

**Development of a Malaria Vaccine Candidate Based on  
Virosome Technology**

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Dekan

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## Summary

Malaria is an infectious disease caused by protozoan pathogens of the genus *Plasmodium*. The most important species affecting humans is *P. falciparum* transmitted by the bite of female *Anopheles* mosquitoes during a blood meal. About 40% of the world's population is exposed to the parasite and 350 to 500 million cases of disease and more than one millions deaths are reported every year. Almost 80% of these cases occur in sub-Saharan Africa, where mainly children younger than five years and pregnant women are affected. Due to problems with arising resistance of mosquitoes against insecticides and parasites against drugs, the development of a malaria vaccine is an urgent need. It has been shown more than 30 years ago that sterile protection against malaria infection is feasible by vaccination with irradiated sporozoites. Since then many malaria vaccine candidates have been developed but there is no vaccine on the market to date.

We have established a strategy to develop synthetic peptides of *P. falciparum* antigens for inclusion in a multi-stage multivalent malaria subunit vaccine based on the immunopotentiating reconstituted influenza virosome (IRIV) technology. IRIVs are an already registered delivery/adjuvant system based on liposomes incorporating influenza surface proteins hemagglutinin and neuraminidase. IRIVs enhance and facilitate the delivery of antigens to antigen presenting cells. This technology allows a stepwise lead peptide optimization based on parasite-binding properties of antibodies elicited after immunization of experimental animals with virosomally-formulated peptide-phospholipid conjugates. In this thesis the development steps of three peptide antigens are described: UK-39 a peptide derived from the sporozoite antigen circumsporozoite protein (CSP), AMA49-CPE derived from the blood-stage protein apical membrane antigen 1 (AMA-1) and FB-23 derived from the blood-stage protein serine repeat antigen 5 (SERA5). All optimized peptides induced parasite cross-reactive antibodies in experimental animals. Virosomal formulations of the antigens UK-39 and AMA49-CPE were carried into a phase 1 clinical trial. Both vaccine components were safe and immunogenic in malaria-naïve volunteers. Two immunizations with appropriate doses of UK-39 or AMA49-CPE were enough to induce high titers of parasite-binding antibodies. Combined delivery of the two peptides did not interfere with the development of an antibody response to either of the two antigens. The antibody responses were affinity matured and long-lived,

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indicating the formation of B cell memory. Purified total IgG from UK-39 immunized volunteers inhibited sporozoite migration, invasion and development in a dose dependent manner. Further clinical trials with this two-component vaccine candidate are ongoing and new peptide candidates like FB-23 have been developed and are now ready for preclinical and clinical profiling.

## Zusammenfassung

Malaria ist eine Infektionskrankheit die durch Protozoen des Genus *Plasmodium* verursacht wird. Die bedeutendste, den Menschen befallende Spezies, ist *P. falciparum*, welche durch den Biss einer blutsaugenden *Anopheles* Mücke übertragen wird. Etwa 40% der Weltbevölkerung lebt in Gebieten, in denen der Parasit vorkommt und jährlich werden zwischen 350 und 500 Millionen Krankheitsfälle und mehr als eine Million Todesfälle gemeldet. Fast 80% aller Fälle treten in Afrika südlich der Sahara auf, wo vor allem Kleinkinder jünger als 5 Jahre und schwangere Frauen betroffen sind. Ein Malaria-Impfstoff wird dringend benötigt, da sich Medikamenten-resistente Parasiten und Insektizid-resistente Mücken ausbreiten. Vor mehr als 30 Jahren wurde gezeigt, dass Menschen eine sterile Immunität entwickeln können, wenn sie mit durch Bestrahlung abgeschwächten Sporozoiten geimpft werden. Seither sind viele Malaria Impfstoffkandidaten entwickelt worden, aber es gibt bis heute keinen effizienten Impfstoff.

Wir haben eine Strategie zur Entwicklung von künstlichen Peptiden, abgeleitet von *P. falciparum* Antigenen, etabliert, die zusammen mit der Virosomen-Technologie (Immunopotentiating Reconstituted Influenza Virosomes=IRIV) zur Herstellung eines multi-Stadien multivalenten Malaria Impfstoffes ermöglichen soll. Virosomen sind ein registriertes Verabreichungs-/Adjuvanssystem, das auf Liposomen, welche die Influenza Proteine Hämagglutinin und Neuraminidase auf der Oberfläche tragen basiert. Virosomen verstärken und erleichtern die Aufnahme von Antigenen durch Antigen-präsentierende Zellen. Phospholipid-Konjugate von Fremdpeptiden können in der Virosomen Membran verankert und so dem Immunsystem präsentiert werden. Diese Technologie erlaubt eine schrittweise Entwicklung und Verbesserung von Peptidantigenen. Als Auswahlkriterium dient hierbei die Fähigkeit der virosomal formulierten Peptide, Parasiten-Bindende Antikörper hervorzurufen. In dieser Dissertation beschreiben wir Entwicklungsschritte von drei Peptid-Antigenen: UK-39 ein Peptid, das vom Sporozoiten Antigen Circumsporozoite Protein (CSP) abgeleitet ist, AMA49-CPE, abgeleitet vom Blutstadien Protein Apical Membrane Antigen 1 (AMA-1) und FB-23 abgeleitet vom Blutstadienprotein Serine Repeat Antigen (SERA5). Alle optimierten Peptide induzierten Parasiten-Bindende Antikörper in Tieren. Virosomale Formulierungen der Peptide UK-39 und AMA49-CPE wurden in einem Phase 1 klinischen Versuch in Malaria-naiven Freiwilligen getestet. Beide

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Impfstoff-Komponenten waren immunogen, ungefährlich und wurden gut toleriert. Zwei Immunisierungen mit einer adäquaten Dosis UK-39 oder AMA49-CPE waren genug, um hohe Parasiten-bindende Antikörper Titer hervorzurufen. Kombinierte Impfung mit UK-39 und AMA49-CPE hatte keinen negativen Einfluss auf die Entwicklung einer Antikörper Antwort gegen die einzelnen Komponenten. Die Antikörper Antworten zeigten Affinitätsreifung und waren langlebig, was für die Entwicklung von langlebigen Gedächtnis B-Zellen spricht. Gereinigtes IgG von UK-39 immunisierten Freiwilligen hemmte abhängig von der Antikörper Konzentration Migration, Invasion und Wachstum von *P. falciparum* Sprozoiten *in vitro*. Weitere klinische Versuche mit diesen beiden Kandidaten sind im Gange und geplant und weitere Komponenten, wie FB-23, stehen bereit für eine detaillierte präklinische und klinische Charakterisierung

## Chapter 1: Introduction

### 1.1 Plasmodium and Malaria

#### 1.1.1 General

Malaria is due to infection with a protozoan of the genus *Plasmodium*, transmitted by a female *Anopheles* mosquito. *Plasmodium* belongs to the phylum of the *Apicomplexa* that contains other human and veterinary pathogens like *Toxoplasma*, *Cryptosporidium*, *Eimeria*, *Babesia* and *Theileria*. *Apicomplexa* are characterized by the presence of an apical complex, which contains an apicoplast, a structure in juxtaposition to the nucleus and related to the chloroplast, a polar ring organizing the microtubules, and vesicles called micronemes, rhoptries and dense granules. These vesicles contain proteins involved in parasite motility, adhesion to host cells, invasion of host cells, and establishment of the parasitophorous vacuole (PV), which are sequentially released during infection of a host cell<sup>1</sup>. The genus *Plasmodium* contains more than 100 species of which four infect humans: *Plasmodium falciparum*, the most important pathogen for humans, *P. vivax*, *P. ovale*, and *P. malariae*. Other species that infect monkeys (e.g. *P. knowlesi*, *P. cynomolgi*) or rodents (*P. yoelii*, *P. berghei*, *P. chabaudi*, *P. vinckei*) are studied and used as animal models.

Malaria remains the most frequent parasitic disease in the world: About 40% of the world's population is exposed to the parasite and 350 to 500 million cases of disease are reported every year. Almost 80% of these cases occur in sub-Saharan Africa, where mainly children younger than five years and pregnant women are affected. With over one million deaths, malaria has one of the highest mortalities of all infectious diseases (WHO World Malaria Report 2005). Moreover malaria is a major social and economic burden in endemic areas<sup>2</sup>. There are multiple channels by which malaria impedes development, including effects on fertility, population growth, saving and investment, worker productivity, absence, premature mortality and medical costs.

#### 1.1.2 Life Cycle of *Plasmodium*

The life cycle of *Plasmodium* is complex (Figure 1). It involves two hosts, a female mosquito of the genus *Anopheles* and a vertebrate (e.g. a human). The cycle can be divided into three consecutive phases of multiplication: Two phases of schizogony

(asexual multiplication) in the vertebrate host first in hepatocytes then in erythrocytes and one phase of sporogony (sexual multiplication) in the mosquito. The vertebrate host gets naturally infected by the bite of a mosquito injecting the parasite in the sporozoite form. Sporozoites rapidly migrate to the liver via the blood circulation. They invade hepatocytes where they develop into hepatic schizonts<sup>3,4</sup>. Every schizont produces up to 10,000 merozoites in a few days, which are released into the blood stream where they infect erythrocytes. In the erythrocyte they multiply giving rise to up to 25 merozoites, which are released and again invade erythrocytes thereby maintaining the erythrocytic cycle. In parallel a few parasites differentiate into male or female sexual forms called gametocytes. Once ingested by a blood sucking mosquito gametocytes give rise to sexual gametes, which fuse in the midgut lumen. The zygotes formed by this fertilization develop into motile ookinetes, which invade and traverse the midgut epithelium. Diploid ookinetes undergo meiosis and, on reaching the basal side of the midgut, transform into oocysts, thereby undergoing several rounds of mitosis as they mature. Each oocyst releases thousands of haploid sporozoites into the mosquito hemocoel, from where they are transported through the hemolymph and invade the salivary glands. Sporozoites are finally transmitted to a new vertebrate host during an infective bite<sup>5</sup>.

The life cycle of *Plasmodium* thus consists of three invasive stages: the ookinetes traversing the intestinal cells in the mosquito, the sporozoites infecting the mosquito salivary gland, and the vertebrate hepatocytes, and the merozoites infecting the vertebrates' erythrocytes. The sporozoites and the hepatic stages are called the pre-erythrocytic stages. The hepatic stage is asymptomatic in humans and takes approximately one week in the case of *P. falciparum*. Clinical symptoms that can be very severe are solely due to infection of erythrocytes. Almost all antimalarial drugs currently in use are directed against this stage<sup>6</sup>.

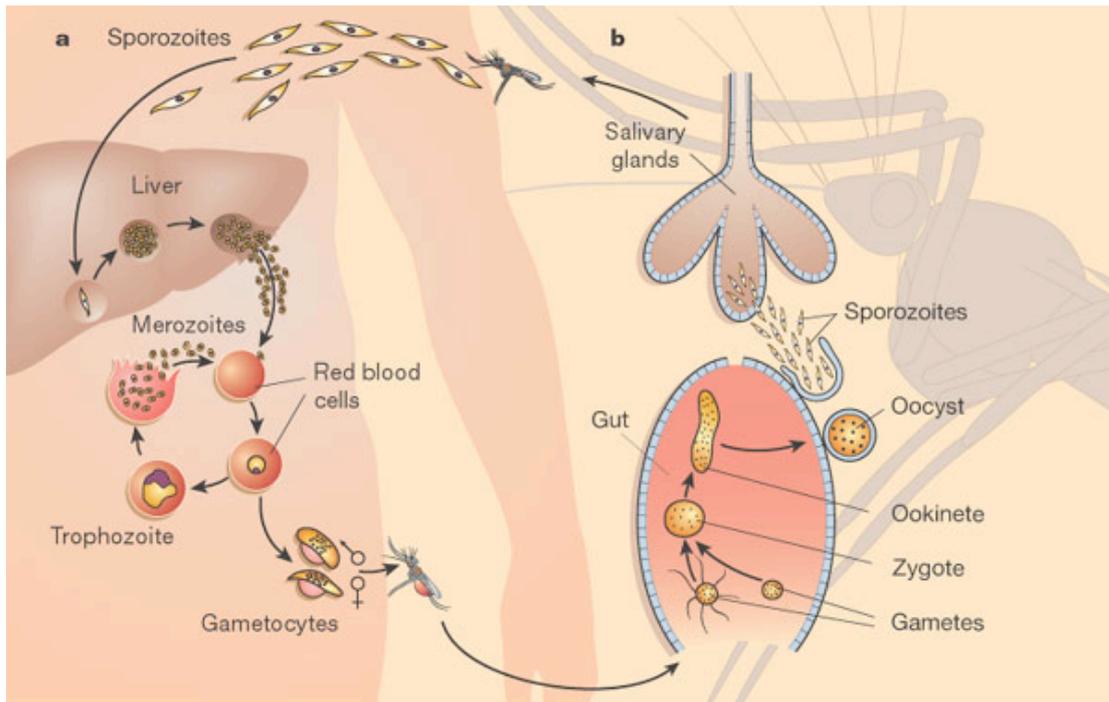


Figure 1. Life cycle of *P. falciparum* (Source Wirth 2002)<sup>7</sup>

## 1.2 Sporozoite Biology

### 1.2.1 General

Sporozoites represent a special life stage of the parasite as they interact with both the mosquito and the vertebrate host. In the mosquito the sporozoites are formed in the oocysts in the midgut cell wall, then infect the salivary glands and once injected into the vertebrate host they rapidly reach the liver and infect hepatocytes. Sporozoites are polarized, elongated cells (Figure 2) measuring about 10 $\mu$ m in length and 1 $\mu$ m in width, and have an apical complex. Until recently the only well characterized sporozoite proteins have been the circumsporozoite protein (CSP) and the thrombospondin-related adhesive protein (TRAP). These two proteins are on the sporozoite surface and contain typical adhesion motifs implicated in ligand receptor interactions in mammals<sup>3,8</sup>.

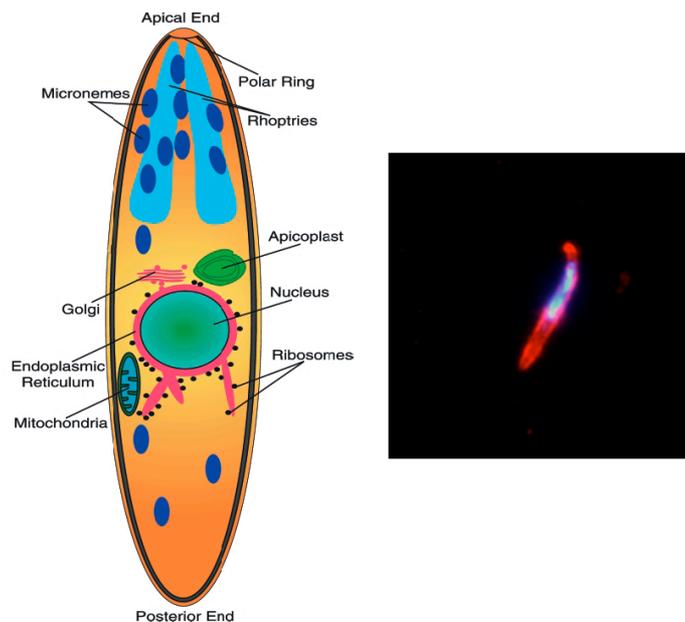


Figure 2. A: Schematic representation of a *Plasmodium* sporozoite showing some of its organelles and sub-cellular structures (Source: Kappe et al. 2004)<sup>4</sup>. B: *P. falciparum* sporozoite isolated from the salivary glands of an infected mosquito, stained with antibodies against circumsporozoite protein (orange) and Hoechst Dye No. 33256 (nucleus in blue) (Photo S. Okitsu).

