV structural proteins. Protein expression, E2 processing, surface localization and VLP formation was analyzed 5 dpi by WB and immunostaining using α-E2 mabs and by TEM (**Fig. 4-6**). The temperature-shift resulted in increased processing of SAV3-E2 (**Fig. 4-6A, lane 3**), as compared to 27°C expression (**Fig. 4-6A, lane 2**), since both E3E2 (~55 kDa) and mature E2 (~48 kDa) were detected. PNGase F treatment led to a decrease in molecular mass, showing that both E2 configurations were glycosylated (**Fig. 4-6A, lane 4**). Immunostaining on non-permeable *Sf*9 cells that were infected following the temperature-shift regime showed that E2 was detected on the surface of infected cells (**Fig.4-6B**), similar to results found after

12°C incubation (**Fig. 4-4**). In contrast, surface staining of the cells infected at 27°C were negative (**Fig. 4-6B**). In addition, medium of infected *Sf*9 cells with the temperature-shift was examined by electron microscopy (**Fig. 4-6C**). Characteristic VLP structures were again detected, which were similar to those found after expression at 15°C (**Fig. 4-5B**).

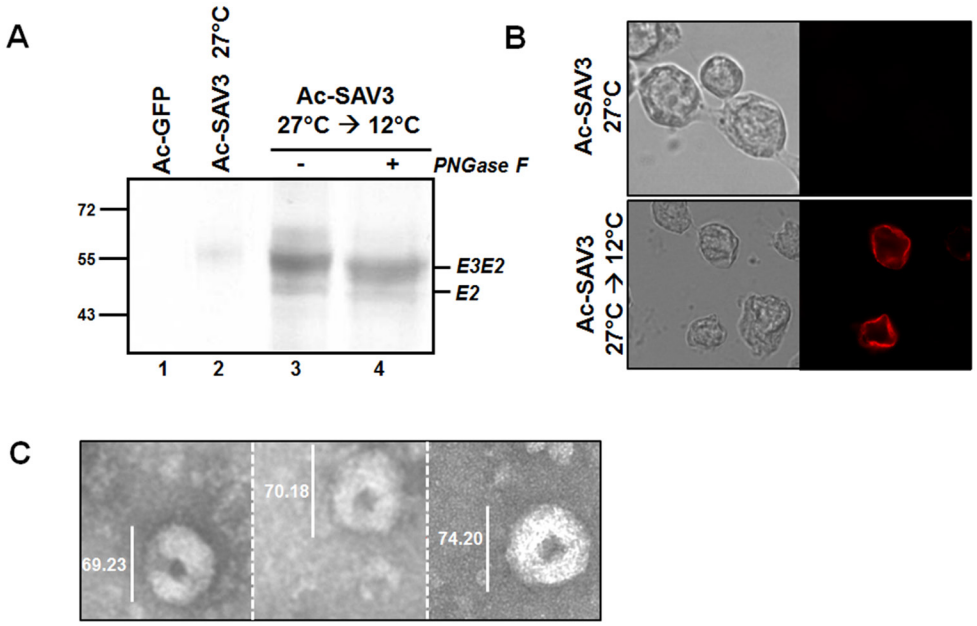

Figure 4-6. SAV3 structural cassette expression and VLP formation by temperature-shift in *Sf*9 insect cells. *Sf*9 cells were infected with *Ac*-SAV3, incubated for 2 days at 27°C and subsequently transferred to 12°C for 3 days. A) Cell cultures were treated with/without PNGase F and analyzed by WB using α-E2 mabs. B) Infected cells were treated with 4% paraformaldehyde and subjected to surface immunostaining with α-E2 mabs. C) The medium fraction the infected cell culture was analyzed by TEM for the presence of VLPs.

To further optimize the VLP formation in a temperature-shift regime, *Sf*9 cells were infected with *Ac*-SAV3 at 27°C and transferred to 12°C, 15°C and 18°C at 1 dpi, 2 dpi and 3 dpi. Cells were subsequently incubated for 3 days at the indicated temperatures. As controls, cells were infected and incubated for a total of 6 days at 12°C, 15°C and 18°C. Both cell and PEG-precipitated medium fractions were analyzed by western analysis using α-E2 mabs (**Fig. 4-7**). SAV3-E2 was not detected in both the cell and medium fraction of cells that were infected at 27°C for 1 day (**Fig. 4-7, lane 1**). However, when cells were subsequently incubated for three days at lower temperatures, E2 was abundantly detected in both cell and medium fractions (**Fig. 4-7, lane 2-4**), indicating a strong increase in processing efficiency due to the temperature shift. Cells that were infected at 27°C for 2 days produced a low amount of E3E2. Although less pronounced than the shift after 1 dpi, the temperature-shift to lower temperatures 2 dpi increased processing and detection of E2 in the cell and

medium fractions (**Fig. 4-7, lane 5-8**). Here, a shift to 15°C appeared optimal (**Fig. 4-7, lane 7**). Infection for 3 days at 27°C, as expected, appeared to be highly disadvantageous for the processing and secretion of E2, since only unprocessed E3E2, but no mature E2 was detected in both cell and medium fractions (**Fig. 4-7, lane 9**). It was concluded from this large temperature-shift experiment that infection for 1 day at 27°C followed by expression for 3 days at 15°C was optimal for E2 expression, processing and VLP secretion into the medium. In addition, this temperature-shift regime of in total 4 days significantly shortens infection time as compared to a 6 day infection at 15°C (**Fig. 4-7, lane 14**).

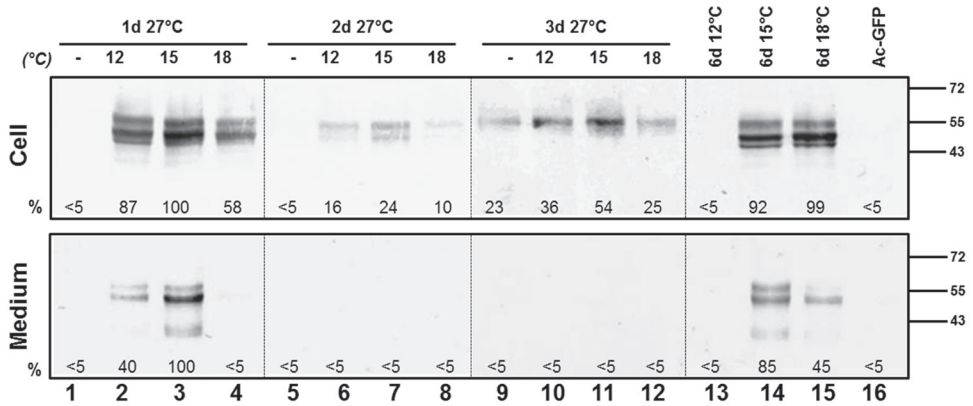

Figure 4-7. Comparative temperature-shift assay on Sf9-cells expressing the SAV3 structural polyprotein by recombinant baculoviruses.

Cells were infected with *Ac*-SAV3 at 27°C for 1, 2 or 3 days. Next, cells were transferred for 3 days to 12°C, 15°C or 18°C. Whole cell lysates and PEG-precipitated medium fractions were analyzed by WB using α-E2 mabs. Protein fraction quantities, relative to 1 day 27°C followed by 3 days 15 °C, are indicated.

Discussion

SAV infections cause severe economic losses in European aquaculture of Atlantic salmon and rainbow trout. Next to common infection control measures, vaccination is a most effective tool to reduce SAV infections in salmonids. The presently available vaccines are based upon inactivated or attenuated-live vaccine strategies. However, a VLP-subunit approach may serve as an elegant and effective alternative to the safety issues that accompany the present way of vaccine production. The recombinant baculovirus-insect cells expression system has proven to have a high potential in the generation of VLP-subunit vaccines (Metz et al., 2011b; van Oers, 2006). In this study, SAV VLPs were generated using recombinant baculoviruses and it was shown that the processing of SAV glycoproteins is a temperature-dependent process. VLP formation in insect cells at the normal production temperature of 27°C is not possible.

The SAV3 structural cassette (C-E3-E2-6K-E1) was cloned downstream of the polyhedrin promoter of *Ac*MNPV (*Ac*-SAV3) and expressed in *Sf*9 insect cells. SDS-PAGE and CBB analysis indicate that the SAV structural (glycol)proteins were expressed in high amounts and that the capsid protein was autocatalytically released from the structural polyprotein, similar as in wildtype alphavirus infection (Saijo et al., 2010; Strauss and Strauss, 1994). Alphavirus capsids are multifunctional proteins that are involved in encapsidation of viral genomic RNA, viral budding and in New-world alphaviruses induce host-cell transcription/translational shut-off (Aguilar et al., 2007; Strauss and Strauss, 1994). Depending on the alphavirus species and the cell type used for expression, capsid localizes in several cellular compartments e.g. cytoplasm, nucleus/nucleoli and mitochondria (Aguilar et al., 2007; Karlsen et al., 2010; Michel et al., 1990; Mitchell et al., 1997). Infection of *Sf*9 cells with *Ac*-SAV3 resulted in the formation of dense nuclear bodies, which appeared to be specific for cells expressing the SAV structural polyprotein. In addition, Hoechst staining showed that nucleic acids co-localized with the dense nuclear bodies and TEM analysis confirmed the in silico predicted nuclear localization of capsid (Karlsen et al., 2010). This is the first report that shows the presence of assembled alphavirus nucleocapsids of ~30 nm in the nucleus of insect cells.

The expression of the SAV-structural cassette in *Sf*9 cells at 27 °C clearly prevented complete processing of E2. In this regard, the efficient glycosylation of SAV glycoproteins is remarkable, since our other work showed that CHIKV glycoprotein expression by recombinant baculoviruses resulted only in partial glycosylation (Metz et al., 2011b).

Processing of SAV-E2 by host-furin cleavage was rescued after decreasing the infection temperatures to 18°C, 15°C or 12°C. Immunostaining indicated that only correctly folded proteins were recognized by the α-E2 mab, since at 27°C E3E2 could be detected under denaturing conditions by western analysis, but was not detected by immunostaining in

permeabilized cells infected with *Ac*-SAV3. It has previously been shown that the 17H23 mab recognizes a discontinuous epitope (aa139-306) (Moriette et al., 2005) on SAV3-E2. Incorrect folding of native PE2 might prevent the binding of mab 17H23 to the epitope, while under denaturing conditions, the conformational epitope is restored, thereby allowing antibody recognition by western analysis. Similar epitope-reformation under standard denaturing conditions has previously been described for other viral denaturation-resistant epitopes (Frost et al., 1995; Wright et al., 1989).

The incorrect folding of PE2, at 27°C that prevents binding of mab 17H23 might also render the SAV-PE2 furin-cleavage signal 68RKKR inaccessible to host furin-like proteases. An alternative explanation is that cellular furin is inactive at 27°C, however, this is highly unlikely given that baculovirus F protein is also activated by furin at similar temperatures (26°C-28°C) (Westenberg et al., 2002). The apparent prevention of furin cleavage itself does not fully explain the absence of E3E2 in the medium at 27°C, because furin cleavage is not a prerequisite for alphavirus budding or E3E2 secretion (Metz et al., 2011b; Ozden et al., 2008; Sjoberg et al., 2011a). The deficiency in E3E2 processing and lack of secreted E3E2 at 27°C, is therefore most likely caused by the retention of misfolded SAV-E3E2 in the ER or secretory pathway, in any case upstream of the trans-Golgi system where furin-dependent maturation takes place (Kuhn, 2007). Misfolded and unfolded proteins usually accumulate in the ER, causing ER stress and thereby disrupting ER functions (Kaufman, 1999), a phenomenom often observed during overexpression of glycoproteins by recombinant baculoviruses (van Oers et al., 2001). It will be important to investigate in future experiments which structural change of E3E2 determines its intracellular retention at 27°C.

We show by electron microscopy that recombinant baculovirus expression of the SAV structural cassette in *Sf*9 cells at lower temperatures results in the formation of SAV VLPs. This result was confirmed by western analysis on the medium fraction of infected cell cultures and by sandwich immune-capture ELISA on infected cells and/or medium, in which SAV proteins were only detected at expression temperatures below 27°C. The spherical particles of ~65 nm in size, morphologically indistinguishable from SAV3 virus particles and other alphavirus VLPs (Akahata et al., 2010), were found exclusively in the medium of *Ac*-SAV3 infected *Sf*9 cells at lower temperatures, but not in the medium of control baculovirus lacking SAV sequences or mock-infected Sf9 cells.

Since total production levels of recombinant SAV protein were the highest at 27°C, while lower temperatures were essential for PE2 processing, a hybrid protein production process, with an infection phase at 27°C followed by a production/processing phase at 12°C was developed. Immunostaining and western analysis showed that recombinant SAV proteins produced following the temperature regime, appeared to be folded and processed correctly, and as expected, SAV VLPs were detected in the medium fraction of *Ac*-SAV3 infected cells. The temperature-shift production and processing regime was further optimized by varying

the production time at 27°C. Both cell and medium fraction analysis showed that a 1 day infection phase at 27°C, followed by a 3 day processing phase at 15°C was optimal for combined protein expression and processing efficiency. In addition, it appeared that the vast majority of VLPs produced at 15°C have mature SAV-E2 incorporated in their envelope. However, PE2 medium detection indicated that, in a small fraction of VLPs, E3 was still associated to the trimeric spikes. This common alphavirus feature does not affect cell receptor recognition by E2 (Lobigs et al., 1990). Thus, we expect VLPs carrying a minor E3E2 fraction still to be sufficiently immunogenic, especially considering that E3 from other alphaviruses harbors protective epitopes (Parker et al., 2010).

In addition to SAV VLPs, also the insect cells expressing correctly folded SAV structurals may be used in veterinary vaccine formulations. Similar to the widely used baculovirus surface display technique, based upon the expression of foreign peptides/epitopes using a chimeric baculovirus GP64 surface glycoprotein (Makela and Oker-Blom, 2006), SAV glycoproteins are anchored in the insect cell membrane and are displayed at its surface. However, the baculovirus surface display technique may find limited use in SAV glycoprotein production, since the preceding alphavirus E3 peptide with signal sequence is required for correct E2 folding and the use of heterologous signal peptides has recently been shown not to enhance alphavirus glycoprotein production (Metz et al., 2011b).

The temperature-shift clearly rescues SAV glycoprotein processing, most likely via restoring upstream protein misfolding. A similar temperature-dependent folding phenomenon has previously been described for a temperature sensitive mutant of the vesicular stomatitis virus glycoprotein (VSV-Gmut). VSV-Gmut accumulated in the ER due to misfolding at 40°C, but was refolded when the temperature was decreased to 32°C, enabling VSV-Gmut to enter the secretory pathway into the Golgi-complex (Presley et al., 1997). In future studies we would like to investigate the molecular mechanisms of presumed SAV glycoprotein misfolding at high temperatures, but it is unlikely that this will lead to a more efficient VLP production process at 27°C in the short term.