### 1.2.2 Circumsporozoite Protein

CSP is expressed only in *Plasmodium* sporozoites and cannot be found in other *Apicomplexa*. It is a major surface protein encoded by a single copy gene and covers

the entire surface of the sporozoite<sup>4,9</sup>. CSP is also present in the micronemes<sup>10</sup> from where it is continuously secreted to the anterior pole of the parasite surface and transported to the sporozoites posterior end, where it is released<sup>11</sup>. Due to its abundance, surface localization, immunogenicity and key role in parasite invasion, CSP is one of the leading candidate molecules for the development of malaria pre-erythrocytic vaccines. CSPs from different *Plasmodium* spp. display common structural features, including a signal peptide, a central domain composed mostly of amino acid repeats, and a C-terminal hydrophobic sequence (Figure 3)<sup>12</sup>.

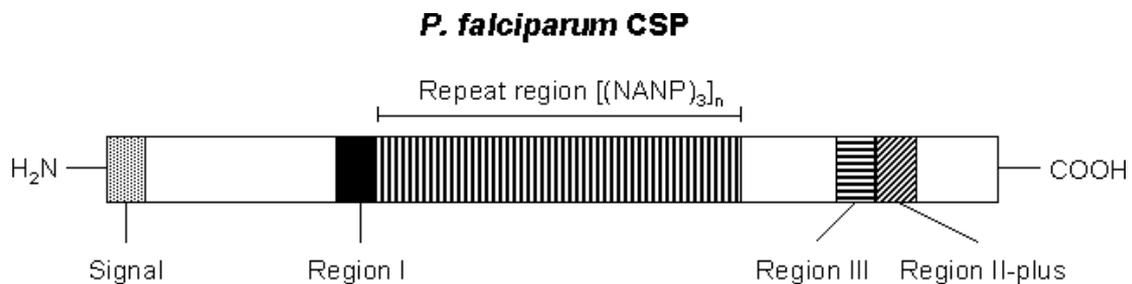


Figure 3. Schematic representation of CSP.

The central repeats are formed by a variable number of in-tandem repeats that contain immunodominant B cell epitopes<sup>13</sup>. The sequence of the CSP repeats is species specific. In *P. falciparum* the repetitive unit consists of tandem repeats of the tetrapeptide NANP interspersed with four copies of NVDP. The repeat motif adopts a type-I b-turn, which is stabilized by hydrogen bonding<sup>14</sup>. The function of these repeats is unknown. They are flanked by two conserved regions called region I, region II-plus and region III. Region I consists of a pentapeptide KLKQP involved in the attachment to the mosquito salivary glands and was shown to be the recognition site for proteolytical cleavage upon contact with hepatocytes<sup>15</sup>. Region II-plus is an 18-amino acid sequence containing a thrombospondin repeat (TSR) type I domain. The TSR is an ancient eukaryotic domain found in a superfamily of adhesive proteins related to thrombospondin. These proteins are involved in immunity, cell adhesion, angiogenesis, and the development of the nervous system (Adams & Tucker Dev.Dyn 2000). The TSR domain mediates adhesion to heparan sulfate proteoglycans (HSPGs) of the liver<sup>16</sup> and is important for sporozoite motility and infectivity<sup>17</sup>. Region

III is predicted to form an amphipathic  $\alpha$ -helix that may provide the proper framework for the neighboring region II-plus adhesion motif<sup>4</sup>. The C-terminus of CSP consists of a hydrophobic stretch of residues that typically encodes a glycosylphosphatidylinositol (GPI) attachment signal, but this has not been formally demonstrated. Recent results in our laboratory have shown that anti-GPI mabs recognize CSP in Western blot with sporozoite lysate (M. Tamborrini personal communication).

### 1.2.3 Gliding Motility

Most apicomplexan invasive stages, called zoites are motile. These parasites have a particular way of moving, called gliding motility, which allows them to glide rapidly (1-10 $\mu$ m/s) on solid substrates and actively invade target cells in a matter of seconds. Gliding motility is based on the redistribution (capping) of adhesion molecules called invasins from the anterior to the posterior end of the parasite. The invasins linking the intracellular actin-myosin motor and the extracellular substrate belong to the TRAP family<sup>18</sup> and act like a tank's caterpillar tracks. The molecular motor pulls the invasins backward, and as the extracellular portion of the protein is anchored to a fixed substrate, this leads to a forward movement of the parasite.

During sporozoite gliding a fragment of TRAP containing only the extracellular portion is left behind as a trail<sup>19</sup>. Large amounts of CSP are also continuously released at the posterior end of the parasite during gliding<sup>20</sup>. As CSP seems to be attached to the parasite membrane via a GPI anchor, it has no cytoplasmic domain and cannot be directly coupled to the actin-myosin motor. It is probably attached to the motor through another transmembrane protein, which might be TRAP although an interaction between these two could not be shown by immunoprecipitation. Supporting such a functional coupling, deletion of region II in the TSR of CSP induces the same phenotype as observed after deletion of TRAP<sup>17</sup>.

### 1.2.4 Invasion of Host Cells

The first ultrastructural analyses on the invasion of host cells by zoites has shown that merozoites enter erythrocytes via formation of a moving junction<sup>21</sup>. A tight junction between the apical end of the parasite and the target cell surface is formed

after initial contact. This junction is moving to the posterior end like a ring around the zoite, which enters into a vacuole in the host cell cytoplasm. This tight junction formation has been observed in several other *Apicomplexa*<sup>22</sup>. Internalization only takes some seconds and is based on a mechanism similar to gliding motility. Ligands are secreted to the surface of the parasite at the anterior pole, attach to the surface of the host cell and are redistributed to the back (Figure 4). This process does not rupture the integrity of the host cells plasma membrane<sup>23</sup>.

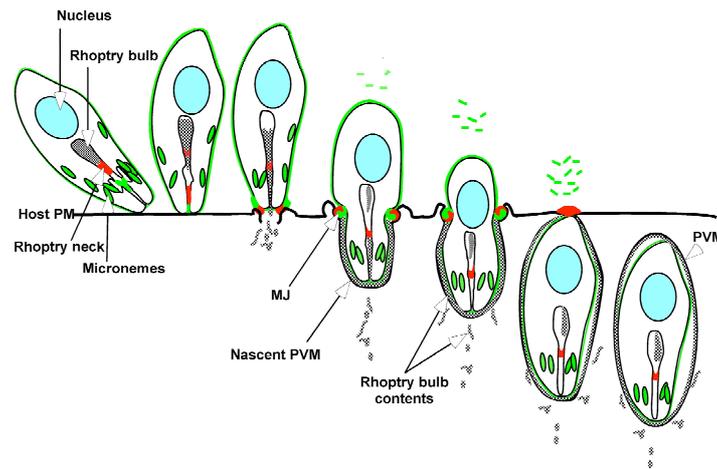


Figure 4. Invasion of a host cell by a zoite. After attachment the zoite establishes a tight junction between micronemal proteins, the neck of the rhoptries and the target cell. The redistribution of this moving junction (MJ) to the posterior end of the zoite moves the parasite forward. This forward movement leads to the formation of an invagination, which gives rise to the parasitophorous vacuole (PV) surrounded by a parasitophorous vacuole membrane (PVM). Once the redistributed proteins reach the posterior pole of the parasite they are proteolytically cleaved and shed into the medium (Source: Alexander et al. 2005)<sup>24</sup>.

### 1.2.5 From the Mosquito to the Liver

In their search for capillaries, infected mosquitoes probe the skin of the host and deposit saliva containing vasodilators, anticoagulants, and *Plasmodium* sporozoites. Although one mosquito can harbor thousands of sporozoites, they normally only inject a low number (10-200) during a blood meal<sup>25</sup>. Intravital microscopy with transgenic parasites expressing GFP has shown that parasites in the skin are highly motile and migrate through the cutaneous tissue before actively penetrating through the vascular endothelium to reach the blood circulation<sup>26</sup>. The presence of anti-

sporozoite antibodies in the skin of mice immunized with irradiated sporozoites can reduce or even block the observed motility of the parasites.

Once in the blood stream the sporozoites reach the liver within some minutes<sup>27</sup>. Most circulating sporozoites are arrested in the liver after a single passage, suggesting that specific receptors are present on the cells lining the liver sinusoids (Figure 5). As CSP covers the entire sporozoite plasma membrane, it is very likely that it contains the postulated liver ligand(s). Numerous observations indicate that the ligand is contained in the stretch of positively charged residues of region II-plus (TSR) of CSP, and that the binding sites in the liver sinusoids are heparan sulfate proteoglycans (HSPGs) from the extracellular matrix, protruding through fenestrations into the sinusoids<sup>17,28</sup>.

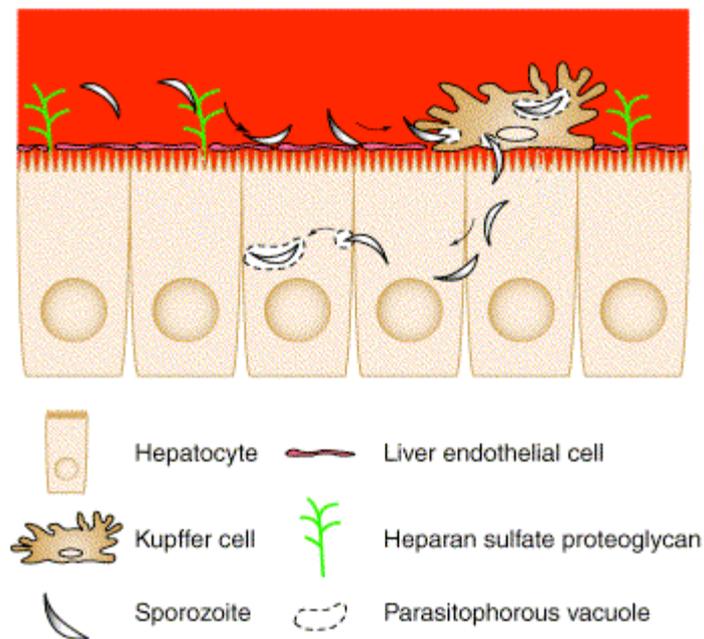


Figure 5. Sporozoite arrest in the liver, passage of the sinusoidal barrier and invasion of hepatocytes (Source Kappe et al. 2003)<sup>29</sup>.

To reach the hepatocytes, sporozoites have to traverse the sinusoidal barrier built by endothelial cells and Kupffer cells (Figure 5). Kupffer cells are macrophages of the liver involved in removal of foreign and degraded substances from the blood stream. Studies in Brown Norway rats and intravital microscopy in mice have shown that *P. berghei* sporozoites traverse Kupffer cells in a vacuole, which does not fuse with lysosomes to reach the underlying hepatocytes<sup>30</sup>.

### 1.2.6 Hepatocyte Infection

According to the current model of hepatocyte infection by *Plasmodium* sporozoites, the parasite migrates through several hepatocytes by disrupting the cell's plasma membrane, i.e. without vacuole formation, before infecting a cell by tight junction and vacuole formation. Migration triggers the secretion of the content of the secretory vesicles at the apical pole of the parasite, needed for tight junction formation between the sporozoite and its target cell and successful infection by vacuole formation<sup>31</sup>. Migration through hepatocytes also induces the release of hepatocyte growth factor (HGF) by wounded cells. HGF-MET signaling in neighboring cells protects them from apoptosis, thereby allowing successful establishment of infection by the parasite<sup>32</sup>. It has to be taken into account that most of these studies have been done in *in vitro* or artificial murine model systems and have not been confirmed *in vivo* or with *P. falciparum* so far.

Once inside a PV in the hepatocyte, the sporozoites differentiate into hepatic schizonts, also called exo-erythrocytic forms (EEFs). Within 2 days in rodent and 6 to 7 days in primate parasites the schizonts grow to a size bigger than the initial size of the hepatocyte containing thousands of merozoites capable of infecting erythrocytes (Figure 6). The merozoites are released to the blood stream in a mechanism not yet completely understood. A recent study with *P. berghei* shows that parasites induce the death and the detachment of their host hepatocytes, followed by the budding of parasite-filled vesicles (merosomes) into the sinusoid lumen<sup>33</sup>.

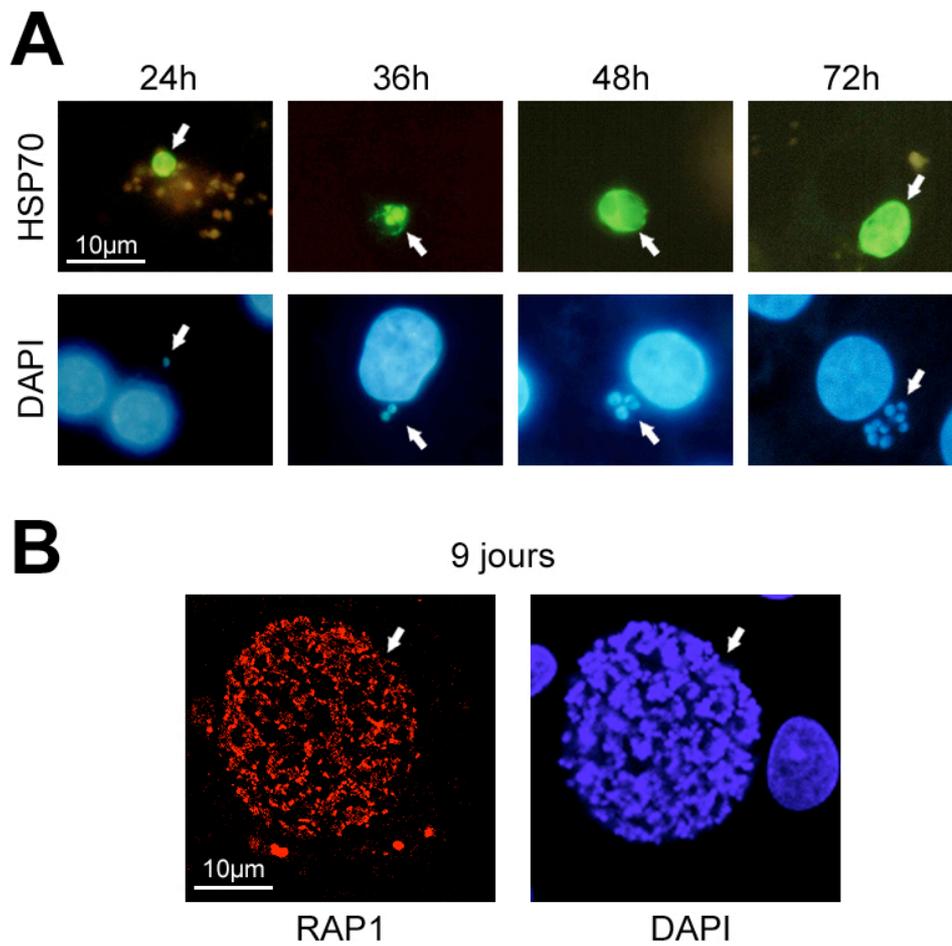


Figure 6. *In vitro* development of *P. falciparum* exo-erythrocytic forms in primary human hepatocytes. A. Intrahepatic parasites, indicated by an arrow, have been stained with an anti-*Plasmodium* HSP70 antibody (green), and the nuclei of the hepatocyte and the parasite with DAPI (blue). B. Mature schizonts 9 days after infection, stained with an anti-RAP1 (rhopty associated protein 1) antibody (red) and DAPI (blue) (Photos O. Silvie).