We clearly show that the initial incubation period for 1 day at 27°C following inoculation with baculovirus is of high importance for efficient VLP production. Nonetheless, extension of the 27°C baculovirus infection phase, negatively influenced SAV-PE2 processing and inhibited the formation of SAV VLPs. The strong CPE associated with baculovirus infection that usually occurs 2-3 dpi is most likely disabling cells to rescue SAV glycoprotein folding and processing after the shift to lower temperatures. However, a one day 27°C infection phase is highly beneficial and combined with a three day production processing phase at 15°C significantly shortens the time it takes to produce similar SAV E2 proteins levels as a six day production period at 15°C. This embodies a major advantage for large scale industrial antigen production in insect cell-bioreactors.

This study provides clear evidence that recombinant SAV VLPs can be produced in insect cells using baculovirus expression but also that SAV glycoprotein processing and folding is strictly temperature dependent and a critical determinant of VLP production. The proposed temperature-shift regime not only optimizes SAV VLP production in insect cells, but also provides a general principle for other vaccine candidates of cold-blooded infectious agents in insect cell systems. We aim to address the immunogencity of our SAV VLPs in a follow up study involving a vaccination trial.

Acknowledgements

We would like to acknowledge Luc Grisez and Petter Frost for continued support of this project and Corinne Geertsema for technical assistance.

Material and Methods

Cells and viruses.

Adherent *Spodoptera frugiperda* (*Sf*9) cells (Invitrogen) were maintained as monolayer cultures in *Sf*-900 II medium (Gibco), supplemented with 5% fetal calf serum. Recombinant baculoviruses were generated using a modified *Autographa californica* multicapsid nucleopolyhedrovirus (*Ac*MNPV) backbone. Promoter sequences and large parts of the coding sequences of cathepsin and chitinase were deleted from the bacmid backbone (Kaba et al., 2004). The resulting *Ac*Δcc was further modified by deletion of the *p10* promoter and ORF. These elements were replaced by a zeocin resistance marker by Lambda Red recombination (Datsenko and Wanner, 2000; Pijlman et al., 2002). Modified LoxP sequences flanking the zeocin resistance marker were used for subsequent removal of the resistance marker by Cre recombinase (Suzuki et al., 2005). The pFastBac1/SAV3 was constructed by cloning the SAV3 structural polyprotein (Genbank accession # AY604235) as a 3957 nt EcoRI-XbaI fragment into pFastBACI (Invitrogen). The recombinant baculovirus *Ac*ΔccΔp10 expressing the structural cassette of SAV3 was generated using the Bac-to-Bac baculovirus expression system (Invitrogen), resulting in *Ac*-SAV3. Recombinant baculovirus titers expressed as tissue culture infectious dose 50 ($TCID_{50}$) per ml, were determined by end point dilution (Vlak, 1979). All infections were performed in serum-free *Sf*-900 II medium.

Recombinant baculovirus infections and temperature-shift assay.

To analyze protein expression and processing, $1.5x10^6$ *Sf*9-cells were seeded in a 6-wells culture plate and infected with *Ac*-SAV3 at a multiplicity of infection (MOI) of 10 $TCID_{50}$ units per cell. Infections were performed at 27°C on a shaking platform. Subsequent

incubations were performed at 12°C, 15°C, 18°C or 27°C for 14, 10, 7 and 3 days, respectively. The medium fraction was removed and cells were harvested, washed once in phosphate buffered saline (PBS), resuspended in 200µl PBS and stored at -20°C. To analyze the effect of a temperature-shift on protein processing, cells were infected and harvested as previously described, but were initially incubated at 27°C. Next, cells were transferred to 12°C, 15°C or 18°C at 24 hpi, 48 hpi or 72 hpi.

Protein analysis.

Secreted protein fractions were precipitated by 7% (w/v) NaCl, 2.3% (w/v) polyethylene glycol (PEG)-6000 precipitation. Pellets were resuspended in 100 µl PBS and stored at -20°C. Whole cell lysates and medium fractions were analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie brilliant blue (CBB) staining. Denatured proteins were transferred to an Immobilon membrane (Millipore) and examined by western analysis. Membranes were blocked with 3% skim milk in PBS-0.1% Tween-60 (PBST) for 1 h at room temperature (RT). Membranes were washed in PBST and incubated for 1h at RT with 1:2000 diluted primary monoclonal antibody (mab) α-E2 17H23 (SAV3) (Moriette et al., 2005) and α-E1 (raised against the N-terminus of SAV3 E1, aa 1-26) in PBST, supplemented with 0.2% skim milk. Membranes were washed 3 times with PBST and incubated for 45 minutes at RT with 1:3000 diluted Alkaline Phosphatase (AP) conjugated secondary antibody, in PBST supplemented with 0.2% skim milk. Membranes were washed three times in PBST and incubated with AP-buffer for 10 min. Proteins were detected by NBT/BCIP staining (Roche). Antigenic mass was determined by a sandwich immune-capture ELISA using the monoclonal α-E2 17H23 (SAV3) antibody (Moriette et al., 2005).

PNGase F treatment.

Protein fractions were treated with PNGase F (New England Biolabs) to analyze the glycosylation status of SAV3-E2. Cell fractions and the precipitated medium fraction were treated with 1µl glycoprotein denaturing buffer in 9 µl MilliQ for 10 min at 100°C. The denatured protein-mix was incubated for 1 h at 37°C with 2 µl 10x G7 reaction buffer, 2 µl 10% NP40, 1.5 µl PNGase F and 4.5 µl MilliQ. Treated proteins fractions were analyzed with SDS-PAGE, western blot (WB) and CBB.

Immunofluorescence assay.

*Sf*9-cells were seeded on glass coverslips in 24-wells plate, infected and incubated at 12°C, 15°C, 18°C and 27°C. Cells were fixed with 4% paraformaldehyde in PBS for 5 min at RT, washed with PBS and subsequently incubated with PBS containing 1:2000 diluted primary monoclonal mouse α-E2 antibody (Moriette et al., 2005) for 1 h at RT. Cells were

washed carefully with PBS and treated with 1:1000 diluted goat α-mouse polyclonal Alexa 546 (Invitrogen) for 1 h at RT in dark conditions. Next, cells were washed and coverslips were fixed on glass slides with a drop of antifade fluoromount-G (Southern Biotech). Cells were analyzed by laser confocal microscopy on a Zeiss LSM 510 Meta, Axiovert 100m.

Electron microscopy analysis.

Copper 400 square mesh grids (Veco) were hydrophillized by Argon gas discharge. Next, 10 µl sample was applied for 2 min and the excess liquid carefully removed. The grid was washed 5x with MilliQ and stained with 2% uranyl acetate for 20 s. Excess dye was removed, grids were air dried and analyzed with a JEOL JEM 2100 transmission electron microscope. Analysis of fixed cell samples was performed as described (Van Lent et al., 1990).

Chapter

Chikungunya virus-like particles are more immunogenic in a lethal mouse model compared to glycoprotein E1 or E2 subunits.

Chikungunya virus (CHIKV) causes acute illness characterized by fever and long-lasting arthritic symptoms. The need for a safe and effective vaccine against CHIKV infections is on the rise due to ongoing vector spread and increasing severity of clinical complications. Here we report the results of a vaccination-challenge experiment in mice using three different vaccine candidates produced in insect cells by recombinant baculoviruses: (*i*) secreted (s)E1 and (*ii*) sE2 CHIKV glycoprotein subunits (2 µg/immunization), and (*iii*) CHIKV virus-like particles (VLPs) (1 µg E2 equivalent/immunization). These experiments show that vaccination with two subsequent administrations of 1 µg of adjuvanted CHIKV-VLPs completely protected IFN-α/β and –γ receptor null mice from lethal CHIKV challenge. The E1 and E2 subunits provided partial protection, with half of the mice surviving but with significantly lower neutralizing antibody titres as compared to the VLP vaccine. This study provides clearly evidence that even a modest neutralizing antibody response is sufficient to protect mice from CHIKV infections and that CHIKV-VLPs provide a superior immune response and protection against CHIKV disease as compared to individual CHIKV-E1 and -E2 subunits.

This chapter is part of a paper in submission as: Stefan W. Metz, Byron Martina, Gorben P. Pijlman (2013). *Chikungunya virus-like particles are more immunogenic in a lethal mouse model compared to glycoprotein E1 or E2 subunits. In preperation*

Introduction

Chikungunya virus (CHIKV), the etiological agent of chikungunya fever, has infected millions of people in Africa and Asia, since it re-emerged in Central Africa in 2004 (Powers and Logue, 2007) and continued to spread in the Indian Ocean region. Recent outbreaks in Italy (2007) (Enserink, 2007) and France (2010) (Gould et al., 2010) exemplify the threat of CHIKV transmission in non-tropical regions including Europe. CHIKV was historically transmitted by *Aedes aegypti*, but recently adapted to a novel transmission vector, the Asian Tiger mosquito *Ae.albopictus* (Tsetsarkin et al., 2007). This vector switch increases the risk of further dissemination in temperate regions (e.g. Europe) or for CHIKV to invade and cause so-called 'virgin-soil' epidemics on continents with no history of CHIKV circulation, such as North America (Caminade et al., 2012; Ruiz-Moreno et al., 2012). Research efforts on the development of a safe and efficacious CHIKV vaccine have not yet resulted in a commercially available product. However, the use of CHIKV-subunits (Kumar et al., 2012), engineered viral vectors (Wang et al., 2011; Wang et al., 2008) and virus-like particles (VLPs) (Akahata et al., 2010) have shown promising results. Large-scale production was not achieved, therefore the baculovirus-insect cell expression system was explored to generate a CHIKV vaccine candidate (van Oers, 2011). Previous work described effective production and purification of secreted CHIKV-sE1 and sE2 subunits in insect cells using recombinant baculoviruses (**Chapter 2**) (Metz et al., 2011b). This expression system was also used to successfully produce CHIKV-VLPs, but their potential as vaccine was not yet explored (**Chapter 3**) (Metz et al., 2013). The current study describes a comparison of the insect cell-derived CHIKV-subunit and VLP vaccine candidates in a lethal IFN-α/β and –γ receptor null (AG129) mouse model for CHIKV vaccine-challenge studies (Partidos et al., 2011). In this mouse model (IFN-α/β and –γ receptor null (AG129)), mice were immunized twice with subunits or VLPs that were formulated in Matrix M adjuvant. These mice were subsequently challenged with a lethal dose of the CHIKV-S27 isolate. Determination of neutralizing antibody titres prior challenge and monitoring of mouse survival and viral RNA titres in brain tissue upon viral challenge indicated that VLPs outperformed the subunits in immunogenicity and conferred complete protection against lethal CHIKV infection.

Results and Discussion

Vaccination of AG129 mice with CHIKV-subunits and CHIKV-VLPs

To analyse the neutralizing activity of the mice sera post vaccination, but prior challenge, mice sera were subjected to a CHIKV neutralization test. All groups, but not all animals within the groups, vaccinated with CHIKV antigen produced neutralizing antibodies (**Fig. 5-1**). The CHIKV VLPs induced the highest neutralizing antibody titres in all of the mice (n=8). The subunits induced significantly lower ($p<0.01$) neutralizing titres in 4 out of 8 (sE1) or 5 out of 8 (sE2) mice. As expected, the control group of mice vaccinated with Bac-ctrl did not produce any neutralizing antibodies against CHIKV. It is not clear whether animals that did not produce neutralizing antibodies and succumbed to infection, produced other types of antibodies, or that they were simply non-responders.

In contrast to other alphavirus E1 and E2 vaccine studies (Hodgson et al., 1999), in which only E2 was sufficiently immunogenic, our results showed that both CHIKV-sE1 and -sE2 induce similar titres of neutralizing antibodies (**Fig. 5-1**). The slightly higher E2 neutralizing antibody titer, although not statistically different, is in line with other reports suggesting that E2 contains the dominant neutralizing epitopes for alphaviruses (Kam et al., 2012b; Strauss et al., 1991; Vrati et al., 1988).

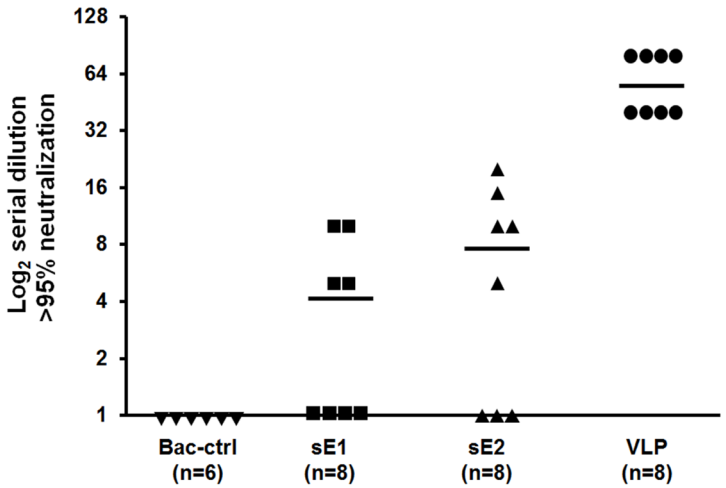

Figure 5-1. CHIKV neutralizing antibody titres after immunization.
Sera of immunized AG129 mice (twice with purified sE1, sE2 (Metz et al., 2011b) or VLPs (Metz et al., 2013), adjuvanted with 5µg Matrix M) were tested for their neutralizing ability. Neutralization was based upon suppression of CHIKV-induced CPE in Vero cells. sE1; subunit E1, sE2; subunit E2, VLP: virus-like particle. Bac-ctrl: negative control. The number of mice per group is indicated, average titres are indicated with a line.

CHIKV-VLPs protect mice against lethal CHIKV-challenge

All immunized and control animals were challenged intraperitoneally with 1000 $TCID_{50}$ units of CHIKV S27 isolate, 6 weeks after the second vaccination. Complete protection against CHIKV-induced death was obtained after immunization with VLP (**Fig. 5-2**). In contrast, immunization with either CHIKV-sE1 or -sE2 only partially protected mice against lethal CHIKV infection, with 4 out of 8 (50%) and 5 out of 8 (62.5%) mice surviving, respectively (**Fig. 5-2**). The mice that died from infection are those that did not develop neutralizing antibodies. Nonetheless, these results do seem to suggest that even low neutralizing antibody titres are sufficient to protect mice from lethal CHIKV challenge.

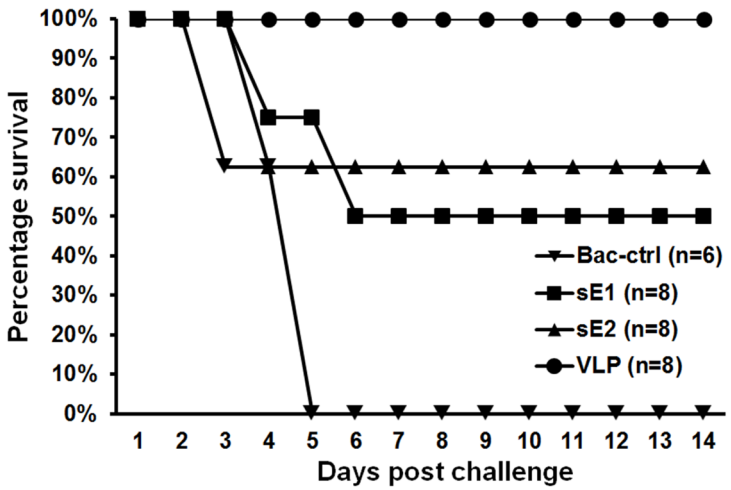

Figure 5-2. Survival curves of vaccinated mice upon lethal CHIKV challenge.
AG129 mice were immunized twice with purified sE1, sE2 (Metz et al., 2011b) or VLPs (Metz et al., 2013), adjuvanted with 5µg Matrix M. Mice were challenged with 1000 $TCID_{50}$ units CHIKV strain S27 6 weeks post vaccination. Abbreviations as in Figure 1.

All mice that succumbed from CHIKV challenge in the groups immunized with Bac-ctrl, sE1 and sE2, had large amounts (10^5-10^6 $TCID_{50}$ equivalents/ gram brain) of CHIKV RNA in their brains (**Fig. 5-3**). In contrast, minor amounts of CHIKV RNA were detected in the brains of surviving mice immunized with the subunits (**Fig. 5-3**) Infectious virus was isolated from mice that received the sE1 and sE2 subunits (data not shown). Significantly less ($p<0.05$) viral RNA was detected in the VLP immunized mice (**Fig. 5-3**) and no infectious virus could be isolated after the homogenization of the brain material (data not shown). This implies that the weak neutralizing response induced after subunit immunization was enough to protect the mice from lethal challenge, but does not completely inhibit virus replication in the brain, after challenge.

The large difference in immune response, i.e. significantly higher titres of neutralizing antibodies in all the VLP versus subunit-immunized mice, might suggest that correct glycoprotein and/or epitope presentation are key features in generating protective immune response against CHIKV. As part of a VLP, epitopes on the exposed domains of E1 and E2 are predominantly presented to the immune system. In the case of subunit immunization, partial incorrect folding (due to lack of E1-E2 interaction) or accessibility of normally hidden domains of sE1 and sE2 may cause a less efficient neutralizing immune response. In addition the particulate nature, particle size and the repetitive presentation of epitopes within the VLP are supposed to be highly active immune stimulatory factors, which are lacking in most subunit formulations (Roldão et al., 2010). Whether combined E1/E2 subunit will provide a better immunogen than a VLP, which is morphologically indistinguisable from live virus, requires further experimentation. In addition to this, the value of adjuvants in this study remains to be determined. For this, different adjuvants, adjuvant doses and VLP:adjuvant ratios are variables that need to be addressed.

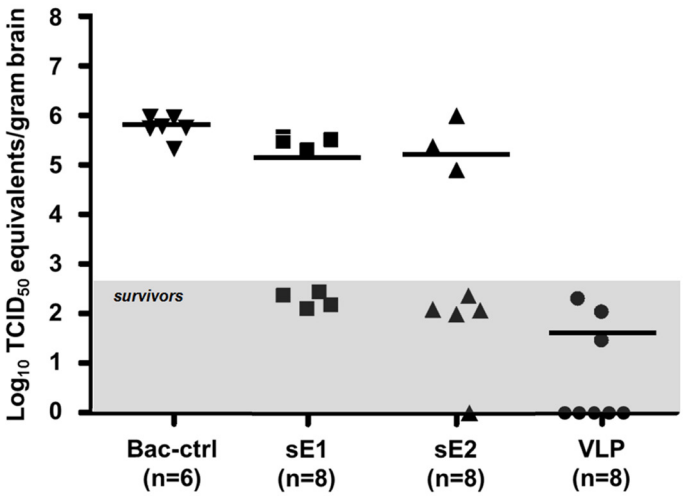

Figure 5-3. CHIKV RNA copies in mouse brain samples.
RNA was isolated from the brain of immunized mice and CHIKV RNA was detected using quantitative RT-PCR. Results are expressed as $TCID_{50}$ equivalents per gram of brain tissue. The shaded area indicates mice that survived lethal CHIKV challenge. Abbreviations as in Figure 1.

The results with the AG129 mice, which are deficient in IFN-α/β and IFN-γ receptors, also indicated that immunity mediated by neutralizing antibodies is sufficient to protect mice against lethal CHIKV infection. These results are in line with the previous finding that the early appearance of neutralizing IgG3 antibodies was associated with viral clearance and long-lived protection against CHIKV in humans (Kam et al., 2012c). However, the role, if any of cell mediated immunity in the protection against CHIKV infections cannot be addressed in

the IFN-α/β –γ receptor null mouse model used. It is known that VLPs are potent stimulators of both B-cell mediated as well as CD4 proliferative and cytotoxic T-cell responses (Noad and Roy, 2003). Which immune pathways are activated by the CHIKV-VLPs and which antigen doses are sufficient to protect mice with one single vaccination, needs to be analyzed in follow up studies using other models, e.g. wild type adult mouse model (Gardner et al., 2010) or macaques (Akahata et al., 2010).

Conclusions

This *in vivo* vaccination-challenge study has shown that CHIKV VLPs produced via insect cells induced high neutralizing antibody titres and provided complete protection against lethal CHIKV challenge in mice. In contrast, immunization with individual CHIKV-sE1 and-sE2 resulted in partial protection, but even at lower levels of neutralizing antibodies in the surviving mice. Our results seem to suggest that CHIKV-VLPs are much more immunogenic than the glycosylated E1 or E2 subunits. The insect cell-derived, CHIKV VLP vaccine is an attractive and effective vaccine candidate to control CHIKV infection and associated disease and should be subsequently tested in more relevant models mimicking CHIKV disease in humans.

Acknowledgements

Albert Osterhaus and Just Vlak are acknowledged for their continued interest and advice. Corinne Geertsema assisted in the purification and quantification of the subunits. This work was performed within the framework of Dutch Top Institute Pharma, project “Development of recombinant live and subunit vaccines against chikungunya virus infections” (project nr T4-301).

Material and Methods

Murine vaccination and challenge

To test the comparative immunogenicity of the CHIKV-sE1 and sE2 subunits and the CHIKV-VLPs, six week old AG129 mice (B&K Universal, East Yorkshire, UK) were vaccinated twice on day 0 and day 21 subcutaneously, with 2 µg of purified CHIKV-sE1, sE2 or negative control antigen and 1 µg (E2 equivalent) of sucrose gradient purified CHIKV-VLPs. As negative control, *Sf*21-cells were infected with *Ac*-GFP and the infection medium was processed under exactly the same conditions as the CHIKV VLPs. The vaccination material was formulated with 5 µg/mouse Matrix M (Isconova, Sweden) adjuvant prior to injection.

Six weeks after the second vaccination, all animals were challenged intraperitoneally with 1000 $TCID_{50}$ units of CHIKV S27 isolate. After the challenge, the mice were monitored daily for morbidity or mortality. The infection was considered lethal when the animals reached humane end-points and needed to be euthanized

CHIKV RNA quantification

In order to quantify viral RNA copies in the brain, half the brain was weighed and homogenized in 1 ml of DMEM containing antibiotics (100 U penicillin, 100 µg/ml streptomycin) using a tissue homogenizer. Hundred µl of brain homogenate was added to 400 µl of lysis buffer (Roche). Viral RNA was extracted from brain samples using the automated MagnaPure method (Total nucleic acid isolation kit, Roche Diagnostics) according to the manufacturer's instructions, and quantified using a one-step RT-PCR TaqMan protocol (EZ-kit, Applied Biosystems) and an ABI PRISM 7500 detection instrument. The primers and probe used for CHIKV RNA quantification were essentially as described (Werneke et al., 2011) except that probe Fam '5-CCAATGTCTTCAGCCTGGACACCTTT-3'Tamra was used. Results are expressed as $TCID_{50}$ equivalents per gram of brain tissue.

Virus neutralization test

To analyse the neutralizing activity of the mice sera post vaccination, but prior challenge, serial two-fold dilutions (1:10 to 1:1280) of heat-inactivated mouse sera were prepared in duplicate in 96-wells plates and 100 $TCID_{50}$ of CHIKV-S27 was added to each well. After 1 h incubation at 37°C, 4 x 10^4 Vero cells were added to each well and plates were incubated for five days. Neutralizing titres were determined microscopically and expressed as the reciprocal of the highest serum dilution giving >95% suppression of cytopathic effect.

Ethics statements

All animal experiments described in this paper were carried out in the BSL3 facilities of the Erasmus Medical Center in accordance with the Dutch guidelines for animal experimentation and were approved by the institute's independent animal ethics committee (EMC 122-11-27).

Chapter

Effective chikungunya virus-like particle vaccine produced in insect cells.

Emerging arthritogenic mosquito-borne alphaviruses, such as the chikungunya virus (CHIKV) cause severe disease and represent serious public health threats, especially since there is no commercially available vaccine against CHIKV infections. Herein we show that CHIKV VLPs, produced in insect cells, using recombinant baculovirus expression, induced high titer neutralizing antibody responses and provided complete protection against viraemia and joint inflammation upon challenge with the Réunion Island CHIKV strain in an adult wild-type mouse model of CHIKV disease, after a single immunization with 1 µg of non-adjuvanted CHIKV VLPs. CHIKV VLPs produced in insect cells using recombinant baculoviruses thus represents a new, safe, non-replicating and effective vaccine candidate against CHIKV infections.

This chapter has been published together with chapter 3 as: Stefan W. Metz, Joy Gardner, Corinne Geertsema, Thuy T Le, Lucas Goh, Just M. Vlak, Andreas Suhrbier, Gorben P. Pijlman (2013). *Effective chikungunya virus-like particle vaccine produced in insect cells. PLoS NTD, 7(3);e2124*

Introduction

Chikungunya virus (CHIKV) is an arthritogenic, mosquito-borne alphavirus (family: *Togaviridae*) that causes severe disease in humans. First isolated in Tanzania in 1952, CHIKV has spread globally, primarily due to vector expansion (Lambrechts et al., 2010). CHIKV is transmitted by the bite of an viruliferous mosquito, originally *Aedes aegypti*. In a recent large-scale epidemic on the Rèunion Island in 2005/2006, CHIKV switched to an alternative vector *Aedes albopictus*, resulting in an estimated 270 000 cases of CHIKV infection (Renault et al., 2007). The epidemic has spread continuously to more than 18 countries (Staples et al., 2009) including India, where millions of people were infected (Schwartz and Albert, 2010). This vector switch enabled CHIKV to become endemic in more temperate regions and has recently caused epidemics in Southern Europe (Grandadam et al., 2011; Rezza et al., 2007). Clinical symptoms that characterize CHIKV infection are rash, sudden onset of high fever and most distinctively severe and chronic arthritis that may persist for years (Solignat et al., 2009).

CHIKV is an enveloped virus of ~70 nm and has a positive single-stranded RNA genome of ~ 11,800 nucleotides long (Khan et al., 2002b). The genome encodes two polyproteins; the 'non-structural' polyprotein and the 'structural' polyprotein. The structural polyprotein is translated from a 26S subgenomic mRNA and is processed into the 5 structural proteins; capsid (C), E3, E2, 6K and E1(Schlesinger and Schlesinger, 2001). The viral RNA is encapsidated in a ~40 nm nucleocapsid, which is tightly enclosed by a host-derived lipid bilayer envelope displaying 80 trimeric spikes, composed of the viral envelope glycoproteins E1 and E2. The trimeric spikes are essential for host receptor recognition and attachment (E2), cell entry via pH-dependent endocytosis (E1) and for virion budding (E2).

At present no licensed vaccine or effective antiviral drug is available for human use for any of the alphaviruses. A number of pre-clinical CHIKV vaccines have been described, including inactivated virus formulations (Gardner et al., 2010; Harrison et al., 1971; Tiwari et al., 2009), live-attenuated virus vaccines (Levitt et al., 1986; Partidos et al., 2012; Plante et al., 2011), chimeric virus vaccines (Wang et al., 2008), DNA vaccines (Mallilankaraman et al., 2011; Muthumani et al., 2008), a recombinant adenovirus vaccine (Wang et al., 2011), subunit protein vaccines (Kumar et al., 2012; Metz et al., 2011b; Metz and Pijlman, 2011) and a virus-like particle (VLP) formulation (Akahata et al., 2010). A formalin-inactivated alphavirus vaccine has been shown to be immunogenic in humans (Edelman et al., 1979). However, growth of large quantities of CHIKV for vaccine manufacture is complicated by the requirement of appropriate BSL3 containment. A live-attenuated CHIKV vaccine (TSI-GSD-218), although immunogenic, caused side effects including arthralgia in a human phase II study (Edelman et al., 2000). DNA vaccines have so far not been particularly effective at generating antibody responses in humans (Kutzler and Weiner, 2008), which is a concern as

neutralizing antibodies are believed to be required for protection against CHIKV infections (Couderc et al., 2009; Gardner et al., 2010).

VLPs mimic the native virus surface architecture and protein conformation, which often makes them potent inducers of protective antibody responses in the absence of adjuvants (Metz and Pijlman, 2011). A CHIKV VLP-based vaccine was recently produced by DNA transfection of mammalian cells, and provided protection in both mice and non-human primates (Akahata et al., 2010; Couderc et al., 2009). Although this VLP approach is promising, the baculovirus-insect cell expression systems out-perform systems utilising DNA plasmid transfection in mammalian cells in a number of aspects, in particular cost and scalability (Roldão et al., 2010).

Here we describe the generation and *in vivo* testing of a CHIKV VLP vaccine generated using the recombinant baculovirus-insect cell expression system (**Chapter 3**). Baculovirus expression in insect cells has proven to be a safe and efficient method for producing heterologous proteins for research, diagnostics and vaccine development. Protein expression in insect cells has the benefit of accurate protein folding and near-native post-translational processing of, for instance, complex glycoproteins (van Oers, 2006). Veterinary baculovirus-produced subunits or VLP vaccines have been on the market for many years (van Oers, 2006). The first human baculo-based vaccine, the cervical cancer VLP vaccine (Cervarix, GlaxoSmithKline) received FDA approval in 2007 (Paavonen and Lehtinen, 2008). A recombinant influenza virus vaccine (FluBlok, Protein Sciences) has recently received approval of the Food and Drug Administration of the USA (Cox and Hollister, 2009). These products pave the way for future licensing of new baculovirus-based pharmaceutical products and/or vaccines. Recombinant baculoviruses have also been used successfully to expressed alphavirus proteins (Hodgson et al., 1999; Oker-Blom and Summers, 1989), functionally active CHIKV subunits (**Chapter 2**) (Metz et al., 2011b) and alphavirus VLPs (**Chapter 3 and 4**) (Metz et al., 2013; Metz et al., 2011a). In this chapter, we test their potential to serve as a vaccine. These vaccines were tested in an adult wild-type mouse model (Gardner et al., 2010).