## 1.3 Vaccine Development

“... let me be permitted to use the word ‘vaccinate’ to express the act of inoculating a chicken with the attenuated virus”

Louis Pasteur, 1880

### 1.3.1 General

Vaccination is an elegant way to induce an adaptive immune response in non-immune individuals by confronting the immune system with important antigenic determinants of a pathogen without the deleterious effects of disease. Some of the first hints of the use of vaccines date back to the eleventh century when variolation (blowing variola crusts into the nostril to immunize against smallpox) was practiced in China. By the end of the 18<sup>th</sup> century Edward Jenner discovered that cowpox, a mild illness in humans, could prevent smallpox. A more rational attempt of vaccine design began in the 19<sup>th</sup> century in the laboratory of Louis Pasteur. He discovered attenuation to reduce the virulence of a pathogen, a cornerstone in modern vaccine development<sup>34</sup>.

Advanced vaccine technologies together with the knowledge provided by modern immunology formed the basis for more deliberate and sophisticated vaccines. Nowadays there are three main approaches for vaccine development: live vaccines, nonliving or subunit vaccines, and vaccines based on genetic engineering.

### 1.3.2 Live Vaccines

After first methods for attenuation like heat, oxygenation, chemical agents, aging, or passage in animal hosts, it was mainly the development of cell culture leading to advances in the field of attenuated live vaccines<sup>34,35</sup> (Table 1).

Table 1. Live vaccines and their approximate times of availability  
(Adapted from Plotkin 2005)<sup>34</sup>

Development strategy	Date	Vaccine or target
Use of related animal virus	1798	Smallpox
Chemical attenuation	1885	Rabies
	1881	Anthrax
Passage in vitro	1927	BCG
	1935	Yellow fever
Cell culture passage	1963	Measles
	1995	Varicella
	2005	Rotavirus 89-12
Cell culture passage with cold adaptation	1969	Rubella
	2003	Live influenza
Auxotrophy	1989	Ty21a typhoid
Use of reassortants	1970s	Inactivated influenza seed
	2005	Rotavirus bovine-human

### 1.3.3 Nonliving and Subunit Vaccines

Table 2 outlines the strategies for the development of inactivated vaccines. The discovery of extracellular bacterial toxins by Roux, Yersin, Behring and Kisato permitted the development of toxoids (inactivated toxins)<sup>36</sup>. As technology advanced, it became possible to separate and use subunits of organisms in the form of extracts of infected tissue (e.g. rabies), capsular polysaccharides (e.g. typhoid Vi and pneumococci) and proteins (e.g. acellular pertussis). Late in the twentieth century conjugation of proteins by polysaccharides became important in vaccines against encapsulated bacteria (e.g. *Haemophilus influenza* type b)<sup>37</sup>. The use of peptides as vaccines has been slowed by the need for strong adjuvants, but is still a focus of research.

Table 2. Nonliving vaccines and their approximate times of availability  
(Adapted from Plotkin, 2005)<sup>34</sup>

Vaccine strategy	Date	Vaccine or target
Inactivated whole organisms	1896	Typhoid
	1896	Cholera
	1897	Plague
	1926	Whole-cell pertussis
	1938	Influenza
	1995	Hepatitis A
Use of extracts and subunits	1944	Japanese encephalitis
	1970s	Influenza
	1960	Anthrax
	1976	Cell-culture rabies
Use of toxoids	1923	Diphtheria
	1927	Tetanus
Use of capsular polysaccharides	1977	Pneumococcal
	1974	Meningococcal
	1995	Typhoid
Use of protein-conjugated capsular polysaccharides	1987	H. influenzae type b
	2002	Pneumococcal
	2002	Meningococcal
Use of purified or recombinant proteins	1986	Hepatitis B
	1996	Acellular pertussis
	1998	Lyme disease

### 1.3.4 Vaccine Development Based on Genetic Engineering

Advances in molecular biology and genetic engineering have had a dramatic effect on vaccine development, providing greater opportunities for construction of inactivated antigens and for rational attenuation of organisms through directed mutation. Several recombinant strategies are possible. To develop live recombinants, genes from heterotypic viruses can be inserted into an attenuated virus. Genes can also be inserted into animal, plant, bacterial, viral, or yeast cells for expression of

proteins used for immunization. Bacterial DNA plasmids containing foreign genes can induce antigen production in muscle cells, inducing a cytotoxic immune response<sup>38</sup>. Another powerful strategy to induce cellular responses uses vectors, microbes that are naturally or artificially attenuated for humans, in which foreign genetic information has been inserted<sup>39</sup>. These vectors have often been employed in a prime-boost configuration, so called because the immune system is primed with proteins expressed by injected DNA plasmids or vector, and then boosted with the same proteins in soluble form or expressed by another vector<sup>40</sup>. The latest approach for vaccine development is called 'reverse vaccinology'. The availability of microbial genome sequences allows for *in silico* search and identification of new protective factors. Candidate genes are recombinantly expressed and used for immunological studies. Another use of the genome information is the generation of mutants to get genetically attenuated organisms as described above. One example is a *P. berghei* knockout for a gene called *uis3*<sup>41</sup>. The mutant sporozoites infect hepatocytes but are unable to establish blood-stage infections *in vivo*, and thus do not lead to disease.

### 1.3.5 Immunopotentiating Reconstituted Influenza Virosomes (IRIVs)

IRIVs are a liposomal carrier system characterized as spherical, unilamellar vesicles with a mean diameter of approximately 150 nm. They consist of 70% egg yolk phosphatidylcholine (EYPC), 20% phosphatidylethanolamine (PE) and 10% envelope phospholipids originating from H1N1 influenza virus (A/Singapore/6/86) (Figure 7A and B). In contrast to liposomes, influenza hemagglutinin (HA) and neuraminidase (NA) are intercalated into the lipid bilayer, which give them their fusogenic activity and thereby play a key role in the mode of action of these virosomes<sup>42</sup>. Virosomes are prepared by detergent removal<sup>43</sup>. Specifically, like native influenza virus, influenza-derived virosomes enter cells through receptor-mediated endocytosis and subsequently fuse with the endosomal membrane (Figure 7A and B).

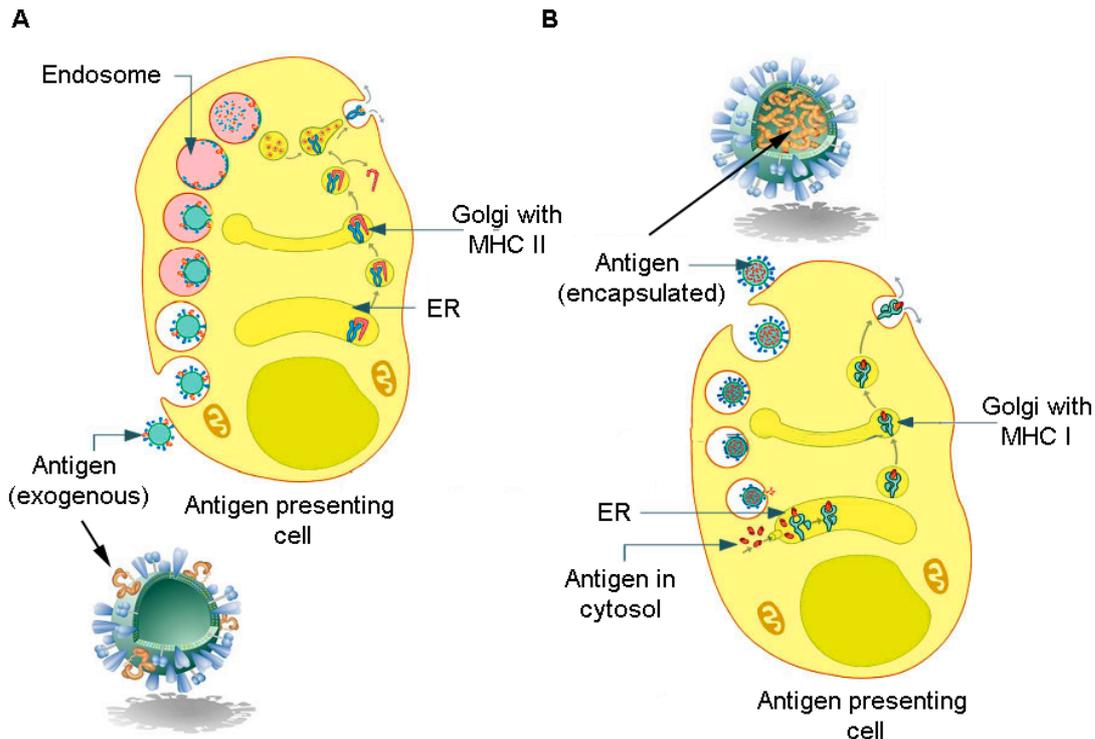


Figure 7. Immunopotentiating reconstituted influenza virosomes (IRIVs). A. Structure and mode of action of virosomes with antigen presented on the surface (exogenous). B. Structure and mode of action of virosomes with antigen encapsulated in the virosome (Adapted from Westerfeld and Zurbriggen, 2005)<sup>44</sup>.

Influenza viruses use a membrane fusion strategy to deliver their genomes to the cytosol of target cells. Hemagglutinin binds to sialic acid residues on glycoproteins or glycolipids on the host cell surface<sup>45</sup>. Receptor binding initiates the uptake of the virus through receptor-mediated endocytosis. In this process, virus particles are engulfed by the host cell plasma membrane. The vesicles thus formed subsequently fuse with endosomes (Figure 7B)<sup>46,47</sup>. The low pH inside the endosomes (pH 5–6) triggers a fusion reaction between the viral envelope and the endosomal membrane, which delivers the viral core to the host cell cytosol<sup>46,48</sup>.

Hemagglutinin and neuraminidase spikes protruding from the virosomal membrane, as well as foreign antigens that may be coupled to the surface of the virosomes via lipid anchors, can be recognized by membrane-associated immunoglobulin receptor molecules on B lymphocytes (Figure 7A)<sup>49</sup>. The repetitive arrangement of the antigens on the virosomal surface presumably enables cross-linking of these immunoglobulin receptors on the B cells which is known to be an exceptionally strong activation signal<sup>50</sup>. The strong immune response induced by peptides presented on

the surface of virosomes is predominantly humoral, whereas a supplementary T-helper immune response is evoked by virosomal influenza-specific glycoproteins<sup>44,51</sup>. Antigen molecules inside virosomes are delivered to the cytoplasm after fusion of the virosomes to the endosomal membrane (Figure 7B). This leads to degradation of the antigen within the cytoplasm by the proteasome and subsequent transport of the antigen peptides into the endoplasmic reticulum (ER) and loading onto MHC class I. Peptide-loaded MHC class I molecules reach the cell surface of the antigen presenting cell and interact with CD8+ T- cells to induce a cytotoxic T-cell response<sup>52</sup>.

The virosome-based technology is validated by two marketed products. These products are registered for human use in more than 38 countries, and were administered to more than 20 million individuals<sup>44</sup>.

### 1.3.6 Malaria Vaccine Development

Malaria vaccine development started in the middle of the 20<sup>th</sup> century. It is based on the observation that a protective immune response eventually develops in people who survive repeated attacks of malaria during early childhood<sup>53</sup>. Older people in endemic areas, although almost always harboring the parasite in the blood during seasons of high transmission, rarely become clinically ill. This immunity primarily impacts the severity of clinical disease, and appears to be linked to continuous antigenic stimulation, waning rapidly when exposure ceases. Moreover passive transfer of purified immunoglobulins from semi-immune adults can protect malaria-naïve children<sup>54</sup>.

The first success in malaria vaccine development was in 1973, when Clyde et al. reported that immunization with sporozoites attenuated by irradiation can protect healthy volunteers against infection<sup>55</sup>. The next milestone was Spf66, a hybrid polymer vaccine combining four different antigens from *P. falciparum*. Spf66 is up to now the only malaria vaccine candidate tested in phase III field trials but failed to show efficacy (summarized in Graves and Gelband, 2006)<sup>56</sup>. During the past 5 years many vaccine approaches have been tested in clinical trials and many potential candidates are now ready for preclinical and clinical assessment (WHO Initiative for Vaccine Research). The most advanced vaccine RTS,S/AS02A is based on recombinant CSP and was the first pre-erythrocytic vaccine candidate to show

protection against malaria in the field<sup>57</sup>. However, key obstacles to the development of a malaria vaccine include the lack of immune correlates of protection, the lack of reliable and predictive animal models, and the developmental and antigenic diversity and variability of the parasite.

The traditional approach to develop malaria vaccines has been the targeting of the different stages of parasite development (pre-erythrocytic, asexual and sexual stages) (Figure 1). Pre-erythrocytic vaccine strategies aim at generating an antibody response, which neutralizes sporozoites and prevents them from invading the hepatocyte, and/or at eliciting a cell-mediated immune response attacking intra-hepatic parasites. This type of vaccine would be ideal for travelers because it should convert sterile immunity, preventing clinical disease.

Asexual blood-stage (erythrocytic) vaccine strategies mainly aim at eliciting antibodies that will inactivate merozoites and/or target malarial antigens expressed on the erythrocyte surface, thus inducing antibody-dependent cellular cytotoxicity and complement lysis. This type of vaccine would mostly serve as a disease-reduction vaccine in endemic countries by decreasing the exponential multiplication of blood-stage parasites.

Vaccines targeting the sexual stage of the parasite, also called transmission-blocking vaccines, do not intend to prevent illness or infection in the vaccinated individual, but to prevent or decrease transmission of the parasite to new hosts. Other novel approaches include the development of multi-stage multi-component vaccines, a commercial irradiated sporozoite vaccine<sup>58</sup>, and an anti-parasite toxin vaccine<sup>59</sup>. This type of potential anti-disease vaccine targets parasite toxins contributing to the disease, such as the glycosylphosphatidylinositol (GPI) anchor.

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## Chapter 2: Goal and Objectives

### 2.1 Goal

To contribute to the development of a malaria vaccine by developing and establishing a technology platform to choose and optimize *P. falciparum*-derived antigens for inclusion in a multivalent virosomal subunit vaccine.