Results

Neutralizing antibody and antibody titers following vaccination

To assess the immunogenicity of the CHIKV-VLPs, C57/BL6 mice were vaccinated once with 0.1 µg or 1 µg of the VLPs, 1 µg of the VLPs formulated with Quil A adjuvant or 10 µg inactivated CHIKV (positive control). The negative controls were PBS and a GFP control. For the GFP control, *Sf*21-cells were infected with *Ac*-GFP and the infection medium was treated under exactly the same conditions as the CHIKV VLPs. All groups receiving CHIKV antigens generated neutralizing antibodies that are significantly different ($P<0.01$) from both control groups (PBS and *Ac*-GFP) but not significantly different from one another (**Fig. 6-1**). The 1 µg VLP dose induced neutralizing antibody titers comparable with those seen after vaccination with 10 µg of inactivated CHIKV (**Fig. 6-1**).

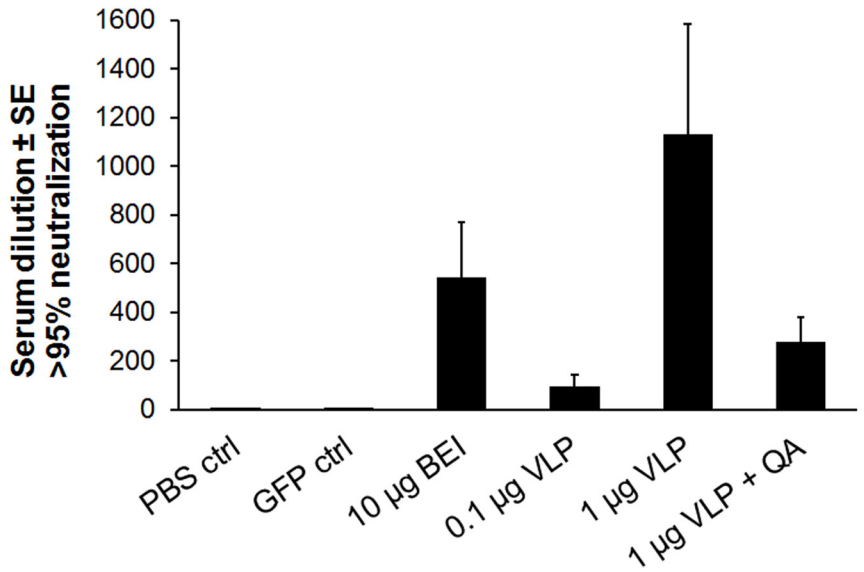

Figure 6-1. CHIKV neutralization.
Serum of immunized mice were collected and tested for their neutralizing ability based on >95% protection against CHIKV induced CPE in Vero cells. BEI corresponds to the inactivated CHIKV positive control. Statistical analysis shows that all CHIKV antigen immunized groups are significantly different from the control groups.

CHIKV-specific IgG1 and IgG2c titers were determined by ELISA, and broadly similar titers were seen for IgG1 and IgG2c (**Fig. 6-2A, B**), which contrasts with natural CHIKV infection where IgG2c dominates (Gardner et al., 2010). At the 1 µg dose, VLPs produced ~10-20 fold significantly lower antibody titers (IgG1 $P<0.05$, IgG2c $P<0.01$) than 10 µg inactivated CHIKV, with the 0.1 µg VLP dose showing a further ~10-20 fold reduction in antibody titers compared to 10 µg inactivated CHIKV (IgG1 $P<0.01$, IgG2c $P<0.01$) (**Fig. 6-2A, B**). The addition of Quil A significantly ($P<0.05$) increased IgG2c but not significantly IgG1 titers by ~5-10 fold (**Fig. 6-2A, B**). In summary, a single 1µg dose of insect-cell derived CHIKV VLPs induced potent CHIKV-specific neutralizing antibody and IgG titers.

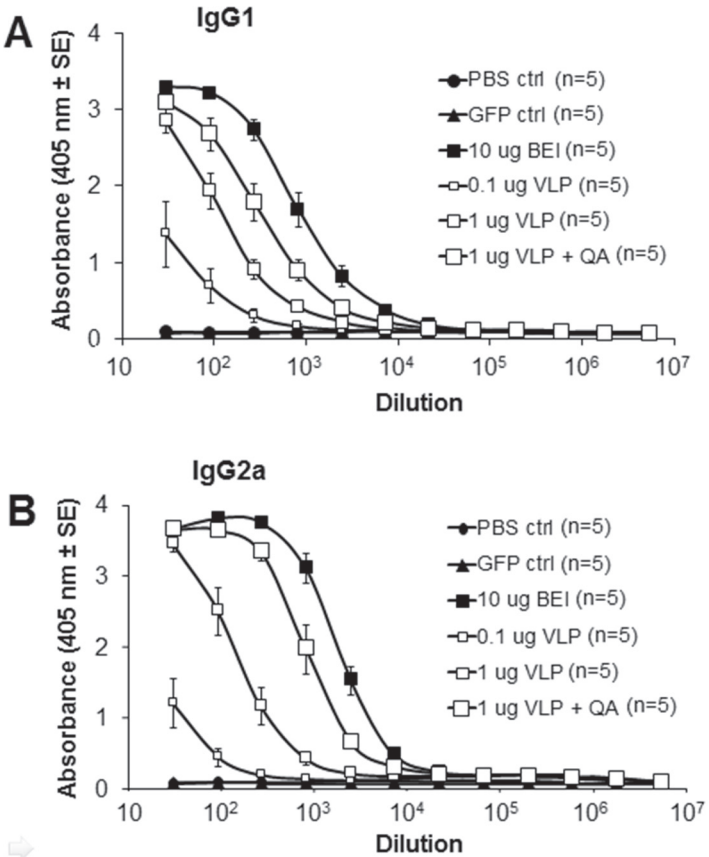

Figure 6-2. IgG-isotyping of immunized mouse serum.
A-B) Immunoglobulin-G1 and -2c isotypes in immunized mouse serum, were determined using ELISA upon serial dilution. Statistical analysis shows that all CHIKV antigen immunized groups are significantly different from the control groups

CHIKV challenge of vaccinated C57BL/6 mice

Vaccinated mice were challenged 6 weeks post-vaccination with a Réunion Island CHIKV isolate using a recently developed adult wild-type mouse model of CHIKV viraemia and arthritis (Gardner et al., 2010). PBS-vaccinated animals and animals vaccinated with the GFP control group showed similar viraemia; > 10^8 $TCID_{50}$/ml at 2d post challenge (**Fig. 6-3**). All animals vaccinated with 1 µg VLPs were completely protected against viraemia (**Fig. 6-3**). Mice vaccinated with 0.1 µg VLPs, showed a ~7 log reduction in viraemia on day 2, with virus undetectable on the other days (**Fig. 6-3**). Vaccination with 10 µg of inactivated virus also provided complete protection against viraemia as described previously (Gardner et al., 2010).

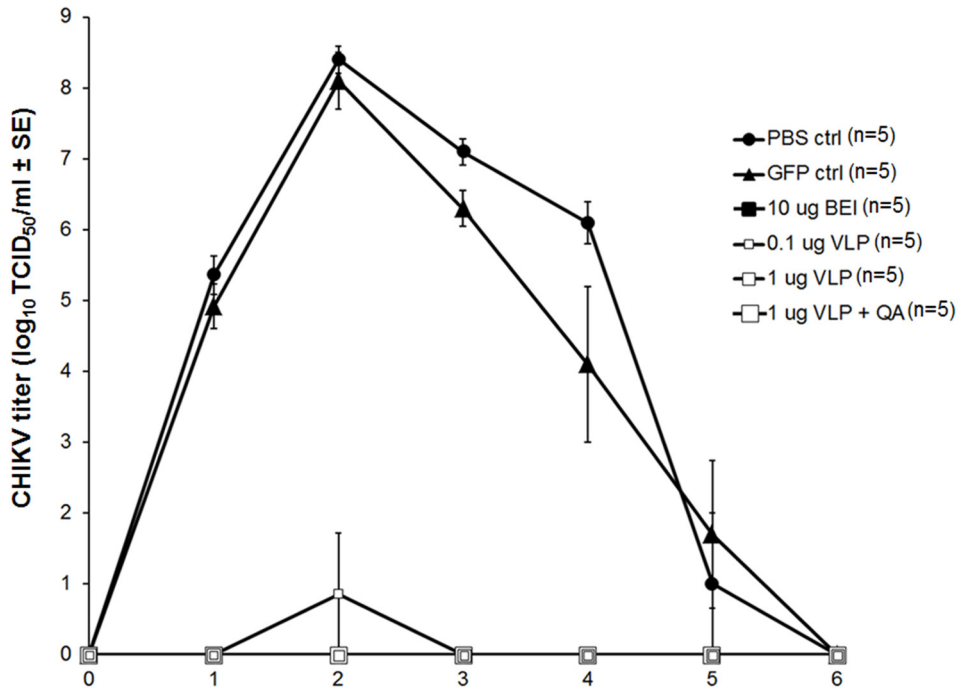

Figure 6-3. CHIKV viraemia after VLP vaccination and CHIKV challenge.
At least 6 weeks old mice were vaccinated with 0.1 µg VLPs, 1 µg VLPs and 1 µg VLPs adjuvanted with Quil A (QA). PBS and GFP were used as a negative control and inactivated CHIKV virus as a positive control (n =6 per group). Mice were challenged with the Rèunion Island isolate 5 w post infection. Viraemia levels were determined over 6 days. Statistical analysis shows that mice vaccinated with inactivated CHIKV virus, 1 µg VLPs and 1 µg VLPs adjuvanted with Quil A display signifact lack of viraemia at times of peak viraemia (1-3 dpi) in the control groups.

Arthritis in this model is readily determined by measuring foot swelling and, as expected (Gardner et al., 2010), the inactivated virus completely protected against foot swelling, whereas animals given PBS showed a mean 60-70% increase in foot swelling (**Fig. 6-4**). The 1 µg dose of VLPs (with or without Quil A) provided complete protection against foot swelling, while the 0.1 µg dose reduced the peak foot swelling from 60-70% to 20-30% (**Fig. 6-4**). These results illustrate that non-adjuvanted VLPs can provide complete protection against CHIKV-induced arthritis.

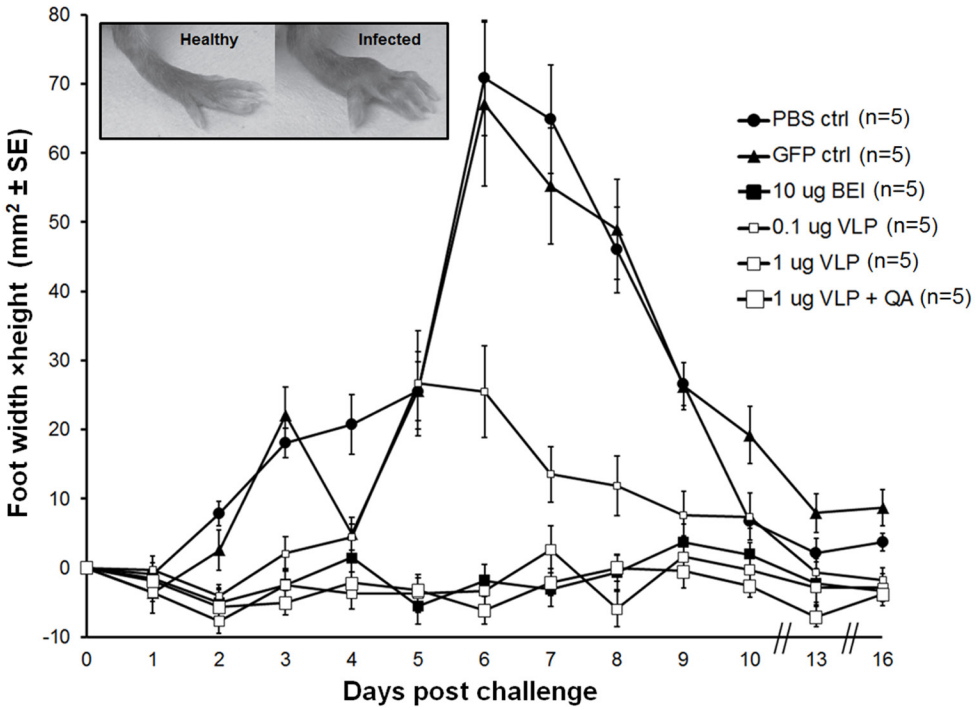

Figure 6-4. CHIKV induced arthritis in immunized mice.
At least 6 weeks old mice were vaccinated with 0.1 µg VLPs, 1 µg VLPs and 1 µg VLPs adjuvanted with Quil A (QA). PBS and GFP were used as a negative control and inactivated CHIKV virus as a positive control (n =6 per group). Mice were challenged with the Rèunion Island isolate 5 w post infection and were monitored for foot swelling as an indicator for arthiritic disease (Gardner et al., 2010). Foot size in mm2 (width×height) were determined over 16 days post challenge. Statistical analysis shows that mice vaccinated with inactivated CHIKV virus, 1 µg VLPs and 1 µg VLPs adjuvanted with Quil A display signifact lack of at times of peak foot swelling (6-8 dpi) in the control groups.

Discussion

The continuous spread of CHIKV and increase in morbidity makes CHIKV one of the most important emerging arboviruses in developed countries. Prevention currently relies on vector control but should be focused on the development of a safe and efficient CHIKV vaccine. Several experimental vaccines have been generated to combat CHIKV infections but none of them has been licensed (Akahata et al., 2010; Harrison et al., 1971; Kumar et al., 2012; Levitt et al., 1986; Mallilankaraman et al., 2011; Muthumani et al., 2008; Plante et al., 2011; Wang et al., 2011; Wang et al., 2008). In the pursuit of developing a new, safe and effective vaccine, we used the recombinant baculovirus-insect cell expression system to produce CHIKV VLPs. This expression system for heterologous proteins is an elegant and promising manner to produce subunits and VLP-based vaccines (Metz and Pijlman, 2011). The complete CHIKV structural cassette was cloned downstream the strong polyhedrin promoter of the *Ac*MNPV baculovirus (*Ac*-S27) and CHIKV VLPs were produced and characterized as described in **Chapter 3** (Metz et al., 2013).