### 2.2 Objectives

1. To optimize and characterize synthetic peptides derived from *P. falciparum* antigens
2. To evaluate virosomal formulations of candidate peptides in experimental animals
3. To show that virosomally formulated synthetic peptides are safe and immunogenic in humans
4. To analyze the human immune response to virosomally formulated synthetic peptides



**Chapter 3: Structure-Activity Based Design of a Synthetic  
Malaria Peptide Eliciting Sporozoite Inhibitory  
Antibodies in a Viroosomal Formulation**

Ready to submit to Chemistry&Biology

**Structure-Activity Based Design of a Synthetic Malaria Peptide Eliciting Sporozoite Inhibitory Antibodies in a Virosomal Formulation**

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Running title: Synthetic peptide malaria vaccine candidate

Key words: malaria vaccine, virosomes, synthetic peptide, circumsporozoite protein

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### Summary

The circumsporozoite protein (CSP) of *Plasmodium falciparum* is a leading candidate antigen for inclusion in a malaria subunit vaccine. We describe here the design of a conformationally constrained synthetic peptide, designated UK-39, which has structural and antigenic similarity to the NPNA-repeat region of native CSP. NMR studies on the antigen support the presence of helical turn-like structures within consecutive NPNA motifs in aqueous solution. Intramuscular delivery of UK-39 to mice and rabbits on the surface of immunopotentiating reconstituted influenza virus-like particles (IRIV) elicited high titers of sporozoite cross-reactive antibodies. Influenza virus proteins were crucially important for the immunostimulatory activity of the IRIV-based antigen delivery system, since a liposomal formulation of UK-39 was not immunogenic. IgG antibodies elicited by UK-39 loaded virosomes inhibited invasion of hepatocytes by *P. falciparum* sporozoites, but not by antigenically distinct *P. yoelii* sporozoites. This approach to optimized IRIV-formulated synthetic peptide vaccines should be generally applicable and amenable for other infectious and non-infectious diseases.

## Introduction

Malaria is the most important parasitic disease in people and may cause as many as 2.5 million deaths per annum [1]. Vaccine development against both *Plasmodium falciparum* and *P. vivax* is ongoing [2] and one candidate vaccine, RTS,S/AS02A demonstrated 30% protection against the first episode of malaria and 58% protection against severe malaria in a clinical trial in Mozambican children [3]. Nevertheless, it is assumed that it will take at least another decade until a malaria vaccine will be available that is more effective and more cost effective than current malaria control tools, such as insecticide treated bed nets and intermittent preventive treatment in infants [4-6].

Apart from plans to develop a radiation-attenuated sporozoite vaccine [7], vaccine development against malaria is focusing largely on subunit vaccine technologies [8]. It is thought that an effective malaria subunit vaccine will have to incorporate antigens against several developmental stages of the parasite. A combination of actions against sporozoites, infected liver cells, merozoites and infected red blood cells may be required to achieve substantial protective activity [8]. The fact that most vaccines currently available are based on attenuated or inactivated whole pathogens or material derived directly from them demonstrates that the technological problems associated with peptide and protein subunit vaccine design are still incompletely solved. Major obstacles include difficulties to retain the native conformation of key antibody epitopes and the need for an effective but safe human-compatible exogenous adjuvant in order to achieve efficient immune responses [9].

We are addressing the problem of protein subunit vaccine design by developing synthetic peptide structures that elicit antibodies against surface epitopes of native malaria antigens [10-12], and coupling them to the surface of immunopotentiating reconstituted influenza virosomes (IRIVs) as a liposomal carrier system [12, 13]. IRIVs are spherical, unilamellar vesicles, prepared by detergent removal from a mixture of natural and synthetic phospholipids and influenza surface glycoproteins. The hemagglutinin membrane glycoprotein of the influenza virus is a fusion-inducing component, which facilitates antigen delivery to immunocompetent cells. IRIVs represent a universal antigen-delivery system for multi-component subunit vaccines, since antigens can be either attached to their surface to elicit

antibody responses or encapsulated in their lumen to elicit CD8 T cell responses. They have an excellent safety profile and are already registered for human use [14].

Sequential rounds of optimization of synthetic peptide structures, as typically applied in drug research, may ideally lead to vaccine candidate antigens, which elicit primarily or exclusively antibodies that contribute to immune protection. In the case of the central repeat region (NPNA)<sub>≈37</sub> of the circumsporozoite protein (CSP) of *P. falciparum* sporozoites, results of clinical trials with a linear (NANP)<sub>3</sub> peptide conjugated to tetanus toxin in alum [15, 16] were disappointing. We have previously described the synthesis and immunological properties of template-bound NPNA peptides, which were superior to their linear counterparts in eliciting sporozoite-binding antibodies [10-12]. Building on NMR and modeling studies we designed and synthesized an improved cyclic NPNA-repeat region mimetic peptide (designated BP-65), which has been shown to efficiently elicit anti-sporozoite antibody responses in mice [11]. Here we describe functional properties of monoclonal antibodies (mAbs) elicited by BP-65, and the properties and preclinical profiling of a synthetically more accessible derivative (UK-39). An IRIV-based formulation of this peptide, designated PEV302, is currently being tested in human clinical trials.

## RESULTS

### Sporozoite-inhibitory activity of anti-BP-65 mAbs

Two mAbs (designated EP3 and EP9) specific for the synthetic compound BP-65 (Figure 1) were generated from spleen cells of mice immunized with BP-65-loaded IRIVs. Both mAbs bind to sporozoites in IFA, and functional inhibitory activity was assessed by performing *in vitro* invasion inhibition assays with *P. falciparum* sporozoites and primary human hepatocytes. At a concentration of 200 µg/ml mAb EP3 caused 82% and mAb EP9 100% invasion inhibition (Figure 2A). The inhibitory activity decreased in a dose dependent manner, when increasing concentrations of BP-66 were added to the antibody-parasite mixture (Figure 2B) confirming the specificity of the inhibitory activity of the mAbs.

### Design of UK-40 and its phosphatidylethanolamine (PE)-conjugate UK-39

In an attempt to minimize the size of the synthetic vaccine antigen, a derivative of BP-65, called BP-125, without the C-terminal PNA and N-terminal NPNA portions was produced and tested for reactivity with the sporozoite-inhibitory anti-BP-65 mAbs EP3 and EP9. Only a strongly diminished reactivity was observed (Figure S1), indicating that BP-65 represents the minimal essential structure.

In BP-65 the folded conformation of the peptide is stabilized by cross-linking an amino group at the  $\beta$ -position of Pro<sup>6</sup> to a spatially adjacent side chain carboxyl of Glu as a replacement for Ala<sup>16</sup> [11]. Since limited availability of orthogonally protected (2S,3R)-3-aminoproline required for the synthesis of BP-65 represented an obstacle for up-scaling of the synthesis for clinical grade material, we synthesized derivatives of BP-65 designated UK-40 and UK-39 (PE- conjugate of UK-40), in which this building block is replaced by (2S,4S)-4-aminoproline (Figure 1). The required orthogonally protected N( $\alpha$ )-Fmoc(2S,4S)-4-(Boc)aminoproline ((2S,4S)-Boc-4-amino-1-Fmoc-pyrrolidine-2-carboxylic acid) is commercially available.

### NMR studies of UK-40

The solution structure of UK-40 was investigated in water (90% H<sub>2</sub>O, 10% D<sub>2</sub>O, pH 5.0, 293 K) by <sup>1</sup>H NMR spectroscopy. The <sup>1</sup>H NMR spectrum of UK-40 indicated the existence of a major and two minor forms in a ratio of 14:3:1, which interconvert slowly on the NMR timescale, caused by *cis-trans* isomerism at Asn-Pro peptide bonds. Similar *cis-trans* rotamers were found in BP-66 in earlier work [11], and in

linear peptides containing multiple NPNA-repeats [17]. The major form can be assigned the all *trans* conformation on the basis of observed NOE connectivities. Resonance overlap prevented an assignment of the minor forms to specific peptide bond rotamers. Chemical shift assignments for the major rotamer (Table S1) were made using standard 2D NMR methods [18]. Side chain proton frequencies in UK-40 exhibited major spectral overlap, although this problem was not as severe as seen in linear peptides containing multiple tandem NPNA-repeats. This is illustrated in Figure S2, where HN-C( $\alpha$ )H cross peaks from 2D DQF-COSY spectra are shown for UK-40 and the peptide Ac-(NPNA)<sub>3</sub>-NH<sub>2</sub>. Notable in the spectrum of UK-40 are the significantly upfield shifted amide HN resonances of Asn<sup>9</sup> and Ala<sup>12</sup>. The amide proton chemical shift temperature coefficients were measured and are given in Figure S3. Notable are the low values for both Ala<sup>8</sup> and Asn<sup>9</sup>, suggesting that both these amide NHs are involved in intramolecular hydrogen bonding. A low value is also seen for Ala<sup>12</sup>, but not for Asn<sup>13</sup>.

2D NOESY spectra of UK-40 showed  $d_{\text{NN}}(i, i+1)$  NOE connectivities between amide protons of Asn<sub>*i*+3</sub>, Ala<sub>*i*+4</sub> and Asn<sub>*i*+5</sub> within each NAN motif, as shown in Figure S3 and Figure S4. The same pattern of NOE interactions was also found earlier in BP-66 [11]. Furthermore, characteristic medium range  $d_{\alpha\text{N}}(i, i+2)$  NOEs were found between Asn<sup>7</sup> and Asn<sup>9</sup>, Asn<sup>9</sup> and Asn<sup>11</sup>, Pro<sup>10</sup> and Ala<sup>12</sup>, Pro<sup>14</sup> and Glu<sup>16</sup> in UK-40. Weak NOE contacts between side chains of the first Asn and the Ala of each NPNA motif are also seen, as predicted in the structure model of an NPNA motif in a helical turn conformation (*vide infra*) (Figure 3A). No other medium/long range NOEs were observed in NOESY spectra.

Using the available short and medium range NOE connectivities, average solution structures for UK-40 were calculated using DYANA. The final ensemble of 20 structures (see Table S2) is shown in Figure 3B. The backbone atoms of residues 6-16 enclosed within the macrocyclic ring can be superimposed with an rmsd of 1.6 Å, whereas the remaining residues at the N- and C-termini show much greater conformational diversity. When each tetrapeptide motif within the macrocycle are considered, the highest structural similarity both to the ANPNA crystal structure and to a model helical turn conformation is seen in the first (N<sup>5</sup>-A<sup>8</sup>) and in particular the central (N<sup>9</sup>-A<sup>12</sup>) tetrapeptide motifs (Figure 4), suggesting that helical turns are significantly populated in this portion of the molecule. Within the third unit N<sup>13</sup>-E<sup>16</sup>,

however, the helical turn conformations are adopted much less frequently, or not at all, in the 20 lowest energy DYANA structures, suggesting either that the turn is not significantly populated or, as seems more likely, that the average NMR structures do not represent accurately these regions of the macrocycle due to the presence of multiple rapidly interconverting helical and extended conformations. The low number and weak intensity of many NOEs are indicative of significant backbone flexibility. Moreover, the occurrence of significant restraint violations, such as from the Glu<sup>16</sup> C( $\alpha$ )H to Asn<sup>17</sup> HN NOE, indicate also the presence of extended ( $\beta$ ) chain forms.

### **Antigenic properties**

The antigenic properties of virosomally formulated UK-39 were compared to those of BP-65 and two previously described [12] template-bound peptides (JL-934 and JL-1036) (see Figure 1). Panels of CSP repeat region specific sporozoite-binding mAbs obtained after immunization of mice with either NPNA peptides or with *P. falciparum* sporozoites were tested in ELISA for cross-reactivity with the first to fourth generation compounds (Table 1). The results demonstrate that antibodies with a range of different fine-specificities can bind to the native CSP on the sporozoite surface. Thus mAb 1.26 the only sporozoite cross-reactive mAb elicited by the antigen JL-934 [12] did not bind to the other peptide antigens and only three of the four sporozoite-binding mAbs elicited by the peptide antigen JL-1036 [12] cross-reacted with the antigens BP-65 and UK-39. Cross-reactivity analysis with a set of twelve CSP repeat region specific mAbs raised against *P. falciparum* sporozoites demonstrated that only two of these mAbs cross-reacted with JL-934, five with JL-1036 and all 12 with BP-65 and UK-39. While cross-reactivity of mAbs Sp4-7H1 and Sp4-1B4 with the antigen BP-65 was only weak, all twelve anti-sporozoite mAbs cross-reacted strongly with UK-39, indicating that this compound better reflects the native structure of the CSP repeat region.

### **Immunogenicity of virosomally formulated UK-39**

Already one immunization of BALB/c mice with UK-39-loaded IRIV elicited detectable titers of anti-UK-39 IgG in ELISA (Figure 5A) and sporozoite cross-reactive IgG in IFA (Figure 5B). While a second immunization led to a strong titer increase, a third immunization had only a moderate further booster effect. In Western blots with a

lysate of salivary glands of *P. falciparum* infected mosquitoes, anti-UK-39 IgG stained a characteristic CSP double-band of 50-55 kDa (not shown).

Comparison of immune responses elicited by a liposomal and an IRIV-based formulation of UK-39 demonstrated the importance of influenza virus proteins for the immunopotentiating activity of IRIVs (Figure 5C). The strong immunogenicity of virosomally formulated UK-39 is virtually abolished when the peptide is presented on liposomes, lacking influenza derived hemagglutinin and neuraminidase. Also no parasite cross-reactive IgG was detected in IFA (not shown). Already one dose of IRIV formulated UK-39 elicited high ELISA and IFA IgG titers in rabbits (Figure S5). Additional immunizations had only a minor booster effect. IgG from immunized animals stained the CSP double-band in Western blots (not shown).

### **Generation of sporozoite inhibitory antibodies by UK-39**

Total IgG purified from UK-39-immunized rabbits showed at a concentration of 1 mg/ml of total IgG, inhibitory activity in sporozoite *in vitro* invasion inhibition assays (Figure 6A). Inhibition decreased in a dose dependent manner. Control IgG preparations from rabbits immunized with empty IRIVs had no inhibitory activity. Sera inhibiting invasion of *P. falciparum* sporozoites did not inhibit invasion of *P. yoelii* into murine hepatocytes (not shown), demonstrating specificity of the inhibitory activity.

Purified IgG from rabbits immunized with UK-39 inhibited parasite gliding motility, as demonstrated by immunofluorescence analysis of the trails of CSP shed by *P. falciparum* sporozoites gliding on glass slides [19]. At a concentration of 1 mg/ml of total IgG, traces were reduced (Figure 6B). Furthermore, CSP was precipitated at the apical ends of the sporozoites as described [20] and sporozoites were agglutinated. Serum of rabbits immunized with empty IRIVs had no effect on gliding motility or on parasite morphology.

### Discussion

An important goal here was the design of a conformationally constrained peptidomimetic of the immunodominant NPNA repeat region of the CS protein, which could be delivered to the immune system on the surface of immunopotentiating reconstituted influenza virus-like particles (IRIVs). Linear peptides are generally unsuitable as immunogens, due to their inherent conformational flexibility. As a result, there are many different ways that linear peptides can be recognized (by B-cell receptors), correlating with the number of accessible conformational states available to the peptide chain. Only a small subset of these will be relevant for cross-recognition of the cognate folded protein. A peptide chain restrained into the biologically relevant backbone conformation, on the other hand, should be a more effective immunogen, since it should be recognized preferentially in a conformation that promotes cross-reaction with the folded protein. There is the further advantage that linear peptides are usually degraded by proteolysis within minutes in biological fluids, which limits the window of accessibility to B-cell receptors.