We show herein that a single vaccination with 1 µg of unadjuvanted CHIKV VLP vaccine was able to completely protect mice from CHIKV-induced viraemia and arthritis in an adult wild-type model of CHIKV arthritis that recapitulates many aspects of human disease i.e. self-limiting arthritis (joint inflammation), tenosynovitus and myositis (Gardner et al., 2010). The value of adjuvants in this system remains to be fully explored, with different adjuvants, adjuvant doses and VLP:adjuvant ratios and their effect on protection needing to be analyzed. The importance of protection studies is highlighted by the ability of Quil A to increase CHIKV-specific IgG titers, but reduce neutralizing titers. A recent human trial also illustrated that aluminum hydroxide adjuvant provided increased immunogenicity for an inactivated Ross River vaccine (Aichinger et al., 2011). The VLPs induced a balanced IgG1/IgG2c response, in contrast with CHIKV infection where IgG2c responses dominate (Gardner et al., 2010). Whether this would have an important effect on protection is unclear. However, we have recently found that there was no difference in viraemia or arthritis following CHIKV infection of mice deficient in the Fc receptor common gamma chain (unpublished data), suggesting the distinct Fcγ receptor-binding properties of the different antibody isotypes (Nimmerjahn and Ravetch, 2006) does not play a major role in protection (at least in mice). Interestingly, the 1 µg non-adjuvanted VLP vaccine elicited a similar neutralizing antibody response as compared to 10 µg of inactivated virus (**Fig. 5A**), yet induced lower IgG2c titers (**Fig. 5C**). This might suggest that chemical inactivation results in generation of antibodies with reduced neutralizing activity, although further experimentation would be required to confirm this.

Previous vaccination studies using CHIKV VLPs have shown that VLP provided protection in mice and non-human primates against CHIKV infection (Akahata et al., 2010).

In those experiments, two immunizations of 19 µg VLPs with adjuvant were needed to induce protection in mice, while our data show that a single vaccination of 1 µg of non-adjuvanted VLPs induces complete protection against viraemia and foot swelling. Although a different CHIKV strain (West-African isotype, also known as strain 37997) was used to produce VLPs in 293F cells (Akahata et al., 2010), the baculovirus-insect cell expression system may also provide VLPs with better immunogenicity. Glycosylation patterns in insect cells differ from those in mammalian cells, in that lepidopteran insect cells do not process N-glycans to terminally sialylated complex-structures. Differences in glycan processing have been shown to influence glycoprotein immunogenicity (Gavrilov et al., 2011; Helle et al., 2010; Tomiya et al., 2004).

Four different CHIKV strains have been described so far, including the East-, Central- and South African (ECSA) strain, the West-African strain, the Asian strain and the recent Réunion Island strain. The produced VLPs are of the ECSA strain, while the immunized mice were challenged with the Réunion Island strain. Even though these strains belong to the same ECSA phylogroup, it has been shown that ECSA strain based vaccines are able to cross-neutralize against other CHIKV strains and even other alphaviruses from the same serogroup (Gardner et al., 2010; Partidos et al., 2012; Wang et al., 2011). Therefore, our recombinant CHIKV VLPs would be expected to provide protection against most, if not all, CHIKV strains.

The favorable properties of the recombinant baculovirus-insect cell expression system renders it extremely powerful in the production of subunit or VLP based vaccines. Even though baculoviral replication is lytic to insect cells and heterologous protein production is therefore not continuous, the sheer expression levels reached are high, if not the highest, of all eukaryotic expression systems (Roldão et al., 2010). Baculovirus expression vectors can be quickly generated and therefore this system is ideally suited for generating emergency vaccines ("pandemic preparedness")(Cox and Hashimoto, 2011). In addition, insect cells can easily be scaled up in serum-free suspension culture to large culture volumes (van Oers, 2011). Conditions for optimal stability of VLPs produced under serum-free conditions should be determined when this vaccine candidate is further developed by the industry, but sofar we have no indications that storage at -80ºC is detrimental to VLP integrity.

In conclusion, we have shown that complex structures such as CHIKV VLPs are produced at high levels and were efficiently processed and glycosylated in insect cells using recombinant baculoviruses. More importantly, this is the first study that shows that a single low-dose immunization with 1 µg of non-adjuvanted CHIKV VLPs provided complete protection against viraemia and foot swelling caused by CHIKV infection. We propose CHIKV VLPs produced by insect cells using recombinant baculoviruses to be further developed as a safe and effective vaccine candidate to protect humans against CHIKV outbreaks.

Acknowledgements

This work was performed within the framework of Dutch Top Institute Pharma, project "Development of recombinant live and subunit vaccines against chikungunya virus infections" (project nr. T4-301).

Material and Methods

Vaccination

The purified CHIKV VLPs were used as a vaccine. Female C57/BL6 mice (6-12 weeks old) were vaccinated once subcutaneously on the back above the base of the tail with 0.1 µg VLPs or 1 µg VLPs in 50 µl RPMI 1640 medium (Gibco). As negative control, a purified fraction of *Ac*-GFP infected culture media was used. Binary ethylenimine (BEI)-inactivated purified CHIKV was used as positive control and produced as described (Gardner et al., 2010). Where indicated, the VLP suspension was formulated with 10 µg/mouse Quil A (Iscotec) prior to injection.

Murine virus neutralization and ELISA antibody assays

The neutralizing ability of the mouse serum was assayed as described (Wang et al., 2011). Serum from each mouse was heat-inactivated at 56°C for 30 min and serially diluted in a 96-well plate. Diluted serum was incubated with 200 $TCID_{50}$/ml CHIKV of the Réunion Isalnd CHIKV isolate for 2 h at 37°C. Vero cells (10^4/well) were added to the plate and incubated for 5 days at 37°C. The serum dilution yielding >95% protection against CPE was determined by staining the cells with crystal violet. Determination of the CHIKV-specific IgG1 and IgG2c antibody titers was performed by ELISA as described previously (Gardner et al., 2010).

Viral challenge and disease monitoring

Female C57BL/6 mice (6 to 12 weeks old) were inoculated with CHIKV (LR2006-OPY1), and viraemia and foot swelling were determined as described previously (Gardner et al., 2010; Rudd et al., 2012). Foot swelling was monitored by measuring the height and width of the metatarsal area of the hind feet using digital callipers and is presented as a group average of the percentage increase in foot height times width for each foot compared with the same foot on day 0. All animal experiments were approved by the Queensland Institute of Medical Research (QIMR) animal ethics committee and adhered to the Australian code of practice for the care and use of animals for scientific purposes (NHRMC, Australia; 7th edition 2004).

Statistical analysis

Statistical analysis on antibody responses was performed using SAS, specifically one way ANOVA with Tukey post-hoc test. Statistical analysis on viraemia and foot swelling was performed using IBM SPSS Statistics 19. For comparison of two samples, the t-test was used when the difference in the variances was less than 4 and skewness was greater than minus 2 and kurtosis was less than 2; otherwise, a non-parametric test was used, specifically, Mann-Whitney U test if variance was less than 4 or Kolmogorov Smirnov test if greater than 4.

Chapter

General Discussion

The CHIKV epidemic of 2004 in East-Africa was the outbreak in which CHIKV emerged from its relative obscurity. Outbreaks in coastal Kenya ultimately lead to epidemics on islands in the Indian Ocean, among which the infamous epidemic on Réunion Island in 2005/2006. Further spread to India and Southern-Europe and the number of traveller infections has boosted the development of a safe and effective CHIKV vaccine. Although this has been the focus of many studies (Akahata et al., 2010; Gardner et al., 2010; Harrison et al., 1971; Kumar et al., 2012; Levitt et al., 1986; Mallilankaraman et al., 2011; Muthumani et al., 2008; Partidos et al., 2012; Plante et al., 2011; Tiwari et al., 2009; Wang et al., 2011), there is still no commercially available vaccine against CHIKV infections. The use of CHIKV subunits and VLPs in vaccine development have shown to be a promising strategy in terms of safety and immunogenicity, but large-scale production has not been documented (Akahata et al., 2010; Kumar et al., 2012). This thesis focusses on the production and characterisation of CHIKV-E1 and -E2 subunits and CHIKV-VLPs in insect cells, using the recombinant baculovirus-insect cell expression system. After purification, the E1 and E2 subunits and VLPs were tested for their ability to induce protective immune responses in mice as a proxy for primates.

Generation of vaccine candidates

The trimeric spikes on the surface of the CHIKV virion are composed of three E1-E2 heterodimers, and carry the major antigenic determinants to which neutralizing antibodies are targeted. To generate the CHIKV subunits, E1 and E2 were cloned downstream the strong *polh*-promoter of baculoviruses, together with their native signal peptides 6K and E3, respectively (**Chapter 2**). Deletion of the C-terminal transmembrane domain of both E1 and E2 resulted in secreted subunits. N-linked glycosylation and furin dependent processing of the final recombinant E1 and E2 subunits produced by recombinant baculoviruses appeared to be similar if not identical to authentic CHIKV processing in mammalians. However, expression in *Sf*21-cells also resulted in partially incomplete processing in the form of E3E2 processing intermediates. Incomplete glycosylation and ER-retention is a common phenomenon seen by high level expression of viral glycoproteins in insect cells (van Oers et al., 2001). This ER-stress induces the unfolded protein response (UPR) and it might be that the massive overexpression of the CHIKV glycoprotein disables the UPR to mitigate ER-stress. Despite this partial incomplete processing, the presence of E3E2 is not expected to cause restrictions on immunogenicity of subunits from insect cells, since E3 harbours neutralizing epitopes as well (Kam et al., 2012a; Parker et al., 2010).

Similar results were found after the expression of the full structural gene cassette (C, E3, E2, 6K, E1) (**Chapter 3**). However, E3E2 processing and glycosylation efficiency were significantly higher than that of individual expressed E2. Only correctly processed E1 and E2 proteins were incorporated in to VLPs. The VLPs were morphologically similar to authentic CHIKV and other alphavirus VLPs and ranged in size of a diameter of 68 ± 14 nm (Akahata et al., 2010; Metz et al., 2011a). The VLP yield (40 mg/L) was based on E2 detection and outweighs the CHIKV VLP production yield of DNA-transfection of 293F cells (10-20 mg/L) (Akahata et al., 2010) by a factor of two. This underscores one of the major advantages of the baculovirus-insect cell system for production of recombinant proteins. A further gain in VLP yield is expected in an optimized large-scale insect-cell bioreactor configuration (Vicente et al., 2011).

The same strategy as was used for CHIKV, was applied to produce SAV VLPs (**Chapter 4**), in order to serve as an alternative vaccine to the currently available inactivated virus vaccine Compact PD® (MSD). However, glycoprotein processing and VLP production of this unusual alphavirus appeared to be highly temperature dependent. At normal expression temperatures of 27°C, E3E2 was misfolded, not processed by furin and undetectable on the surface of infected cells. Since wild type SAV replicates at low temperatures (10-12°C) in Atlantic salmon, a temperature-shift regime was designed to enable correct E1/E2 processing and VLP production. Recombinant SAV-VLP expression appeared to be highly adaptable to lower temperatures, since both baculovirus infection and heterologous gene expression was observed at temperatures as low as 12°C. However, the yields were relatively low and further tailoring is required to be competitive with Compact PD®.

Immunogenicity of CHIKV subunits and VLPs

Vaccination with the CHIKV-E1 and -E2 subunits only partially protected IFN-knockout mice from lethal CHIKV challenge. The VLPs induced a full protective immune response after a single immunization, with significant higher neutralizing antibody titers compared to when subunits are involved (**Chapter 5**). Particle size, repetitive antigen presentation and protein folding most likely explain the reduced immunogenicity of the individual subunits. These data suggest that even a mild neutralizing immune response is sufficient to protect mice from lethal infection. However, VLPs are known to activate both B-cell mediated and cytotoxic T-cell responses (Noad and Roy, 2003). This may explain the results from testing the CHIKV VLPs in an adult wild type mouse model (**Chapter 6**) (Gardner et al., 2010). A single immunization with 1 µg of non-adjuvanted VLPs protected mice against CHIKV infection and arthritic disease symptoms. In addition and in contrast to what was found previously (Gardner et al., 2010), the VLPs induced a balanced IgG1-IgG2c response. The antigen dose required for complete protection, can most likely can be reduced even further, especially in combination with a potent adjuvant. This would increase the number of doses

that can be retrieved from a production batch. Successive studies should further focus on VLP doses and the effect of adjuvant on protection, but these first results indicate that full protection is achieved using significant lower doses of insect cell-derived VLPs as compared to VLPs produced by DNA transfection in mammalian cells (Akahata et al., 2010).

Upstream processes

Several expression or production systems, such as bacteria, yeast or mammalian cells are suitable for VLP production. However, the choice of the appropriate expression system is restricted if VLPs are enveloped or are composed of e.g. glycoproteins. Insect cell expression using recombinant baculoviruses, has many advantages over other expression systems and remains a versatile and robust expression platform for the production of VLPs. In the current research, CHIKV-VLPs were produced in monolayer *Sf*21-cell cultures, which is not a suitable setup for large-scale production. However, insect cells have fast growth rates in serum-free and protein-free culture media and are perfectly adaptable to growth in suspension culture (O'Reilly et al., 1993). This makes these cells well suited for large-scale upstream processes such as VLP production. The appropriate production scale depends highly on the specific VLP production yield, which can range from 0.2 mg/L of SARS coronavirus VLPs (Mortola and Roy, 2004) to 662 mg/L rotavirus VLPs (Vieira et al., 2005). The highest CHIKV-VLP yield, 40 mg/L, was derived from a 30 ml suspension culture. It was shown that 1 µg of VLPs (E2-equivalents) is sufficient to provide complete protection in mice. However, the antigen levels necessary to induce protection in man, can at this stage only be estimated based on the comparison with other human vaccines against enveloped viruses, such as Flublok® (Protein Science Corp.) This trivalent influenza vaccine is composed of 45 µg of each of the in total three types of recombinant hemagglutinin (rHA) protein subunit of different influenza strains (Treanor et al., 2007). If similar CHIKV-VLP antigen levels are assumed to be required to provide protection in man, this would indicate that approximately 440 thousand doses can be derived from a 500 L bioreactor. However, these yields are not representative for the yields that will be reached after stringent downstream processing to e.g. remove and inactivate baculovirus particles, necessary for human application. To make a reasonable estimate of the yields that can be reached for this purpose, a pilot production process of at least 1 L should be executed.

Single-use bioreactors are very suitable for the large scale production of the CHIKV-VLPs. These disposable bioreactors (e.g. WAVE Bioreactor®, GE Healthcare) have many advantages over stainless-steel vessels such as low risk of cross contamination, no cleaning and sterilization costs, lower upstream investment costs and they utilize little laboratory space. Single-use bioreactors have limitations on culture volumes, pressure and high temperature usage, but these limitations do most likely not apply for the production of the CHIKV VLPs described in this thesis. Although insect cells are very robust, bioreactor parameters such as

dissolved oxygen concentrations, agitation rates, temperature, pH and inlet gas flow, should be carefully optimized.