Our strategy for the design of a multi-stage multi-component subunit malaria vaccine is to focus immune responses onto protection-relevant structural elements of key parasite proteins. Moreover, synthetic peptides and proteins with native-like folds can be presented to the immune system on IRIVs, thereby bypassing many of the problems associated with the production of stable recombinant protein-based vaccine formulations. A key parameter used for selecting vaccine candidates was the cross-reactivity of elicited antibodies with the native target antigen on the cell surface of the parasite. IRIV formulations of the peptide antigens evaluated here were immunogenic in mice and rabbits when coupled to virosomes. The optimized structure, UK-39, has remarkable structural and antigenic similarity with the native repeat region of the key sporozoite vaccine antigen CSP and is suitable for large scale GMP production.

Key structural information used to guide the design of UK-39 came from earlier NMR and modeling studies [11]. Already NMR studies on linear peptides containing one or several tandemly linked NPNA motifs suggested the presence of turn-like structures based on the NPNA cadence, stabilized by hydrogen bonding, but in rapid dynamic equilibrium with extended chain forms [17]. Recently, an X-ray crystal structure was reported of the pentapeptide Ac-ANPNA-NH<sub>2</sub>, which confirmed that the NPNA unit adopts a type-I  $\beta$ -turn conformation in aqueous solution [21]. Nevertheless, a key question remains how individual  $\beta$ -turns in a longer (NPNA)<sub>n</sub>

oligomer might be interconnected to form a repeat structure. In earlier work, we were strongly influenced by the observation in NOESY spectra of various (NPNA)<sub>n</sub>-containing cyclic and linear peptides, of strong sequential backbone NH-NH NOEs between both Asn<sup>*i*+2</sup> and Ala<sup>*i*+3</sup> as well as Ala<sup>*i*+3</sup> and Asn<sup>*i*+4</sup>. This led us to consider backbone conformations of extended (NPNA)<sub>n</sub> oligomeric peptides, in which Pro<sup>*i*+1</sup>, Asn<sup>*i*+2</sup> and Ala<sup>*i*+3</sup> are in the α-helical region, leaving only Asn<sup>*i*</sup> in the β-region of φ/ψ space; a combination called here a helical-turn. A computer model of such a turn is shown in Figure 3A, where the close approach of the backbone amide NHs of Asn<sup>*i*+2</sup> and Ala<sup>*i*+3</sup> as well as Ala<sup>*i*+3</sup> and Asn<sup>*i*+4</sup> can be seen. With these φ/ψ angles for each NPNA motif, and assuming *trans* peptide bonds, a cross-linked peptidomimetic was designed using a computer model of an extended (NPNA)<sub>n</sub>-oligomer, as described earlier [11]. In our first effort, (2S,3R)-3-aminoproline was used to synthesize the mimetic BP-66, and the phospholipid conjugate BP-65. Here, we have investigated the related peptidomimetics UK-39 and UK-40 (Figure 1), which use instead the synthetically more accessible (2S,4S)-4-aminoproline.

NMR studies on UK-40 in aqueous solution provide insights into the preferred backbone conformations of this mimetic, and confirm a close similarity to those deduced earlier for BP-66 [11]. As expected, the residues contained within the macrocyclic ring of UK-40 are more restrained in their conformation than the NPNA units outside the macrocycle at the N- and C-termini (Figure 3B). The Asn<sup>5</sup>-Ala<sup>8</sup> and Asn<sup>9</sup>-Ala<sup>12</sup> motifs, in particular, appear to populate helical turns, as seen in the average solution structures calculated using NOE-derived distance restraints (Figure 4). However, especially the Asn<sup>13</sup>-Glu<sup>16</sup> motif within the macrocycle appears to be more disordered and to exist in dynamic equilibrium with extended forms. It is less clear what role (if any) the observed minor *cis*-(Asn-Pro) peptide bond conformers might play in eliciting antibodies cross-reactive with the CS protein

As far as the antigenic properties are concerned, UK-39 was recognized by all tested CSP repeat region specific mAbs elicited by sporozoite-immunization of mice, and by the majority of sera from human donors living in malaria endemic regions (data not shown). One major limitation in using peptide antigens as vaccine components is their poor immunogenicity. We demonstrate here that presentation of UK-39 on the surface of virus-like particles elicited a strong parasite cross-reactive antibody response both in mice and in rabbits. Lack of immunogenicity of a liposomal

formulation demonstrated the importance of influenza protein antigens for the immunopotentiating activity of IRIVs. We have observed better immune responses with liposomal formulations of PE-conjugates of larger peptides, but also in these cases IRIV formulations were far superior (unpublished results). While two immunizations were needed to reach high IgG titers in mice, one immunization was sufficient in rabbits. Seroconversion was not only observed in all inbred mice, but also in all rabbits with diverse immunogenetic backgrounds. Genetic restriction of the response in humans may therefore not represent a serious limitation for IRIV-based peptide vaccines. As previously observed for other antigens [13, 22], pre-immunization of animals with an influenza vaccine improved the titers of antibodies elicited by the IRIV formulation (data not shown). Possible explanations for this phenomenon are (i) opsonization of IRIVs with pre-existing anti-influenza antibodies, leading to enhanced uptake by antigen presenting cells and (ii) activation of influenza-specific memory T cells, providing T cell help to UK-39-specific B cells. Results of the animal immunogenicity studies described here suggested that two immunizations with a 10 µg dose of UK-39 will elicit a potent antibody response in influenza pre-exposed humans, irrespective of their immunogenetic background. Preliminary immunogenicity data of clinical trials with two IRIV-based malaria vaccine components, UK-39 and a peptide loop derived from the *P. falciparum* Apical Membrane Antigen-1 [10, 22] support this assumption and the general concept of delivering peptides as IRIV-bound phosphatidylethanolamine (PE)-conjugates to the human immune system. Widespread outbreaks of influenza have been reported for Africa [23], making the virosomal antigen delivery system applicable in malaria endemic areas of Africa.

Antibodies against BP-65 and its easier to synthesize derivative UK-39 inhibited sporozoite invasion into hepatocytes *in vitro*. Inhibition with mAbs against BP-65 was up to 100% whereas only lower levels of inhibition were observed with purified IgG from UK-39-immunized rabbits. This reduced effect can be explained by the fact that we used total IgG from immunized animals. Thus, despite using high concentrations of IgG (1 mg/ml), only a minor fraction of the antibodies is specific for the NPNA repeats. Additionally, physiological serum IgG concentrations of rabbits are higher than 1 mg/ml, pointing towards an increased inhibitory effect *in vivo*.

The mechanism of this inhibition is not clearly understood. The observed inhibition of gliding motility suggests interference with parasite motility, which is

necessary to invade the target cell, as described before [24]. Whether the gliding inhibition is based on a specific block of the mechanism promoting movement or just the result of steric hindrance remains to be elucidated, as the involvement of CSP in the process of gliding motility is still controversial [25]. The protective potential of anti-CSP repeat antibodies has been shown *in vivo* with passive transfer of anti-CSP repeat antibodies, which can protect mice from experimental sporozoite challenge [26]. The mechanism of this protection is at least in part due to antibody-mediated immobilization of sporozoites in the skin after injection by the mosquito [27].

It is not likely that antibodies against UK-39 alone would result in complete protection against malaria. However, in the context of a multi-stage vaccine, they may reduce the number of sporozoites entering liver cells and thus support immune protection elicited by components directed against the liver and blood stages of the malaria parasite. Moreover it is thought that reduction of the number of merozoites released from the liver by anti-pre-erythrocytic vaccination [3] or reduction of the sporozoite inoculum by the use of insecticide treated bed-nets [28] can reduce the incidence of severe malaria.

The universal IRIV-based antigen delivery platform described in this report is highly suitable for combining antigens specific for the different development stages of the parasite into a multi-component malaria subunit vaccine.

### **Significance**

With increasing global prevalence of malaria and emerging resistance of *P. falciparum* to drug treatment, the need for an efficient malaria vaccine is greater than ever. Although it was shown more than 30 years ago that man can be protected against malaria by vaccination [29], a safe, effective and affordable vaccine is still many years away [4]. Especially the lack of safe and potent adjuvant/delivery systems and the technical and regulatory problems associated with recombinant protein production has hampered vaccine development. We have developed a technology platform based on the design of conformationally constrained synthetic peptides and the IRIV delivery system, which allows for the rational development of a malaria vaccine. The parasite-binding capacity of antibodies elicited by immunization of mice was used as a key indicator for the optimization of peptide antigens. The universal IRIV-based antigen delivery platform is highly suitable for combining antigens specific for the different development stages of the parasite into a multi-component malaria subunit vaccine. As the IRIV system is already registered for use in humans, this platform can contribute to the rapid development of a safe, efficient and cost-effective malaria vaccine.

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## Experimental procedures

### Synthesis of peptides

Synthesis of JL-934, JL-1036 and BP-65 has been described previously [11, 12, 30]. For UK-40, a linear peptide was first assembled on Rink Amide MBHA resin (0.73 mM/g) (*Novabiochem*) using an *Applied Biosystems ABI433A* peptide synthesizer, and Fmoc-protected amino acids (Fmoc-Asn(Mtt)-OH, Fmoc-Pro-OH, Fmoc-Ala-OH, Fmoc-Glu(*t*Bu)-OH and Fmoc-(4*S*,2*S*)-4-aminoproline(Boc)-OH) and HBTU/HOBt (4 eq.) for activation. After cleavage of the linear peptide from the resin with 95% TFA, 2.5% TIS and 2.5% water over 3 h, the peptide was precipitated using  $i\text{Pr}_2\text{O}$  and dried. The linear peptide was then stirred overnight with HATU (4eq.) and HOAt (4 eq.) in DMF with 1 % v/v  $i\text{Pr}_2\text{EtN}$  (2 mg/ml peptide). After drying in vacuo the peptide was stirred in 20 % piperidine in DMF for 15 min. The solvent was evaporated and peptide was precipitated using  $i\text{Pr}_2\text{O}$ , dried in vacuo, and then purified by reverse phase HPLC (C18 column using a gradient of MeCN/H<sub>2</sub>O (+0.1% TFA; 5 to 95% MeCN ;  $t_{\text{R}}$  = 11 min). ES-MS  $m/z$ : 2276 (M+H)<sup>+</sup>.

The cyclic peptide (40 mg) was coupled to PE-CO-(CH<sub>2</sub>)<sub>2</sub>-COOH (PE-succinate; 4 eq.) in DMF (5 ml), CH<sub>2</sub>Cl<sub>2</sub> (5 ml) and 1%  $i\text{Pr}_2\text{EtN}$  using HATU and HOAt with stirring overnight at rt. The solvent was then removed and the product purified by reverse phase HPLC (C4 column *Vydac 214 TP 1010, 25cmx10mm*) using a gradient of 50 % ethanol in water to 100 % ethanol (+0.1% TFA) over 15 min. UK-39 appears as a broad peak at about 90 % ethanol. ES-MS  $m/z$ : 1427 (M+2H)<sup>2+</sup>. Alternatively, the cyclic peptide was acetylated with acetic anhydride in MeOH and NH<sub>4</sub>HCO<sub>3</sub> buffer and UK-40 was purified by reverse phase HPLC (C18 column *Vydac*) using a gradient of 0 to 100% MeCN in water. ES-MS  $m/z$ : 1049 (M+2H)<sup>2+</sup>.

### NMR studies

For NMR studies, UK-40 was dissolved in 90%H<sub>2</sub>O/10%D<sub>2</sub>O, pH 5.0, at a concentration of ca. 10 mg/ml. 1D and 2D <sup>1</sup>H NMR spectra were recorded at 600 MHz (Bruker AV-600 spectrometer). Water suppression was by presaturation. The sequential resonance assignments were based on DQF-COSY, TOCSY and NOESY spectra. Distance restraints were obtained from NOESY spectra with mixing times of 80 and 250 ms. Spectra were collected with 1024 x 256 complex data points zero-filled prior to Fourier transformation to 2048 x 1024, and transformed with a cosine-

bell weighting function. Data processing was carried out with XWINNMR (Bruker) and XEASY. [31]

The structure calculations were performed by restrained molecular dynamics in torsion angle space by applying the simulated annealing protocol implemented in the program DYANA. [32] Starting from 100 randomized conformations a bundle of 20 conformations were selected with the lowest DYANA target energy function. The program MOLMOL [33] was used for structure analysis and visualization of the molecular models.

### **Preparation of peptide-loaded virosomes**

For the preparation of IRIV loaded with phosphatidylethanolamine (PE)-peptide conjugates, a solution of purified Influenza A/Singapore hemagglutinin (4 mg) in phosphate buffered saline (PBS) was centrifuged for 30 min at 100 000 g and the pellet was dissolved in PBS (1.33 ml) containing 100 mM octaethyleneglycolmonodecylether (PBS-OEG). Peptide-PE conjugates (4 mg), phosphatidylcholine (32 mg; Lipoid, Ludwigshafen, Germany) and PE (6 mg) were dissolved in a total volume of 2.66 ml of PBS-OEG. The phospholipid and the hemagglutinin solutions were mixed and sonicated for 1 min. This solution was then centrifuged for 1 hour at 100 000 g and the supernatant was filtered (0.22  $\mu$ m) under sterile conditions. Virosomes were then formed by detergent removal using BioRad SM BioBeads (BioRad, Glattbrugg, Switzerland).

### **Mouse immunogenicity studies**

BALB/c mice were pre-immunized intramuscularly with 0.1 ml of the commercial whole virus influenza vaccine Inflexal Berna<sup>TM</sup> (Berna Biotech, Bern, Switzerland). At least three weeks later they were immunized with PE-peptide conjugate-loaded IRIVs (containing 10  $\mu$ g PE-peptide) in intervals of at least two weeks. Blood was collected before each immunization and two weeks after the final injection.

### **Generation of hybridomas and production of mAbs**

Hybridomas were generated from spleen cells of mice three days after a booster immunization with BP-65 loaded IRIVs using PAI mouse myeloma cells as a fusion

partner. Hybrids were selected in HAT medium and cells that secreted anti-BP-65 mAbs were identified by ELISA. For large-scale mAb production, hybridoma cell lines were cultured in 175 cm<sup>2</sup> flasks and mAbs were purified by protein A or G affinity chromatography (Protein A Sepharose<sup>TM</sup> CL4B or HiTrap<sup>TM</sup> Protein G HP, Piscataway, NJ). Purified mAbs were dialyzed against PBS, aliquoted and stored at -80°C. Generation of anti-*P. falciparum* sporozoite mAbs has been described previously [34].

### **Rabbit immunogenicity studies**

Rabbits were pre-immunized intramuscularly with 0.5 ml of the commercial whole virus influenza vaccine Inflexal Berna<sup>TM</sup> (Berna Biotech, Bern, Switzerland). At least three weeks later they were immunized with peptide-loaded IRIVs (containing 10 µg PE-peptide) in intervals of at least two weeks. Blood was collected before each immunization and two weeks after the final injection. Rabbit IgG was purified from immune sera by protein A affinity chromatography.