Optimizing CHIKV VLP-production

Besides optimizing the insect cells environment, improving protein expression by tailoring molecular changes to the transcription, translation and processing machinery of the insect cells, can have strong effects on production yields. Currently, most baculovirus expression constructs use the very late *polyhedrin* and/or *p10* promoters (Smith et al., 1983b). However, the nature of the promoter can have substantial impact on productivity of the protein product. Late and very late promoters initiate protein expression at the final stages of baculoviral replication, which is accompanied by strong protease activity and cell death. Early promoters in general induce lower protein yields because the enzymes and transcription factors necessary for protein processing are not yet fully active (Roldao et al., 2007). The expression of CHIKV-glycoproteins from the *polh* promoter resulted in the accumulation of E3E2 processing intermediates in the ER. Provided that expression levels are high enough, putative beneficial effects of earlier transgene expression from baculoviral early and late (e.g. *Ac*-gp64 or *Ac*-p6.9) promoters may be used to prevent accumulation of heterologous protein and thereby increasing processing efficiency and VLP production.

Insect cells are dramatically transformed by baculovirus infection, have enlarged nuclei and are specialized to support nuclear baculovirus DNA replication and virion production. Small amounts of only two baculoviral glycoproteins, i.e. GP37 and GP64 (Wang et al., 2010), are produced to be incorporated in the envelope of budded viruses. In this respect, the environment in baculovirus-infected cells is very different from the cellular transformation induced by CHIKV, which replicates its viral RNA in virus-induced, proliferating membraneous structures in the cytoplasm of infected cells (Mackenzie, 2005). The production of CHIKV virions and thus CHIKV VLPs is accompanied by high level expression of complex viral glycoproteins in the ER. Solving this apparent discrepancy in the requirements for baculovirus replication vs. CHIKV glycoprotein production in the same cell is a key point for future technological improvements. Co-expression from the baculovirus expression vector of ER-resident chaperones, foldases, or ER proliferating genes may further boost the potential for CHIKV glycoprotein production (van Oers, 2011).

During expression of the structural genes of both SAV and CHIKV, dense capsid bodies, composed of assembled nucleocapsids were observed in the nucleus of infected insect cells (**Chapter 4**). Alphavirus capsid (C) proteins are known to translocate to the nucleus (Aguilar et al., 2007; Karlsen et al., 2010; Michel et al., 1990; Mitchell et al., 1997), but assembly in to nucleocapsids in the nucleus has not been reported. The formation of capsid bodies consisting of accumulation of nucleocapsids, have so far only been observed after high level expression of capsid by recombinant baculoviruses (**Fig. 7-1**). Normally, the assembly of the

nucleocapsid takes place in the cytoplasm of infected cells (Kuhn, 2007). By the interaction with the intracellular tail of the trimeric spikes, the nucleocapsids bud out from the cells, taking along the host plasma membrane with the trimeric spikes attached to it. This is not expected to be different in VLP formation. Thus, nuclear capsid body formation is most likely an artefact of the very high baculovirus expression levels of CHIKV-C. Using GFP-capsid fusion constructs it was confirmed that these dense nuclear bodies are composed of C, which indicates that there is a substantial amount of C accumulating in the nucleus and therefore not available for VLP budding (Metz, unpublished results). Interestingly, nucleic acid staining by Hoechst reagent, showed that nucleic acid co-localizes with the capsid. In theory, by preventing capsid to go to the nucleus, there may be more cytoplasmic capsid available for the formation cytoplasmic nucleocapsids as well as for the formation of VLPs, provided that the amount of E1/E2 on the cell surface is not a limiting factor. This might be an avenue for further research on optimising VLP yields.

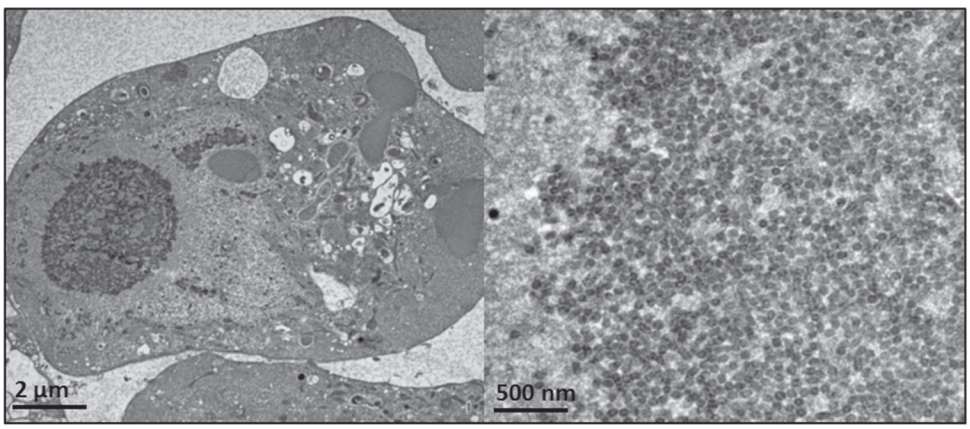

Figure 7-1. Nuclear capsid body formation and nuclear localisation of baculovirus-expressed CHIKV capsid. Transmission electron micrograph of Ac-capsid infected cells showing the nuclear capsid boy and assembled nucleocapsids.

Downstream processes.

One of the potential drawbacks of using recombinant baculoviruses for the expression of VLPs is the presence the production of budded viruses (BV) during infection. Although BVs may induce synergistic effects on the VLP-induced immune responses due to their adjuvant activity (Hervas-Stubbs et al., 2007; Margine et al., 2012). While this may not be a problem for development of veterinairy vaccines, in order to use the CHIKV-VLPs as a human vaccine, the purity, efficacy and consistency become crucial factors in the development of large-scale production processes, thus including the removal or inactivation of BVs.

The entire downstream process can be divided into three processing steps *i*: clarification, *ii*: intermediate purification/concentration and *iii*: polishing. The sucrose gradient ultracentrifugation method used to purify the CHIKV-VLPs is well suited for laboratory scale processes, but is non-scalable and laborious. Although centrifugation is still used by industry, scalable membrane processes such as depth-filters or tangential flow microfiltration are more attractive methods to remove large aggregates and cell debris from the VLP-rich medium fraction (Peixoto et al., 2007; Saha et al., 1994). During laboratory-scale purification, it was shown that 0.2 µm retention pores allow passage of the VLPs, and have the additional advantage of retaining the fraction of BVs larger than 200 nm (Metz, unpublished results). In terms of recovery, membrane and filter processes do fairly well, reaching recovery yields over 90% (Peixoto et al., 2007).

In order to reduce investment and consumable costs, the processing volume of the VLPs should be reduced as early as possible. Ultrafiltration or diafiltration processes are frequently used for concentration, but adsorptive chromatography is a popular alternative. If the capacity of the chosen carrier is significant for the VLPs in question, high concentration factors can be reached when chromatography is operated as a capture step (Vicente et al., 2011). The use of disposable ion-exchange membrane layers or monoliths is preferred over packed-bed matrixes. The specific surface area of the porous membranes is significantly higher than those of the packed-bed resin beads. Pore sizes in membrane matrices are larger (up to > 1µm) compared to the pores of conventional resin beads (10-20 nm), resulting in an more optimal usage of the internal binding surface by VLPs within a 20 nm- 200 nm size range (Trilisky et al., 2009). Before large-scale concentration can be performed by using ion-exchange membrane matrices, the optimal operating conditions and CHIKV-VLP electrostatic properties have to be determined, by e.g. dynamic light scattering and surface plasmon resonance (Vicente et al., 2010a; Vicente et al., 2010b).

As determined by the US Food and Drug Administration (FDA), residual host-cell protein debris and host-cell DNA, need to be removed below acceptable thresholds, which are set in the Center for Biologics Evaluation and Research (CBER) guidelines. This means that during the polishing step, most of the BVs need to be removed, which can be done by ion-exchange chromatography. However, this might be challenging in case of enveloped VLPs with similar electrostatic properties. Recent research towards a BV-free production platform showed that deletion of the *Ac*MNPV VP80 protein prevented the formation of BVs, while heterologous gene expression was not affected (Marek et al., 2011). However, this technology is not yet suitable for large-scale production processes. Purified VLPs produced by recombinant baculoviruses often undergo chemical inactivation by treatment with detergents and for alkylation with binary ethylenimine (BEI) to eliminate the present BVs (Rueda et al., 2000). A common and final step in the purification process is sterile filtration through a 0.2 µm filter.

Arbovirus VLP vaccine development using baculovirus vectors.

Similar to CHIKV, many other pathogenic mammalian viruses are transmitted by arthropods. These arboviruses are found in four families of RNA viruses: *Togaviridae* (genus *Alphavirus*), *Flaviviridae* (genus *Flavivrus*), *Bunyaviridae* (genus *Nairovirus, Orthobunyavirus, Phlebovirus*) and *Reoviridae* (genus *Orbivirus*). Highly pathogenic arboviruses that are transmitted by mosquitoes include CHIKV, dengue virus (DENV), West Nile virus (WNV) and Rift valley fever virus (RVFV). They have a significant worldwide impact on human health by causing a variety of diseases including (hemorrhagic) fever, hepatitis and encephalitis, leading to hundreds of thousands of deaths each year (Whitehead et al., 2007). Increased global trade, transport and travel in combination with changing climate and increasing population density are considered important factors in the emergence and increased incidence of arbovirus-related illnesses worldwide, particularly in the temperate regions of Western Europe and North America. A dramatic, recent example of global vector distribution is the Asian tiger mosquito, *Aedes (Ae) albopictus*, which originated in Asia but is now endemic in large parts of the USA and Southern Europe (Paupy et al., 2009). In September 2010, 2 autochthonous cases of DENV and 2 cases of CHIKV in the South of France were reported, most likely the result of arbovirus circulation in the established, local population of *Ae. albopictus* (Gould et al., 2010). The ongoing DENV epidemic in Maderia, Portugal has caused 1891 cases of infection within one month. This event represents the first european DENV epidemic since 1928. Recent models have indicated temperate regions in the US as high risk areas for CHIKV epidemics (Ruiz-Moreno et al., 2012), indicating potential threats for global arbovirus spread. Epidemics of animal arboviruses, such as Venezuelan equine encephalitis virus (VEEV), Bluetongue virus (BTV) and the recent Schmallenberg virus are on the increase as well and can cause dramatic losses of livestock in short periods of time (Paessler and Weaver, 2009; Savini et al., 2008).

Vaccination is the most effective tool to protect humans and livestock against arboviral disease. At present, only a few arboviral vaccines are licensed for use in humans, they are directed against yellow fever virus (YFV), Japanese encephalitis virus (JEV) and tick-borne encephalitis virus (TBEV) (family *Flaviviridae*). For animals, WNV, RVFV and BTV vaccines are currently available. For all arbovirus families, recombinant baculoviruses and insect cells have been used to produce (glyco)protein subunits or VLPs, of which many provide full or partial protection against subsequent viral infection in mice (Metz and Pijlman, 2011). Although the baculovirus-insect cell expression system serves as an elegant platform to produce arboviral VLPs (**Fig.7-2**), this is still a fairly uncharted area and few studies have focused on the immunogenicity of arbovirus VLPs produced in insect cells. Alphavirus VLP production using baculovirus expression has been studied most extensive among the arboviruses.

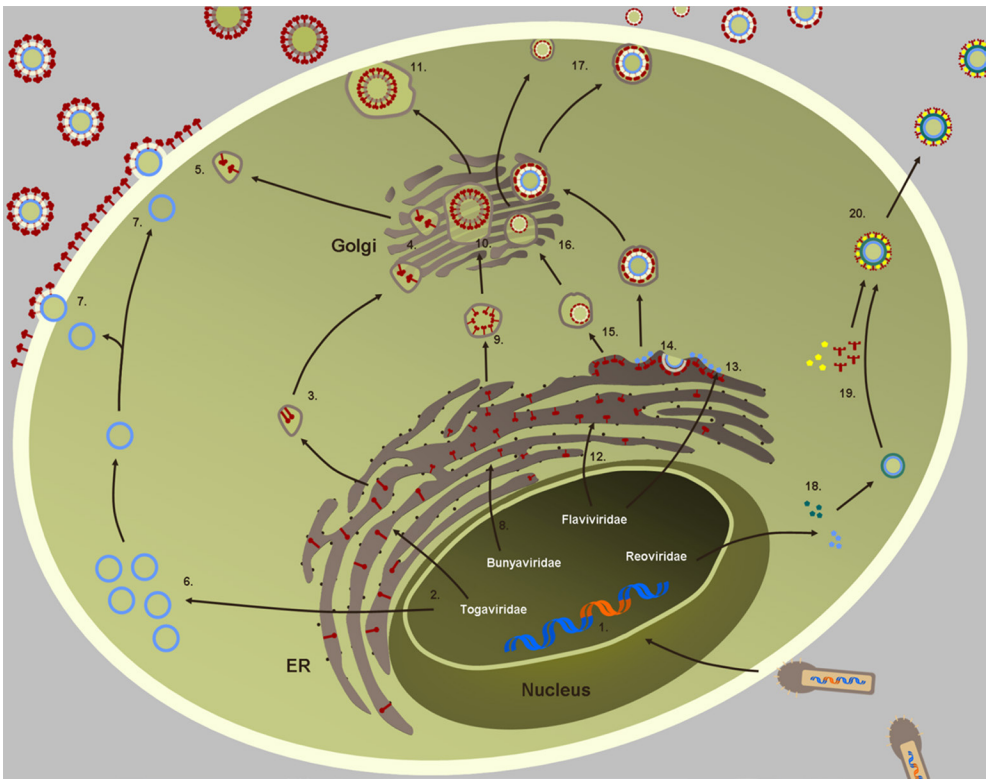

Figure 7-2 Arbovirus VLP formation and maturation in insect cells after recombinant baculovirus expression. The heterologous genes encoding the structural proteins of (2-8) *Toga*-, (9-12) *Bunya*-, (13-17) *Flavi*- and (18-20) *Reoviridae* are transcribed in the nucleus. 1) The *Togaviridae* envelope glycoproteins E1 and E2 are transported to the ER, complex into heterodimers (2) and are transported to the Golgi (3, 4) where three heterodimers complex into trimeric spikes and furin processing takes place. 5) The mature trimeric spikes are transported to the plasma membrane and are exposed on the surface of the cell. 6) The capsid protein assembles into nucleocapsids in the cytoplasm. 7) The nucleocapsids bud out from the plasma membrane, taking along the trimeric spikes anchored in the lipid bilayer. 8) *Bunyaviridae* glycoproteins Gn and Gc are transported to the ER and complex into heterodimers. 9) The dimers are transported to the Golgi, where they assemble into new viral particles (10). 11) The particle containing membrane vesicles bud out from the plasma membrane. 12) *Flaviviridae* glycoproteins M and E are transported into the ER and form heterodimers. 13) The capsid protein assembles into nucleocapsids in the cytoplasm and buds into the ER (14). In addition, subviral particles are formed, that lack nucleocapsids (15). Both viral particles are transported through the Golgi (16) in membrane vesicles which eventually fuse with the plasma membrane. 17) All *Reoviridae* structural proteins are translated in the cytoplasm (18, 19) First, the inner capsid is assembled from VP3 and VP7 (18), after which the particle matures with the anchoring of VP5 and VP2 (19). 20) The particle is released from the cell through cell lysis or membrane penetration.