### **ELISA**

ELISA analyses with peptide-PE conjugates were performed essentially as described before [10]. Briefly, Polysorp<sup>TM</sup> plates (Nunc, Fisher Scientific, Wohlen, Switzerland) were coated overnight at 4°C with 100 µl of a 10 µg/ml solution of UK-39 in PBS (pH 7.2). After three washings with PBS containing 0.05% Tween-20 wells were blocked with 5% milk powder in PBS for 30 min at 37°C and washed three times again. Plates were then incubated with serial dilutions of anti-peptide mouse or rabbit sera or anti-peptide mAbs in PBS containing 0.05% Tween-20 and 0.5% milk powder for 2 h at 37°C. After washing, plates were incubated with alkaline phosphatase-conjugated goat anti-mouse IgG (Fc-specific) antibodies (Sigma, St. Louis, Mo) for 1 h at 37°C. After washing again, phosphatase substrate solution (1 mg/ml p-nitrophenyl phosphate (Sigma, St. Louis, Mo) in a pH 9.8 buffer solution containing 10% [vol/vol] diethanolamine and 0.02% MgCl<sub>2</sub>) was added and the plates were incubated in the dark at room temperature until the colorimetric reaction had progressed sufficiently. The optical density was measured at 405 nm on a Titertek Multiscan MCC/340 reader (Labsystems, Helsinki, Finland). For experiments with rabbit sera, horseradish peroxidase-conjugated goat anti-rabbit IgG heavy and light chain antibodies (Bio-Rad

Laboratories, Hercules, CA) were used as secondary antibody. The TMB microwell peroxidase substrate system (KPL, Gaithersburg, MD) was used according to the manufacturer and optical density was measured at 650 nm. After stopping the reaction by addition of 50  $\mu$ l 1N HCl per well OD was measured at 450nm.

### **Indirect immunofluorescence assay (IFA)**

Air-dried unfixed *P. falciparum* (strain NF54) salivary gland sporozoites attached to microscope glass slides were incubated for 15 min at room temperature with 25  $\mu$ l blocking solution containing 1% fatty acid-free bovine serum albumin (BSA) in PBS. Immunostaining was performed by incubating the wells with 25  $\mu$ l of an appropriate serum dilution in blocking solution in a humid chamber for 1 h at room temperature. After five washes with blocking solution 25  $\mu$ l of 5  $\mu$ g/ml cyanine dye (Cy3)-conjugated affinity-pure F(ab')<sub>2</sub> fragment goat anti-mouse IgG (Fc-specific) antibodies (Jackson Immuno Research Laboratories, West Grove, Pa.) or Cy3-conjugated donkey anti-rabbit IgG heavy and light chain antibody (Jackson Immuno Research Laboratories, West Grove, PA), diluted in blocking solution containing 0.01 mg/ml Hoechst dye no. 33256 (Sigma, St. Louis, Mo) were added to the wells and incubated for 1 h at room temperature. Finally, wells were washed five times with PBS, mounted with mounting solution (90% [vol/vol] glycerol containing 0.1 M Tris-Cl, pH 8.0 and 2 mg/ml *o*-phenylenediamine) and covered with a coverslip. Antibody binding and DNA staining were assessed by fluorescence microscopy on a Leitz Dialux 20 fluorescence microscope and documented with a Leica DC200 digital camera system.

### **SDS-PAGE and immunoblotting**

100  $\mu$ l of an *Anopheles stephensi* salivary gland lysate containing about 100'000 *P. falciparum* sporozoites were diluted with an equal volume of 2x loading buffer (1.7ml, 0.5M Tris-HCl pH 6.8, 2 ml glycerol, 4.5 ml 10% SDS, 1 ml  $\beta$ -mercaptoethanol, 0.8 ml 0.3% w/v bromophenol blue) and heated to 95°C for 10 minutes. Proteins were separated on a 10% SDS PAGE mini-gel. Separated proteins were electrophoretically transferred to a nitrocellulose filter (Protran® Nitrocellulose, BA85, Schleicher & Schuell) by semi-dry blotting. Blots were blocked with PBS containing 5% milk powder and 0.1% Tween-20 over night at 4°C. The filter was cut into strips

and incubated with appropriate dilutions of immune sera in blocking buffer for 2 h at room temperature. Filter strips were then washed three times for 10 minutes in blocking buffer and incubated at room temperature for 1 h with alkaline peroxidase-conjugated goat anti-mouse IgG (Fc-specific) antibodies (Sigma, St. Louis, Mo) diluted 1:30'000 in blocking buffer or horseradish peroxidase-conjugated goat anti-rabbit IgG heavy and light chain antibodies (Bio-Rad Laboratories, Hercules, CA) diluted 1:6'000 in blocking buffer. After washing again, blots were finally developed using ECL™ Western blotting detection (Amersham Biosciences, Buckinghamshire, England) reagents to visualize bands.

### ***P. falciparum* and *P. yoelii* in vitro invasion inhibition assay**

Inhibition assays were performed as described before [35, 36]. Briefly, primary human or mouse hepatocytes were isolated as described [37, 38], and inoculated with *P. falciparum* (NF54 strain) or *P. yoelii* (265BY strain) sporozoites (1 x 10<sup>5</sup>/Labtek well) obtained from salivary glands of infected *Anopheles stephensi* mosquitoes. After 3 h at 37°C, the cultures were washed, further incubated in fresh medium for 3 days (*P. falciparum*) or 2 days (*P. yoelii*) before fixation in methanol. Quantification of exoerythrocytic forms was done by immunofluorescence. To determine the effects of anti-CSP antibodies on sporozoite infectivity, sporozoites were incubated with hepatocytes in the presence of increasing concentrations of mAbs or purified polyclonal rabbit IgG from immunized animals. The percentage of inhibition was determined in comparison to PBS control.

### **Gliding inhibition**

To analyze sporozoite motility, 30,000 sporozoites were deposited on multispot glass slide wells precoated with anti-*P. falciparum* CSP (*PfCSP*) mAb E9 (100 µg/ml 1 h at 37 °C) and incubated at 37 °C for 1 h. The slides were then washed, and the deposited CSP trails were fixed with 4% paraformaldehyde for 15 min. The trails were then labeled using the anti-*PfCSP* mAb E9 conjugated to Alexa Fluor® 488 and visualized under a fluorescence microscope.

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## Figure legends

Figure 1. Structures of peptidomimetics discussed in the text.

Figure 2. Hepatocyte invasion inhibition by anti-BP-65 mAbs. A: Inhibition of parasite invasion into primary human hepatocytes by mAb EP3 and mAb EP9. Shown is the mean percentage of inhibition compared to PBS control  $\pm$  SD deviation for duplicates. B: Competition of invasion inhibition by increasing concentrations of BP-66 peptide (BP-65 without PE). Inhibitory mAbs were used at a constant concentration of 20  $\mu\text{g/ml}$ .

Figure 3. A: An NPNA motif is depicted in a helical turn conformation (see text). N-atoms dark blue, O-atoms red, C-atoms grey, amide H-atoms light white, H-bonds pale blue broken line. Here the first Asn has  $\phi/\psi$  angles in the  $\beta$ -region, and the following Pro, Asn and Ala in the  $\alpha$ -region. A green ribbon traces the backbone. B: Superimposition of the final 20 DYANA structures for UK-40. The backbone only is represented, superimposed using all heavy atoms from Apro6 to Glu16 (region in blue).

Figure 4. Backbone superimposition of the 20 DYANA structures of UK-40 with a helical turn conformation (red, A - C), and with the ANPNA crystal structure (green, D - F). In A and D only residues 5-8 in UK-40 were used for the superimposition, for B and E only residues 9-12, and for C and F only residues 13-16.

Figure 5. Immunogenicity of virosomal and liposomal formulations of UK-39 in mice. A: Anti-UK-39 IgG response in ELISA after immunization with IRIV-formulated UK-39. Shown are mean ELISA readouts  $\pm$  SD obtained with serial dilutions of mouse sera taken pre-immune and two weeks after the first, second and third immunization. Sera from 10 animals were analyzed at every time point. B: Induction of *P. falciparum* sporozoite cross-reactive IgG upon immunization with UK-39. Shown are IFA endpoint titers of 10 individual mice. Sera have been taken pre-immune and two weeks after first, second and third immunization. C: Anti-UK-39 IgG response in ELISA of mice immunized with UK-39 presented either on liposomes or on

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Table 1. Cross-reactivity of anti-NPNA mAbs.

### Supplementary material

Figure S1. BP-125 a shortened version of BP-65. ELISA with anti-BP-65 mAbs EP3 and EP9 on CSP mimetics BP-65 and the shortened version BP-125. Shown are serial dilutions of the mAbs on constant concentrations of the two peptides.

Figure S2. Sections from 2D DQF-COSY spectra in aqueous solution for: A, UK-40; and B, the linear peptide Ac-(NPNA)<sub>3</sub>-NH<sub>2</sub>. Cross-peaks for HN-C( $\alpha$ )H <sup>3</sup>J couplings are indicated.

Figure S3. Results from 2D COESY spectra of UK-40. NOE connectivities from NOESY spectra, and temperature coefficients ( $-\Delta\delta/\Delta T$  ppb/K) of peptide amide NH resonances from 2D TOCSY spectra of UK-40 measured in the range 279 -314 K. The thickness of the lines for the sequential distances  $d_{NN}(i,i+1)$ ,  $d_{\alpha N}(i,i+1)$  and  $d_{\beta N}(i,i+1)$  is inversely proportional to the squared upper distance restraint.

Figure S4. Backbone amide HN-HN cross peaks in a NOESY spectrum of UK-40 in aqueous solution. The residue assignments are shown for each cross peak.

Figure S5. Immunogenicity of UK-39 in rabbits. A: Anti-UK-39 IgG levels measured in ELISA. Shown are the means  $\pm$  SD of 3 rabbits. Sera have been taken pre-immune and two weeks after first, second and third immunization. B: Cross-reactivity of UK-39 induced IgG with *P. falciparum* sporozoites. Shown are IFA endpoint titers of 3 individual animals pre-immune and two weeks after first, second and third immunization. Arrows indicate immunizations with IRIV-formulated UK-39.

Table S1. <sup>1</sup>H Chemical shift assignments for UK-40 in 90% H<sub>2</sub>O/10% D<sub>2</sub>O, pH 5, 293 K.

Table S2. Experimental distance restraints and statistics for the final 20 NMR structures calculated for UK-40.

Figure 1. Structures of peptidomimetics discussed in the text.

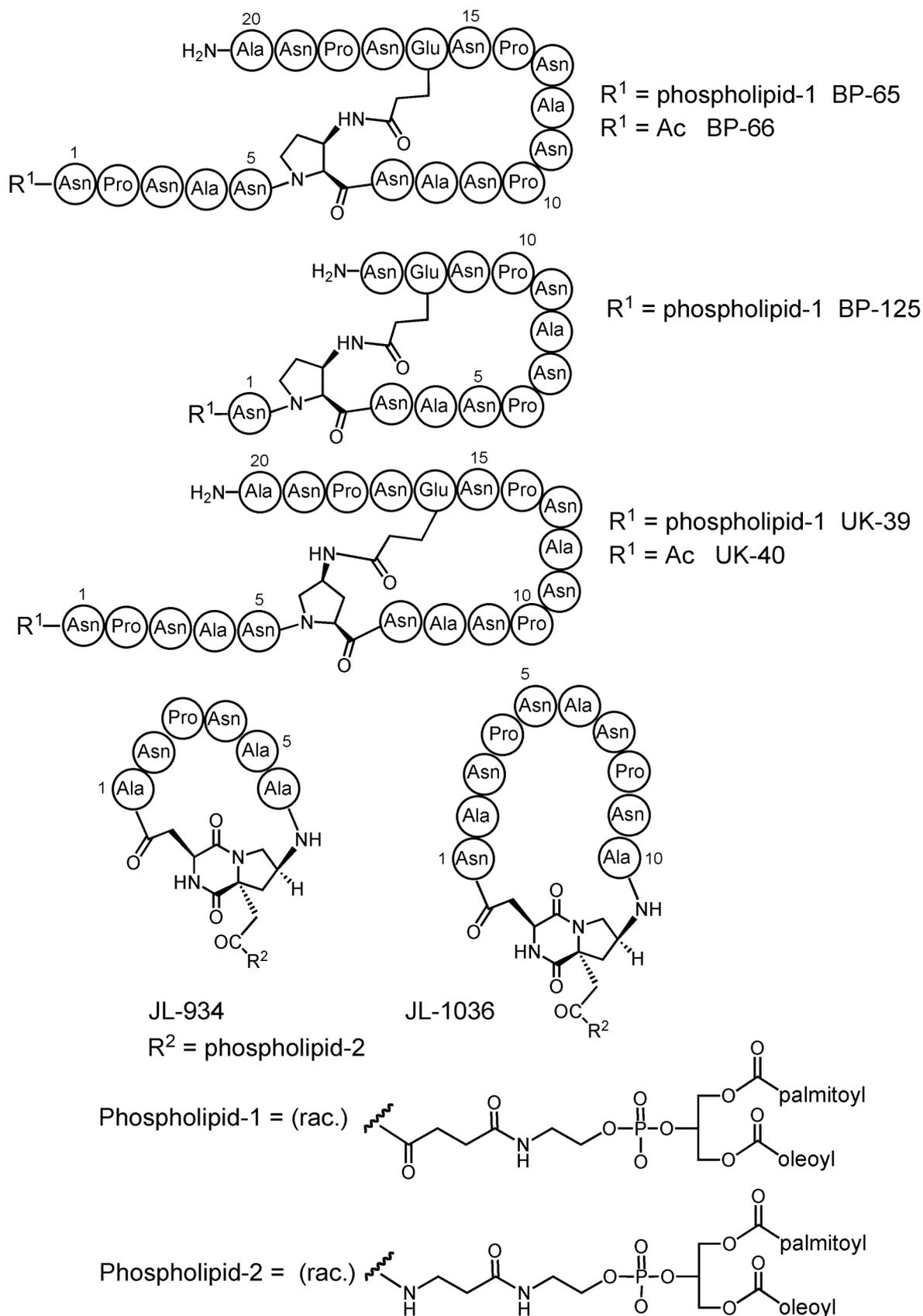


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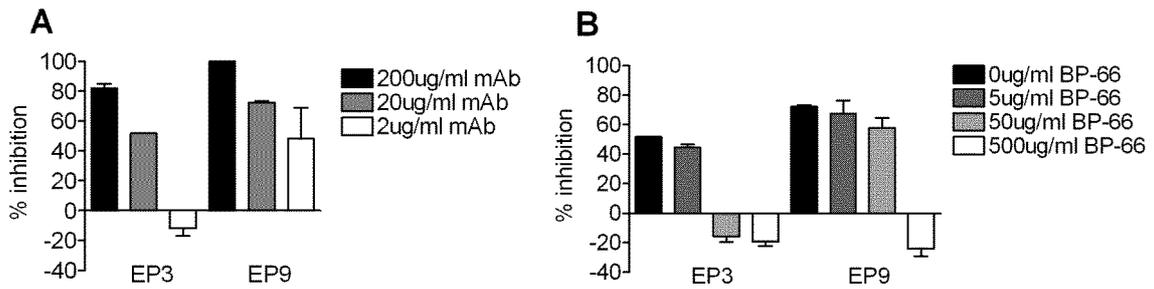