To date, VLPs have been produced of CHIKV (**Chapter 3**) (Metz et al., 2013), salmonid alphavirus (SAV) (**Chapter 4**) (Metz et al., 2011a) and VEEV (Hodgson et al., 1999), by expressing the full structural polyprotein (capsid, E3, E2, 6K, E1) in insect cells. Correct SAV glycoprotein processing (E1 and E2) and SAV VLP-production required a temperature-shift regime involving a production stage of 12°C. This low-temperature production exemplifies the versatility of the baculovirus-insect cell expression system. The CHIKV and VEEV VLPs were shown to provide full protection against viral challenge.

For flaviviruses, neutralizing responses focus more efficiently on the exposed epitopes on the envelope protein (E) when it is embedded in virus particles (Zlatkovic et al., 2010), indicating that VLPs should be able to induce a more efficacious neutralizing antibody response, compared to E-subunits. Recombinant baculoviruses have been used to produce flavivirus VLPs and subviral like particles (sVLP), by expressing CprME or prME, respectively (**Fig. 7-2**) (Qiao et al., 2004). Mice immunized with WNV sVLPs produced in insect cells, developed protective neutralizing antibody responses (Qiao et al., 2004).

More recent studies focus on the generation of VLPs for bunyavirus vaccine purposes. Co-expression of RVFV-N, -Gc, and -Gn results in the formation of VLPs from baculovirus infected insect cells (**Fig. 7-2**) (Liu et al., 2008). VLP immunization in mice elicited a high titer neutralizing antibody response and mice were fully protected against subsequent challenge (Naslund et al., 2009).

The co-expression of the African horse sickness virus (AHSV, family *Reoviridae*) inner capsid proteins VP3 and VP7 by recombinant baculoviruses, resulted in the formation of core-like particles (French and Roy, 1990; Maree and Paweska, 2005). These double-shelled VLPs are able to induce a high titer neutralizing antibody response (French et al., 1990). Recently, an improved strategy for rapid BTV VLPs production has been presented. A baculovirus expressing VP3 of BTV-10 and VP7 of BTV-17, was used as a basis for insertion of outer-capsid proteins VP2 and VP5 from different serotypes. This strategy was demonstrated to be fast in production, safe, and highly effective in sheep (**Fig. 7-2**) (Stewart et al., 2010). The successes obtained in VLP generation of BTV with baculovirus expression opens the way to develop similar vaccines for other, non-arboviral, members in the family *Reoviridae.* VLPs of the other arbovirus families contain two or more viral (glyco)proteins anchored in a host-derived envelope. It is interesting to note that for arboviruses from the families *Flaviviridae* and *Bunyaviridae*, VLPs can be made by expression of glycoproteins in the absence of the nucleocapsid protein (de Boer et al., 2010; Konishi et al., 1992; Kuwahara and Konishi, 2010; Qiao et al., 2004). Flavivirus subviral or prME particles have been shown to be immunogenic, and sometimes give higher immune responses than the corresponding nucleocapsid-containing VLPs (Qiao et al., 2004). For the *Togaviridae*, VLP formation without C expression has not been reported, which is not entirely unexpected taking into account that the direct protein-protein interaction between E2 and C is required for virion

assembly (Kuhn, 2007). However, new studies have shown that CHIKV and SINV trimeric spikes can also be efficiently produced via the replacement of 6K by a short peptide linker (Li et al., 2010; Voss et al., 2010). These complex yet stable polypeptides were secreted from the cells and were suitable for crystallization and structure analysis by X-ray diffraction. These artificial spikes turned out to adopt a very similar conformation as the trimeric spikes found on the surface of alphavirus virions. They can be considered a compromise between true subunits and VLPs and may provide a promising novel approach in alphavirus vaccine design.

Concluding remarks

This thesis describes the development of an effective and safe-to-produce CHIKV-VLP vaccine produced in insect cells using recombinant baculovirus expression and has led to more insight into alphavirus VLP formation. The characterisation of the CHIKV and SAV structural (glyco)proteins, clearly showed the applicability of baculovirus vectors for correctly processed and functional glycoprotein processing. Our CHIKV VLP vaccine provided protection after a single low-dose immunization, and is amenable to large-scale production. It is a novel vaccine candidate in the battle against CHIKV infections, and is ready for further safety and efficacy studies.

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Summary

Chikungunya virus (CHIKV) is an arthropod-borne alphavirus (family *Togaviridae*) and is the causative agent of chikungunya fever. This disease is characterised by the sudden onset of high fever and long-lasting arthritic disease. First identified in Tanzania in 1952, CHIKV has re-emerged in the last decade causing large outbreaks throughout Africa, Asia and Southern Europe. Increased CHIKV spread is mainly caused by its adaptation to a new mosquito vector, the Asian tiger mosquito *Ae. albopictus*, which is able to colonize more temperate regions. Currently, there are no antiviral treatments or commercial vaccines available, to prevent CHIKV infections. However, increased vector spread and clinical manifestations in humans, have triggered vaccine development. A broad range of vaccine strategies have been proposed and described, including inactivated virus formulations, live-attenuated virus, chimeric virus vaccines, DNA vaccines, adenoviral vectored vaccines, subunit protein vaccines and virus-like particle (VLP) formulations. However, these vaccination strategies have specific limitations in manufacturing, immunogenicity, safety, recombination and large scale production. Many, if not all safety problems do not apply for subunit or VLP based vaccines, except for the recombinant origin of the vaccine.

Recently, a CHIKV VLP-based vaccine was developed and provided protection in both mice and non-human primates. Even though this VLP approach is a safe, efficient and promising alternative to other vaccine strategies, large scale DNA plasmid transfection into mammalian cells and VLP yield of transfected cells remains challenging in terms of industrial production. These problems are alleviated by using the recombinant baculovirus-insect cell expression system.

In this thesis, recombinant baculoviruses were constructed to produce CHIKV glycoprotein E1 and E2 subunits and VLPs. For the production of CHIKV-E1 and E2 subunits, both protein genes were cloned downstream the polyhedrin gene (*polh*) promoter of in an A*utographica californica* multiple nucleopolyhedrovirus backbone, together with their authentic signal peptides 6K(E1) and E3(E2). Deletion of the C-terminal transmembrane domain, generated secreted versions of E1 (E1ΔTM or sE1) and E2 (E2ΔTM or sE2). A substantial amount of recombinant protein was glycosylated and processed by furin. The secreted CHIKV subunits were purified from the medium and were able to induce neutralizing antibodies in rabbits. For the production of the VLPs, the complete structural polyprotein (capsid, E3, E2, 6K, E1) was cloned downstream the *Ac*MNPV polh promoter. E3E2 precursor processing and glycosylation appeared to be more efficient when E3E2 were expressed as part of the whole structural polyprotein cassette, compared to the individually expressed E3E2. The VLPs were isolated from the medium fraction and were morphologically similar to wild type CHIKV. A similar strategy was used to produce VLPs from another alphavirus, the salmonid alphavirus (SAV). Here, however, the normal baculovirus expression temperature of 27°C appeared to be detrimental for SAV-E3E2 furin cleavage and SAV-VLP production. E2-glycoprotein

processing was shown to be temperature dependent and a tailored temperature-shift regime was designed in which *Sf*9-cells were infected with a recombinant baculovirus expressing the SAV structural proteins, and incubated at 27°C for 24 h, followed by a processing phase of 72 h at 15°C. Using this temperature regime, SAV-VLPs were produced that were morphologically indistinguishable from wild type SAV and underscores the flexibility of the baculovirus-insect cell expression system.

The immunogenicity of purified CHIKV-sE1 and -sE2 subunits and purified CHIKV-VLPs were then tested in a lethal vaccination-challenge mouse model, in IFN α/β, -γ receptor null AG129 mice. The innate immune system of these mice was made dysfunctional. This vaccine-challenge study clearly showed that VLPs provided superior protection, compared to their subunit counterparts. The subunits provided only partial protection and induced low neutralizing antibody titres. Immunization with the VLPs fully protected mice against lethal challenge and induced significant higher neutralizing antibody titress. Even though neutralizing antibody titres were lower after subunit immunization, this study showed that a minor neutralizing antibody response is sufficient to protect mice from lethal CHIKV challenge. Next, the CHIKV VLPs were tested for their ability to induce complete protection in an adult wild-type immune-competent mouse model, in which mice develop arthritic disease after CHIKV infection. The VLPs were able to induce full protection after a single immunization of 1 µg VLPs, without the use of adjuvants. In addition, IgG isotyping revealed a balanced IgG1-IgG2c response, suggesting a role for both humoral and cellular immunity in the protection against CHIKV infection. Mice served as a proxy for primates and vaccination trials in primates are next on the agenda.

This thesis is a typical example of the opportunities for the recombinant baculovirus-insect cell expression system in viral vaccine development, especially in vaccine development for other arboviruses. Although the CHIKV-VLPs produced in insect cells are amenable for large-scale production, the production process and downstream processing need to be carefully designed and optimized before CHIKV VLPs can be produced on an industrial scale. However, the data presented in this thesis show that CHIKV-VLPs produced in insect cells using recombinant baculoviruses represents as a new, safe, non-replicating and effective vaccine candidate against CHIKV infections.

Het chikungunyavirus (CHIKV) is een alphavirus (familie *Togaviridae*) en veroorzaakt de chikungunyagriep. De ziekte wordt gekarakteriseerd door een plotselinge hoge koorts die samengaat met ernstige gewrichtspijnen. De ziekteverschijnselen kunnen chronisch van aard zijn en houden soms jarenlang aan. CHIKV werd voor het eerst geïdentificeerd in Tanzania in 1952, waarna het in de laatste decennia verscheidene grote epidemieën in Afrika, Azië en Europa heeft veroorzaakt. De sterke toename in de geografische verspreiding van CHIKV wordt voornamelijk veroorzaakt door de nieuwe transmissievector, de Aziatische tijgermug *Aedes albopictus*. Heden ten dage is er geen antivirale behandeling of commercieel verkrijgbaar vaccin beschikbaar. De uitbreiding van het leefgebied van de vector en het verergeren van klinische complicaties hebben voor een verhoogde aandacht en activiteit in vaccinontwikkeling gezorgd. Een scala aan vaccinstrategieën is inmiddels beschreven, waaronder geïnactiveerde en geattenueerde virusformuleringen, chimaere virusvaccins, DNA-vaccins, adenovirusvectorvaccins, subeenheid- en 'virus-like particle' (VLP)-vaccins. Elk vaccinplatform heeft echter specifieke beperkingen op het gebied van ontwikkeling, immunogeniciteit, veiligheid, recombinatie en productie op industriële schaal. Veel van deze beperkingen gelden niet of in mindere mate voor het gebruik van virale subeenheden of VLP's.

Recentelijk is aangetoond dat een CHIKV-VLP vaccin volledige bescherming kan bieden bij zowel muizen als makaken. Hoewel VLP's een veilig en effectief alternatief zijn ten opzichte van andere vaccins, is het op grote schaal transfecteren (inbrengen) van zoogdiercellen met DNA-plasmiden en de beperkte VLP opbrengst daarvan, niet direct geschikt voor industriële productie. Deze problematiek wordt omzeild door gebruik te maken van het opschaalbare baculovirus-insectencelexpressiesysteem.

In dit promotieonderzoek zijn recombinante baculovirussen gemaakt om CHIKV glycoproteïne E1 en E2 subeenheden en CHIKV VLP's te produceren in insectencellen. De genetische codes van E1 en E2 zijn inclusief hun signaalpeptides 6K(E1) en E3(E2) achter de polyhedrine (*polh*) promoter gekloneerd in een *Autographica californica* multiple nucleopolyhedrovirus (*Ac*MNPV) expressievector. Het verwijderen van de C-terminale transmembraandomeinen van E1(E1ΔTM of sE1) en E2 (E2ΔTM of sE2) resulteerde in uitscheiding van de E1 en E2 subeenheden uit de cellen in het medium van de celcultuur. Een substantiële fractie van de subeenheden werd op de juiste wijze voorzien van suikergroepen en geknipt door furine-proteases. De uitgescheiden subeenheden zijn gezuiverd en waren in staat om neutraliserende antilichamen op te wekken in geïmmuniseerde konijnen. CHIKV VLP's werden geproduceerd door het genetische deel van CHIKV, dat de informatie voor capsid, E3, E2, 6K en E1 eiwitten bevat, als DNA-cassette onder de regie van de *Ac*MNPV *polh* promoter in insectencellen tot expressie te brengen. De rijping (maturatie) van E3E2 was efficiënter wanneer dit eiwit als onderdeel van de structurele cassette tot expressie werd gebracht in vergelijking met expressie van de individuele E3E2 subeenheden. De VLP's

werden met succes uit het medium geïsoleerd en waren qua uiterlijk niet te onderscheiden van CHIKV-virusdeeltjes. Eenzelfde strategie werd toegepast om VLP's te produceren van een ander alphavirus, het salmonid alphavirus (SAV). Hier bleek echter dat de normale expressie via een baculovirus bij een temperatuur van 27°C niet resulteerde in VLP-productie en de maturatie van E3E2 door furine niet plaatsvond. De correcte vouwing en maturatie van SAV-E2 bleken temperatuurafhankelijk te zijn. Daarop werd een specifiek temperatuurregiem ontwikkeld, waarin insectencellen geïnfecteerd werden met recombinante baculovirussen, die de SAV cassette met structurele genen tot expressie brachten gedurende 24 uur bij 27°C. Vervolgens werden gedurende 72 uur bij 15°C de SAV VLP's geproduceerd, qua uiterlijk niet te onderscheiden van infectieuze SAV-virusdeeltjes. Deze temperatuuraanpassing onderstreept nog eens de veelzijdigheid van het baculovirus-insectencelexpressiesysteem.

De immunogeniciteit van gezuiverde CHIKV E1/E2 subeenheden en gezuiverde CHIKV VLP's werd getest in een muizenmodel, waarbij muizen (AG129) bij infectie doodgaan omdat ze de IFN α/β, -γ-receptor missen en alleen beschermd kunnen worden via vaccinatie. Hieruit bleek dat de VLP's superieure bescherming boden in vergelijking met CHIKV subeenheden. De subeenheden boden slechts gedeeltelijke bescherming en induceerden lage neutraliserende antilichaamhoeveelheden. VLP-vaccinatie daarentegen, induceerde een significant sterkere neutraliserende immuunreactie en leidde tot volledige bescherming tegen CHIKV-infectie. Deze studie laat overigens zien dat zelfs een zwakke neutraliserende immuunrespons voldoende is om muizen te beschermen tegen lethale CHIKV-infecties. Vervolgens is het VLP-vaccin getest om te zien of het complete bescherming kan bieden in een muizenmodel, waarbij muizen artritis ontwikkelen als gevolg van CHIKV-infectie. Hieruit bleek dat een enkele vaccinatie met 1 µg zuivere VLP's volledige bescherming bood, zonder het gebruik van adjuverende hulpstoffen. IgG-serotypering wees daarnaast uit dat een evenwichtige IgG1-IgG2c respons werd geïnduceerd, wat duidt op een rol voor zowel humorale als cellulaire afweer in de bescherming tegen CHIKV-infecties.