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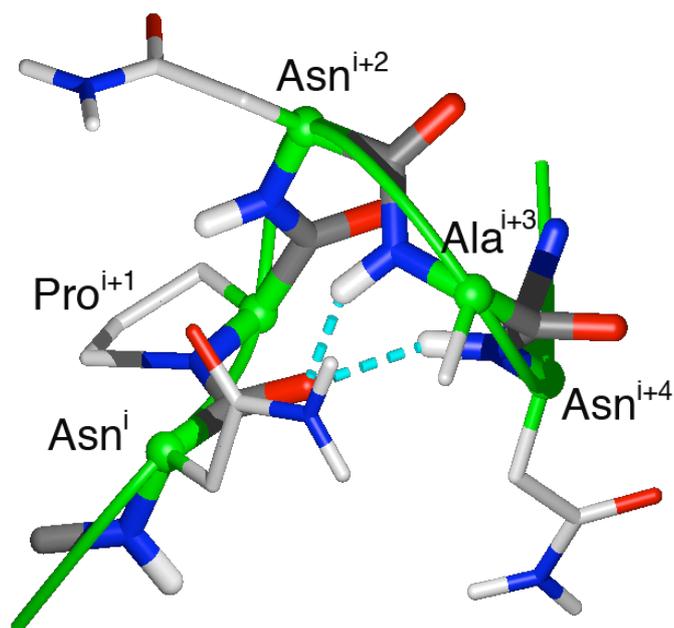
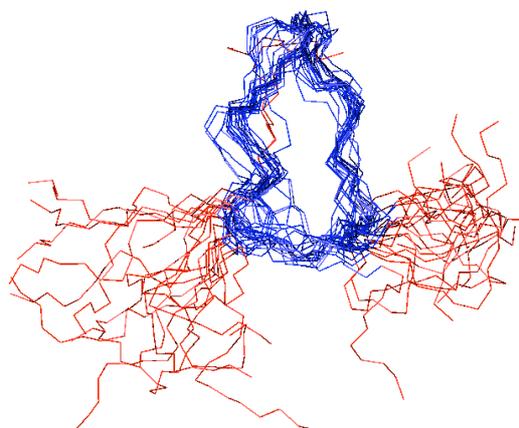
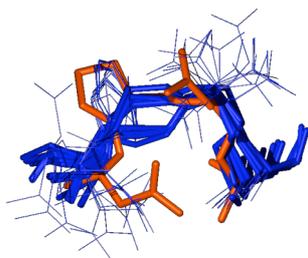
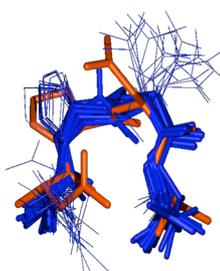
**A****B**

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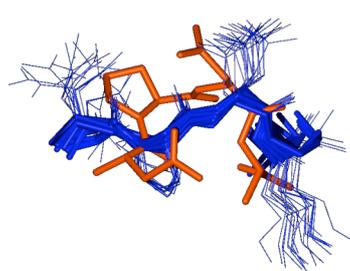
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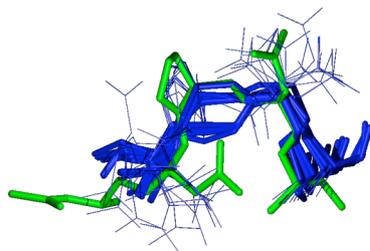
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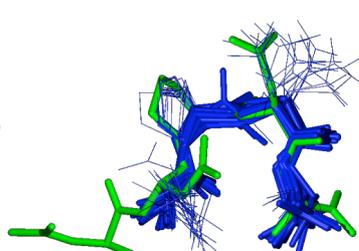
**C** (res. 13-16)



**D** (res. 5-8)



**E** (res. 9-12)



**F** (res. 13-16)

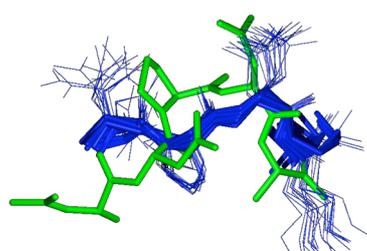


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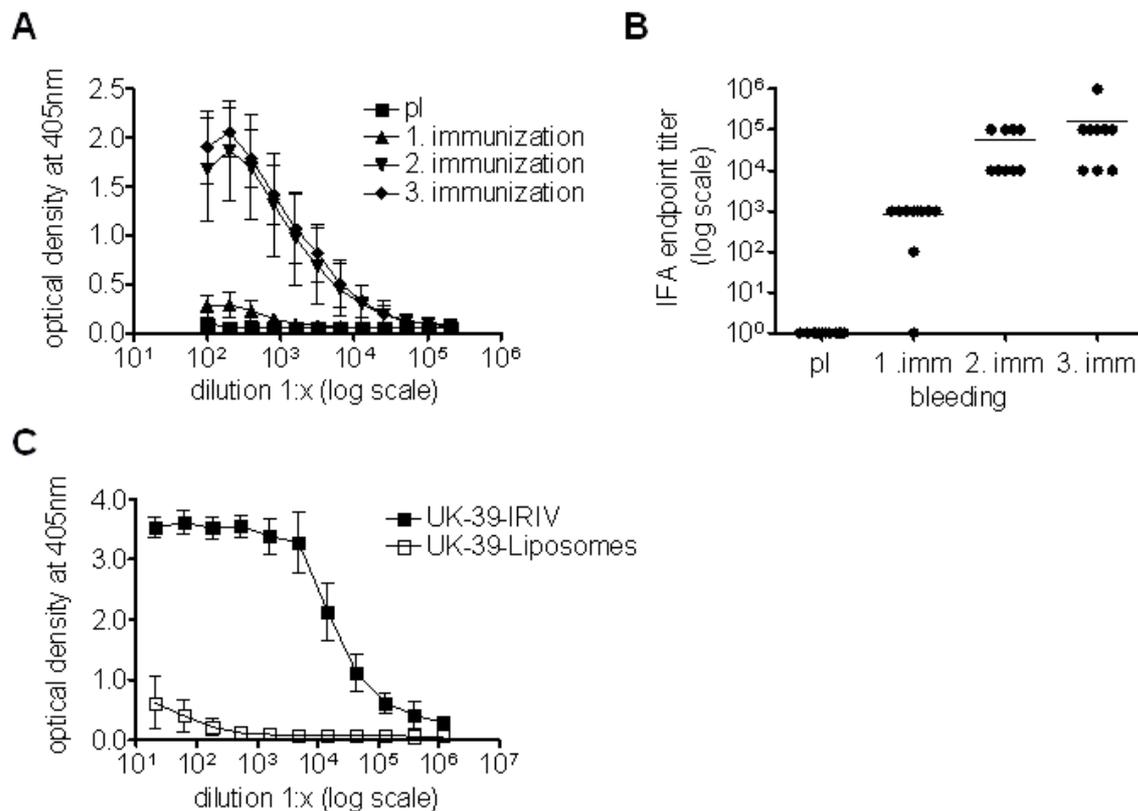


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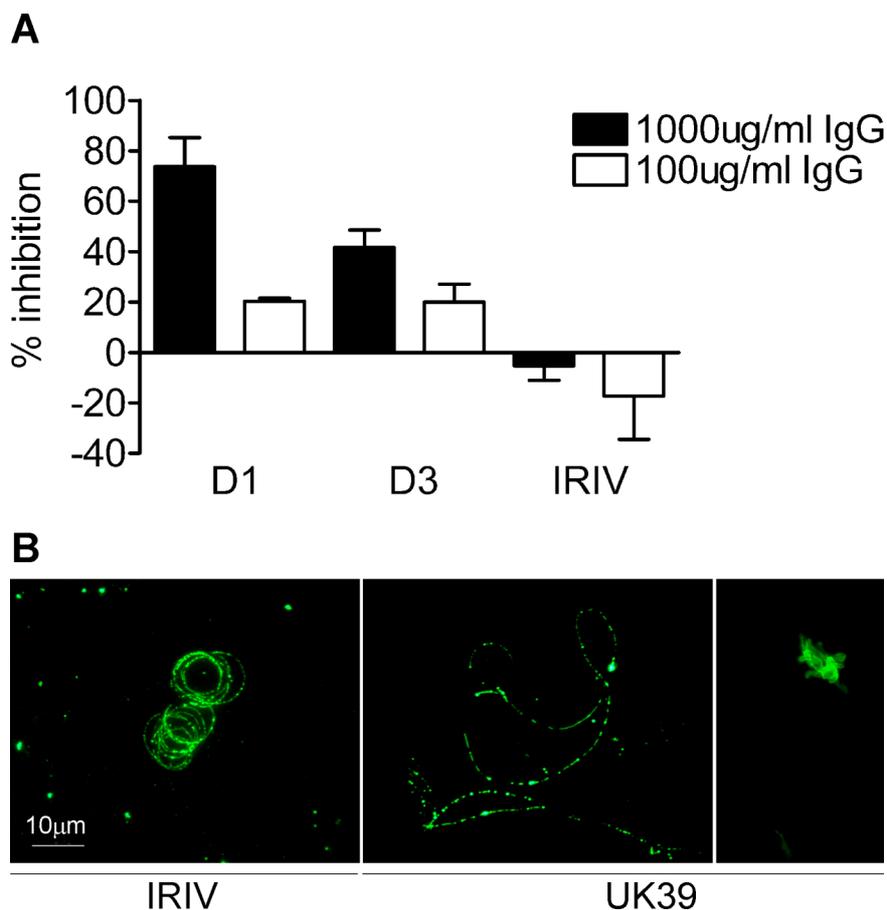


Table 1. Cross-reactivity of anti-NPNA mAbs.

mab	Immunogen	Cross-reactivity with				
		JL934 <sup>c</sup>	JL1036 <sup>c</sup>	BP-65 <sup>c</sup>	UK-39 <sup>c</sup>	Sporozoites <sup>d</sup>
1.26 <sup>e</sup>	JL934 <sup>a</sup>	+	-	-	ND	+
1.7 <sup>e</sup>		+	-	ND	ND	-
1.15 <sup>e</sup>		+	-	ND	ND	-
2.1 <sup>e</sup>	JL1036 <sup>a</sup>	-	+	+	+	+
3.1 <sup>e</sup>		-	+	+	+	+
3.2 <sup>e</sup>		-	+	-	-	+
3.3 <sup>e</sup>		-	+	+	+	+
3.4 <sup>e</sup>		-	+	ND	ND	-
3.5 <sup>e</sup>		-	+	ND	ND	-
EP3	BP-65 <sup>a</sup>	-	-	+	+	+
EP9		-	+	+	+	+
Sp4-5F2	sporozoites <sup>b</sup>	(+)	+	+	+	+
Sp4-2H1		(+)	+	+	+	+
Sp3-E6		-	+	+	+	+
Sp3-C6		-	(+)	+	+	+
Sp3-E9		-	(+)	+	+	+
Sp4-4B6		-	-	+	+	+
Sp4-7C2		-	-	+	+	+
Sp4-7E4		-	-	+	+	+
Sp4-7H1		-	-	(+)	+	+
Sp4-4D7		-	-	+	+	+
Sp3-B4-C12		-	-	+	+	+
Sp4-1B4		-	-	(+)	+	+

<sup>a</sup>mice were immunized with IRIVs loaded with the respective peptide

<sup>b</sup>mice were immunized with *P. falciparum* sporozoites from *A. stephensi* salivary glands in Freund's adjuvant

<sup>c</sup>ELISA reactivity to the peptides

<sup>d</sup>IIFA reactivity to *P. falciparum* sporozoites

<sup>e</sup>Described by Moreno et al (Moreno et al. 2001)

**Supplementary material**

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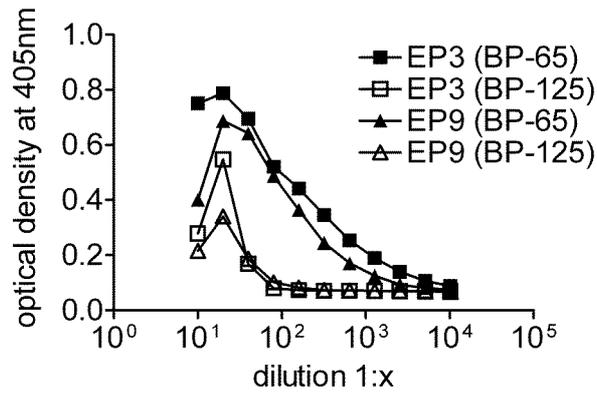


Figure S2. Sections from 2D DQF-COSY spectra in aqueous solution for: A, UK-40; and B, the linear peptide Ac-(NPNA)<sub>3</sub>-NH<sub>2</sub>. Cross-peaks for HN-C( $\alpha$ )H  $^3J$  couplings are indicated.

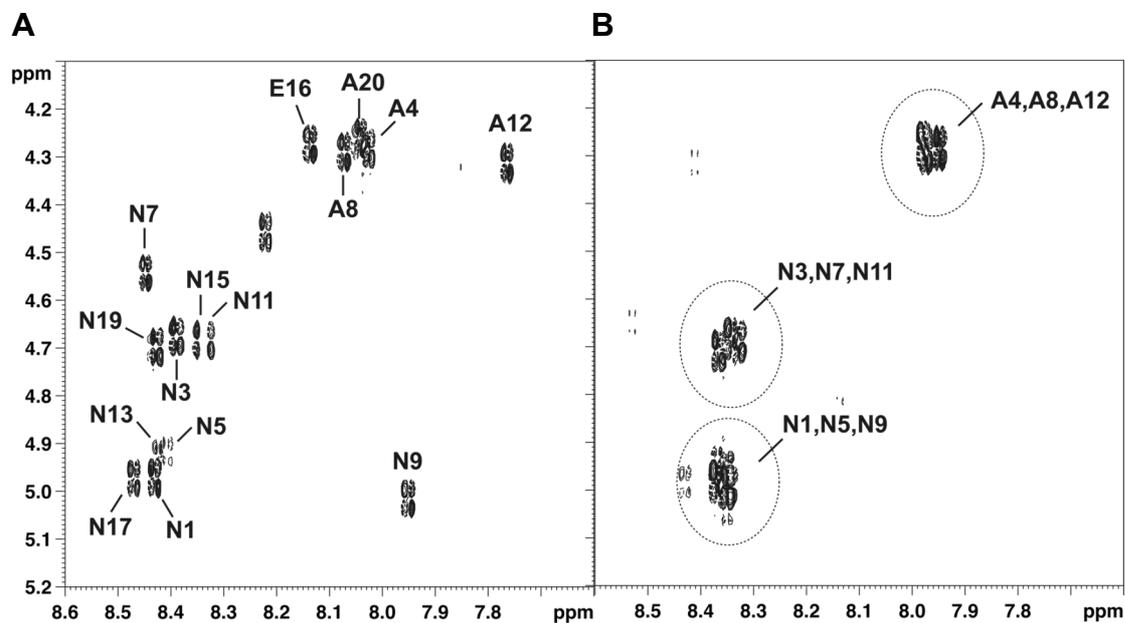


Figure S3. NOE connectivities from NOESY spectra, and temperature coefficients ( $-\Delta\delta/\Delta T$  ppb/K) of peptide amide NH resonances from 2D TOCSY spectra of UK-40 measured in the range 279 -314 K. The thickness of the lines for the sequential distances  $d_{NN}(i,i+1)$ ,  $d_{\alpha N}(i,i+1)$  and  $d_{\beta N}(i,i+1)$  is inversely proportional to the squared upper distance restraint.