Dit proefschrift is een mooi voorbeeld van de toepassing van het baculovirus-insectcelexpressiesysteem bij de ontwikkeling van virale vaccins. Hoewel de productie van CHIKV VLP's in insectencellen in principe op industriële schaal kan plaatsvinden, zal het gehele productieproces nog een ontwikkelfase moeten doormaken voordat de VLP's op deze schaal kunnen worden geproduceerd. Echter, deze studie laat duidelijk zien, dat de CHIKV VLP's, geproduceerd in insectencellen via het baculovirus-insectencelexpressiesysteem, een nieuwe, veilige en effectieve vaccin kandidaat vormen tegen CHIKV-infecties.

Abbreviations

AAV1	Adeno-associated virus serotype 1
*Ac*MNPV	*Autographa californica* multicapsid nucleopolyhedronvirus
AP	Alkaline phosphatase
Arbo	Arthropod borne
BEI	Binary Ethylineimine
BHK	Baby hamster kidney
BSL3	Biosafety level 3
BV	Budded virus
CBB	Coomassie brilliant blue
CBER	Center for Biologics Evaluation and Research
CHIKV	Chikungunya virus
CPE	Cytopathogenic effect
DNA	Deoxyribonucleic acid
ECSA	East-Central-South African
ELISA	Enzyme linked immunosorbent assay
EMC	Erasmus Medical Center
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
FDA	US Food and Drug Administration
GFP	Green fluorescent protein
GTNE	*200nm* Glycine, *50mM* Tris/HCl, *100mM* NaCl, *1mM* EDTA,
HBM	Honey bee mellitin
His	Histidine
Hpi	Hours post infection
IgG	Immunoglobulin Gamma
IRES	Internal ribosomal entry site
kDa	kilo Dalton
LSM	Laser scanning microscope
Mabs	Monoclonal antibodies
MOI	Multiplicity of infection
NBT/BCIP	Nitro blue tetrazolium/(5-bromo-4-chloro-1H-indol-3-yl) dihydrogen phosphate
NIAID	National Institute for Allergies and Infectious diseases
nsP	non-structural protein
OB	Occlusion body
ODV	Occlusion derived virus
ON	Overnight
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis

PAS	Periodic acid stain
PBST	Phosphate buffered saline Tween
PD	Pancreas disease
PEG	Polyethylene glycol
PE2	Precursor E2
PNGase F	Peptide-N-glycosidase F
QIMR	Queensland Institute of Medical Research
RdRp	RNA-dependent RNA polymerase
rHA	Recombinant hemagglutinin
RNA	Ribonucleic acid
RRV	Ross River virus
RT	Room temperature
RT-PCR	Reverse transcriptase- polymerase chain reaction
SAV	Salmonid alphavirus
SD	Sleeping disease
SDS	Sodium dodecyl sulphate
SFM	Serum free medium
SFV	Semliki Forest virus
*Sf*21	*Spodoptera frugiperda* 21
*Sf*9-ET	*Spodoptera frugiperda* 9 easy titer
SINV	Sindbis virus
SPDV	Sleeping disease virus
TCID50	Tissue culture infectious dose 50%
TEM	Transmission electron microscopy
TM	Transmembrane domain
UPR	Unfolded protein response
USAMRIID	U.S. Army Institute of Infectious Diseases
VEEV	Venezuelan equine encephalitis virus
VLP	Virus-like particle
WB	Western Blot
WNV	West Nile virus

Nawoord

Als al het officiële schrijfwerk erop zit is het tijd voor deze laatste woorden, die meestal het eerste en waarschijnlijk ook het meest gelezen zullen worden. Een zeer belangrijk onderdeel van het proefschrift dus, waarin duidelijk gemaakt kan worden hoe dit proefschrift tot stand is gekomen en zonder welke personen het nooit zou zijn gelukt. In niet geheel onwillekeurige volgorde:

Gorben. We mogen toch wel zeggen dat we terug kunnen kijken op een zeer geslaagde samenwerking in dit promotietraject. Tijdens mijn MSc thesis werd me al duidelijk dat ik in zeer goede handen was en ik twijfelde geen moment om bij je aan de slag te gaan. Hier heb ik in de afgelopen 4 jaar geen seconde spijt van gehad. Een betere begeleider had ik me niet kunnen wensen. Je gaf advies en sturing wanneer dit nodig was, en vrijheid en vertrouwen waar het kon. Onze R&O gesprekken waren iedere keer weer van zeer korte duur, zodat er weer snel over onderzoek kon worden gepraat. Naast de vele wetenschappelijke discussies, was je ook nooit te beroerd om mee te doen aan borrels en verjaardagen. Dit alles heeft er voor gezorgd dat ik mezelf enorm heb kunnen ontwikkelen zowel binnen als buiten het onderzoek. Je bent een onuitputtelijke bron van creatieve ideeën en kennis en een groot voorbeeld voor al je AIOs. Het was een eer om je eerste keer te zijn. Van chickiwawa tot chikinango, bedankt Chef!

Just. Vier jaar geleden had niemand zien aankomen dat jouw inbreng en betrokkenheid bij het project zo groot zouden worden. Na het overlijden van **Rob** heb je op bijzondere wijze het gehele lab onder je hoede genomen en het door een onzekere tijd geloodst. Heel erg bedankt voor je input, interesse, adviezen en sturing.

Rob. Ook al was onze direct samenwerking van veel te korte duur, jouw invloed en nalatenschap waren desalniettemin van groot belang voor mij en het project.

Corinne. Ik kan me onze 'kennismaking' tijdens onze MSc thesis nog goed herinneren. "Oh nee, hij/zij?" is wat we allebei dachten. Maar nadat bleek dat we allebei afstand hadden gedaan van een waardevol bezit (jouw dreadlocks en mijn paarse blouse) was er al snel een duidelijke klik. Ik was dan ook erg blij dat je ons team kwam versterken. Ik kon je op het lab altijd om een gunst vragen, en je was altijd erg betrokken bij het project en met mij. Daarnaast stond bij jou en **Simon** de deur altijd open voor een heerlijk diner met voortreffelijke empanadas! Ook al ben ik nu niet meer 'jouw AIO' en scheiden hier onze wegen, toch hoop ik nog regelmatig bij elkaar over de vloer te komen. Bedankt voor alle gezelligheid en vriendschap binnen en buiten het lab en voor het feit dat je ook tijdens het laatste kunstje naast me staat/zit.

Jelke. We hebben samen inmiddels vele kilometers afgelegd, afgewisseld bij elkaar in de slipstream. Een mooie parallel naar de vele discussies die we hebben gehad tijdens werkbesprekingen en in de gezelligste hoek van het Radix. Je bent een waardevolle toevoeging aan het arbo-team en je competitiviteit zal je ver brengen. Hier houdt het natuurlijk niet op, maar in ieder geval bedankt tot nu toe en ik kijk uit naar de velen tochten en gezelligheid die nog in het verschiet liggen.

Mia. Het zal effe wennen zijn om je straks niet meer tegenover me te hebben, want op de een of andere manier was jij gedurende mijn Wageningen periode altijd aanwezig. Zelfs tijdens werk bij De Kater wist je me te vinden en zeer vrolijk en vooral luid te begroeten. Al snel daarna was schreeuwen niet meer nodig, want zagen we elkaar iedere dag. Je krijgt steeds meer controle over dat aparte zalmenvirus en daarnaast breng je altijd een hoop gezelligheid en hilariteit met je mee. Ik hoop nog vaak samen een dansje te kunnen doen op een lekker nummertje.

Stineke. We begonnen ongeveer op hetzelfde moment aan ons promotieonderzoek en we sluiten het ook op ongeveer hetzelfde moment af. Ook al hield je jezelf met totaal andere dingen bezig, je toonde altijd interesse in de vorderingen van het vaccin project. Samen met **Edze** hebben we aan de Buissteeg onder het genot van een heerlijke maaltijd, een goed glas alcohol met op de achtergrond een draaiende langspeelplaat van over- en ondergewaardeerde artiesten de belangrijke dingen des levens en des onderzoek blootgelegd. Ook al zit je nu zelf tot aan je oren in het schrijfwerk om je eigen thesis af te krijgen en heb ik je een slechte arm bezorgd, toch vond je het geen probleem om samen met **Corinne** mijn paranimf te zijn. **Edze,** voor kritisch en creatief reflecteren zijn >8 cm ijs, tartiflette en goede whisky onmisbaar. Helaas gaan we er niet harder van lopen. Heel erg bedankt voor de afgelopen 4 jaar en allebei veel plezier en succes in Engeland.

A lot of the work in the past 4 years has been performed by a small army of students. It was always a pleasure to work with you guys, on a daily basis. Unfortunately, not all of your efforts are represented in this thesis, but they are appreciated nonetheless. **Hannelie**, **Mia**, **Femke**, **Tom**, **Hans**, **Maya**, **Rik**, **Sarah**, **Wouter** and **Jana**, thanks for your help and contributions.

De goede atmosfeer op het lab heeft er altijd voor gezorgd dat ik altijd met veel plezier naar m'n werk ging. **Vera**, **Dryas**, **Paulus** en **Patrick**, bedankt voor de gezelligheid binnen en buiten het lab en voor de vele koffietafel discussies. We hebben regelmatig krom gelegen als we samen met **Corinne**, **Jelke**, **Mia**, **Stineke** (en vele gastoptredens) wederom een legendarisch promotie filmpje in elkaar draaiden. We waren uitstekende detectives, Duitsers,

Chinezen en “Occlusion Buhdies”. Velen zijn gekomen, gebleven en gegaan **Richard**, **Jan**, **Monique**, **Esther**, **Ke**, **Christina**, **Dick L**, **Marcio**, **Amaya**, **Marleen** en alle andere virologen, heel erg bedankt voor deze onvergetelijke jaren.

Besides working at the lab, I have learned a lot and enjoyed my time within the **PPC** and **WPC**. The regular meetings offered a nice break in daily routine. Thanks to all the people I worked with throughout the years. I appreciated the collaborations and good times during meetings, dinners and PE&RC days. I wish you all the best in your future careers.

In addition to the people within Wageningen, I would also like to thank all the other collaborators who contributed to this thesis. **Byron**, **Ab**, **Han**, **Luuk**, **Jacco**, **Sjo**, **Mariëlle**, **Luc**, **Stephane**, **Andreas** and **Lucas**. Thanks for all the efforts, help and fruitfull meetings that gave rise to this thesis and I am looking forward to more collaborations in the future.

Lieve **Pa** en **Ma**. Deze thesis komt jullie misschien nog wel het meest toe. Zonder jullie onvoorwaardelijke steun had ik het nooit zo ver kunnen schoppen. Jullie gelden als een fantastisch voorbeeld en dankzij jullie hulp en investering heb ik ongestoord kunnen werken aan mijn eigen toekomst en daar ben ik jullie eeuwig dankbaar voor. Het resultaat van die investering ligt nu voor jullie. Ook al was het niet altijd duidelijk wat ik nu precies aan het doen was, toch heb ik me altijd door jullie gesteund gevoeld en heb ik alle interesse ontzettend gewaardeerd. Je kunt niet vaak iemand bedanken voor alles, maar hier is dat zeker van toepassing. Bedankt voor alles!

Gerben en **Gabriëlle**, jullie zullen je regelmatig hebben afgevraagd waar ik allemaal mee bezig was. Hoewel onze interesses waarschijnlijk niet verder uit elkaar hadden kunnen liggen, kijk ik als trotse broer naar hoe jullie het doen. Ook jullie bedankt voor de enthousiaste belangstelling en interesse in jullie broertje.

Femke. Zowel voor als achter de schermen heb je de afgelopen jaren een dikke stempel op mij en mijn thesis weten te drukken. Ook al is het op het werk nog zo leuk en gezellig, toch ben ik altijd weer blij om naar huis te gaan. Na het eten samen nog even aan de slag of even naar buiten om de frustraties van een dag labwerk kwijt te raken. Wie weet waar we ooit nog eens terecht komen, maar ik heb ontzettend veel zin in de tijd die we samen nog voor ons hebben liggen.

Curriculum Vitae

Stefan Willem Hendrik Metz was born in Oud-Beijerland on October 5th, 1985. In 2003, he finished secondary school (Bracbant HAVO) and studied Biology and Medical Laboratory Research at Fontys Applied Science in Eindhoven. After graduation in 2007, he continued studying Biotechnology at Wageningen University, specializing himself on a cellular and molecular level. He performed his MSc-thesis research in the arbovirus group of Dr. Gorben Pijlman at the Laboratory of Virology on the identification of RNAi suppressors of the West Nile virus. After receiving his MSc in Biotechnology in 2009, Stefan continued his research as a PhD-candidate on the development of a recombinant subunit vaccine against chikungunya virus infections, supervised by Dr. Gorben Pijlman and Prof. dr. Just Vlak at the Laboratory of Virology at Wageningen University. During his PhD-study, Stefan presented his work at various international scientific meetings and conferences and was amongst others member of the Production Ecology & Resource Conservation PhD-council and chair of the Wageningen PhD-council. He finished his PhD-research within 4 years and his research resulted in 5 published papers, one patent and many ongoing research projects. Stefan will continue his career as a molecular virologist at the Laboratory of Experimental Virology of the Academic Medical Center in Amsterdam in the group op Prof. dr. Ben Berkhout and Dr. Atze Das.

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European patent number 11159807.4

Review of literature

Arbovirus vaccine; opportunities for the baculovirus-insect cell expression system

Writing of project proposal

Development of a recombinant chikungunya virus vaccine

Invited review of journal manuscript

PLoS one

Journal of Virological Methods

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International conferences, symposia and other scientific meetings

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*American Society of Virology; Minneapolis, MN USA (2011) **

*Virus-like particle and nanoparticle vaccine conference; Cannes FR (2012) ***

*Society of Virology; Essen GER (2012) **

*NBV workmeeting; Nijmegen, NL (2012) ***

*Experimental Evolution Discussion Group ***

*TI Pharma Springmeeting ***

Dutch Annual Virology Symposium

Teaching activities

Molecular Virology

Immunotechnology

Supervision of 11 MSc/BSc thesis students

** Poster ** Oral presentation*

The research described in this thesis was carried out at the Laboratory of Virology at Wageningen University, Wageningen, The Netherlands, and was financially supported by Top Institute Pharma.

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Cover by Stefan W.H. Metz
Thesis layout by Stefan. W. H. Metz
Printed by Gildeprint Drukkerijen, Enschede, The Netherlands