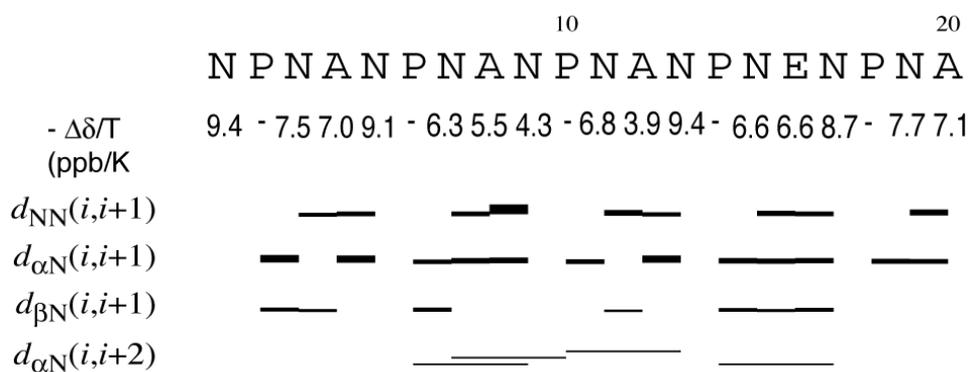


Figure S4. Backbone amide HN-HN cross peaks in a NOESY spectrum of UK-40 in aqueous solution. The residue assignments are shown for each cross peak.

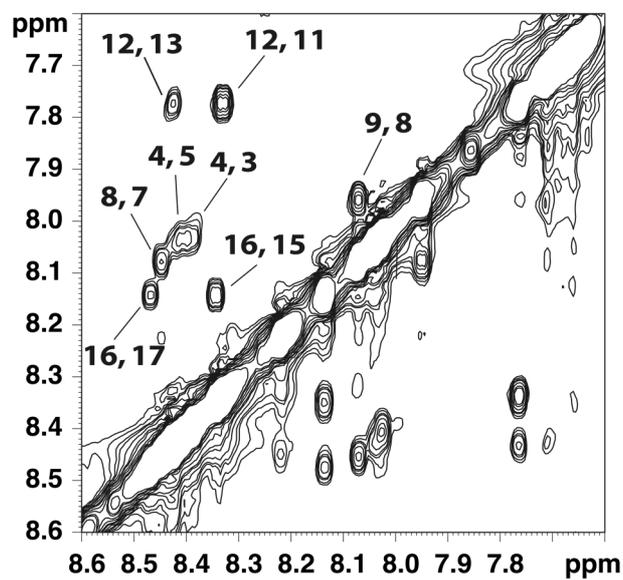


Figure S5. Immunogenicity of UK-39 in rabbits. A: Anti-UK-39 IgG levels measured in ELISA. Shown are the means  $\pm$  SD of 3 rabbits. Sera have been taken pre-immune and two weeks after first, second and third immunization. B: Cross-reactivity of UK-39 induced IgG with *P. falciparum* sporozoites. Shown are IFA endpoint titers of 3 individual animals pre-immune and two weeks after first, second and third immunization. Arrows indicate immunizations with IRIV-formulated UK-39.

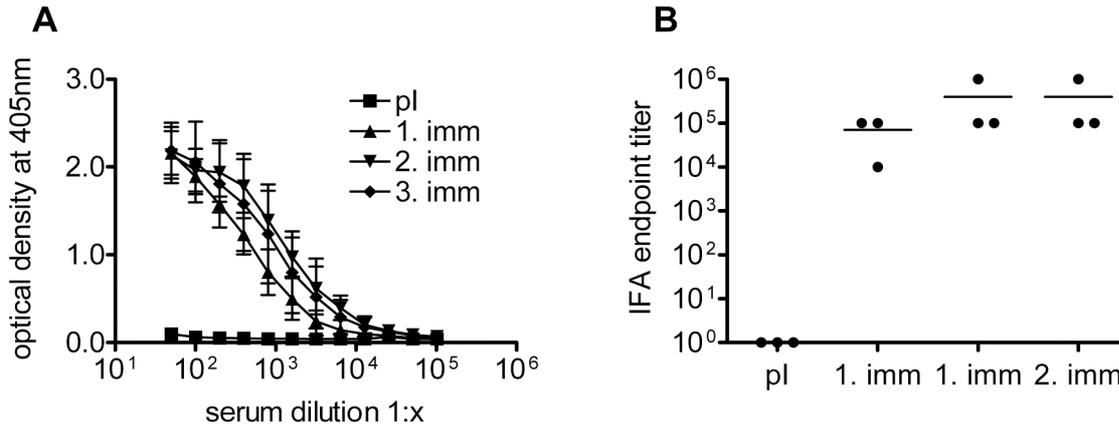


Table S1. <sup>1</sup>H Chemical shift assignments for UK-40 in 90% H<sub>2</sub>O/10% D<sub>2</sub>O, pH 5, 293 K.

Residue	NH	H-C(α)	H-C(β)	Others
<b>UK-40</b>				
Asn <sup>1</sup>	8.43	4.97	2.86, 2.71	NH(δ) <sup>a</sup>
Pro <sup>2</sup>	-	4.43	2.31, 1.98	CH(γ) 2.04 – 1.98; CH <sub>2</sub> (δ) 3.81, 3.81
Asn <sup>3</sup>	8.38	4.67	2.85, 2.74	NH(δ)α <sup>a</sup>
Ala <sup>4</sup>	8.02	4.28	1.39	
Asn <sup>5</sup>	8.41	4.91	2.87, 2.74	NH(δ) <sup>a</sup>
Apro <sup>6</sup>	8.21 <sup>b</sup>	4.51	2.66, 2.00	CH(γ) 4.45; CH <sub>2</sub> (δ) 4.24, 3.62
Asn <sup>7</sup>	8.44	4.54	2.80, 2.80	NH(δ) 7.63, 6.95
Ala <sup>8</sup>	8.07	4.29	1.40	
Asn <sup>9</sup>	7.95	5.009	2.92, 2.74	NH(δ) 7.71, 7.08
Pro <sup>10</sup>	-	4.40	2.33, 1.96	CH <sub>2</sub> (γ) 2.04 – 1.98; CH <sub>2</sub> (δ) 3.81, 3.81
Asn <sup>11</sup>	8.33	4.68	2.88, 2.76	NH(δ) <sup>a</sup>
Ala <sup>12</sup>	7.76	4.31	1.40	
Asn <sup>13</sup>	8.42	4.92	2.87, 2.76	NH(δ) <sup>a</sup>
Pro <sup>14</sup>	-	4.43	2.31, 1.99	CH <sub>2</sub> (γ) 2.04 – 1.98; CH <sub>2</sub> (δ) 3.80, 3.80
Asn <sup>15</sup>	8.34	4.68	2.84, 2.74	NH(δ) 7.66, 6.98
Glu <sup>16</sup>	8.13	4.27	2.12, 1.99	CH <sub>2</sub> (γ) 2.34, 2.34
Asn <sup>17</sup>	8.47	4.97	2.88, 2.74	a(δ) <sup>a</sup>
Pro <sup>18</sup>	-	4.43	2.30, 1.99	CH <sub>2</sub> (γ) 2.04 – 1.98; CH <sub>2</sub> (δ) 3.80, 3.80
Asn <sup>19</sup>	8.42	4.69	2.87, 2.75	NH(δ) <sup>a</sup>
Ala <sup>20</sup>	8.04	4.25	1.41	

<sup>a</sup> Overlapping resonances. Range H(δ) (Z) 7.75-7.65 and H(δ)E 7.11-6.96

<sup>b</sup> The C(γ)-NH proton, Apro<sup>6</sup> = (2S,4S)-4-aminoproline

Table S2. Experimental distance restraints and statistics for the final 20 NMR structures calculated for UK-40.

	<b>UK-40</b>
NOE upper-distance limits	99
Intraresidue	27
Sequential	60
Medium- and long-range	12
Residual target function value ( $\text{\AA}^2$ )	$0.16 \pm 0.05$
Mean rmsd values ( $\text{\AA}$ )	
All backbone atoms	$3.74 \pm 1.02$
Backbone atoms of residue 6-16	$1.59 \pm 0.54$
Residual NOE violation	
Number $> 0.2 \text{ \AA}$	13
Maximum ( $\text{\AA}$ )	0.34



**Chapter 4: Preclinical Profiling of the Immunogenicity of a  
Two-Component Subunit Malaria Vaccine Candidate  
Based on Virosome Technology**

Ready to submit to Vaccine

**Preclinical profiling of the immunogenicity of a two-component subunit malaria vaccine candidate based on virosome technology**

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Running title: Preclinical profiling of a virosomal malaria vaccine candidate

Key words: malaria vaccine, virosomes, synthetic peptides, apical membrane antigen 1, circumsporozoite protein, preclinical profiling

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### **ABSTRACT**

Presentation of synthetic peptides on immunopotentiating reconstituted influenza virosomes is a promising technology for subunit vaccine development. An optimized virosomally delivered peptide representing 4 NPNA repeats of *P. falciparum* CSP is highly immunogenic in mice. Antibodies against this peptide, designated UK-39, inhibit sporozoite invasion of primary human hepatocytes. A second peptide, AMA49-CPE, is based on domain III of apical membrane antigen 1 and induces high titers of parasite-binding antibodies in mice. Monoclonal antibodies against AMA49-CPE inhibit blood-stage parasite growth *in vitro*. The present study shows a detailed pre-clinical profiling of these two virosomally-formulated peptides alone and in combination in mice and rabbits. Two immunizations with virosomally formulated UK-39 or AMA49-CPE were enough to elicit high titers of parasite cross-reactive antibodies in mice and rabbits. No dose-dependency was observed for UK-39 in rabbits, where a low dose of 10µg was enough to induce maximal titers. AMA49-CPE induced higher titers at 25 and 50µg peptide. Combination of UK-39 and AM49CPE did not have any negative effect on anti-peptide antibody titers elicited in mice nor rabbits. No MHC restriction was observed in the development of humoral responses in outbred rabbits with different immunogenetic backgrounds. Taken together these results show that low amounts of synthetic peptides delivered on IRIVs induce high antibody titers. Different peptides on separate virosomes can be combined without interfering with individual anti-peptide responses, augmenting the value of the IRIV system for the development of a multi-component subunit malaria vaccine.

### Introduction

With emerging resistance of parasites against drugs and mosquitoes against insecticides the development of a malaria vaccine remains an urgent need. Despite worldwide efforts no successful vaccine has come to market to date. It has been shown more than 30 years ago that sterile protection in humans can be achieved by immunization with x-irradiated sporozoites (Clyde et al., 1973). The mechanisms of this protection are still not fully understood but include humoral as well as cellular components of the immune system (Schofield et al., 1987; Doolan and Martinez-Alier, 2006; Hafalla et al., 2006). Circumsporozoite protein (CSP) is the major surface protein on sporozoites and one of the best studied antigens of *P. falciparum* (Kappe et al., 2004). Anti-sporozoite antibodies are mainly directed against the immunodominant central NANP-repeat region (Zavala et al., 1983; Dame et al., 1984). Different vaccine candidates are based on this protein and have shown partial or total protection from disease in artificial challenge experiments (reviewed in (Targett, 2005; Hill, 2006). A recent field trial with the recombinant CSP vaccine RTS,S/AS02A in children in Mozambique has shown promising results in terms of protection from severe disease (Alonso et al., 2004; Alonso et al., 2005). One of the leading vaccine candidates in blood-stage parasites is apical membrane antigen 1 (AMA-1), an 83-kDa protein. During the course of merozoite release, an additional N-terminally processed 66-kDa form of AMA-1 appears on the merozoite surface (Peterson et al., 1989; Narum and Thomas, 1994). Several passive and active immunization studies have indicated that AMA-1 is involved in eliciting protective immune responses (Deans et al., 1982; Deans et al., 1988; Collins et al., 1994; Crewther et al., 1996; Anders et al., 1998; Kocken et al., 2000; Narum et al., 2000; Xu et al., 2000; Burns et al., 2004) and serves as a target for invasion-blocking antibodies (Deans et al., 1982; Deans et al., 1988; Kocken et al., 1998; Kocken et al., 2000; Hodder et al., 2001). Loop 1 of domain III binds to the erythrocyte membrane protein Kx, and invasion of Kx<sub>null</sub> erythrocytes is reduced, indicating a significant role of AMA-1 and Kx in parasite invasion of erythrocytes (Kato et al., 2005).

We have previously shown the development of two synthetic peptides based on these two proteins for inclusion into a multivalent subunit malaria vaccine (Bisang et al., 1998; Moreno et al., 2001; Mueller et al., 2003; Pfeiffer et al., 2003) (Okitsu et al. submitted). Peptide UK-39 is based on the NANP repeats of CSP (Okitsu et al. submitted) and peptide AMA49-CPE on the erythrocyte-binding loop 1 of domain III

from AMA-1 (Mueller et al., 2003). Both peptides, designated UK-39 (CSP) and AMA49-CPE (AMA-1), were developed in a stepwise optimization process and the optimized peptides were able to induce high antibody titers in mice. Purified IgG from UK-39-immunized rabbits and anti-AMA-1 monoclonal antibodies (mAbs) inhibited *in vitro* growth of sporozoites and blood-stage parasites respectively. Both peptides were delivered on immunopotentiating reconstituted influenza virosomes (IRIVs) for animal immunizations. Virosomes are immunogenic liposomes incorporating the influenza virus derived surface proteins neuraminidase and hemagglutinin, facilitating antigen uptake and presentation by antigen presenting cells. Peptides from other sources, such as *P. falciparum* antigens, can be incorporated by adding phosphatidylethanolamine (PE) tails, anchoring the peptide in the phospholipid bilayer. Antigens presented on the IRIV surface induce an MHC II restricted antibody-oriented immune response, whereas incorporation of antigens into the virosome, leads to induction of a MHC I restricted CD8 T cell responses. Experience with two licensed vaccines based on virosomes has shown that the technology is very safe and immunogenic (Westerfeld and Zurbriggen, 2005).

In this report we show a detailed dose-finding analysis of UK-39 and AMA49-CPE alone and in combination in mice and rabbits. UK-39 and AMA49-CPE both induced high titers of peptide-specific IgG in mice and rabbits irrespective of MHC restriction. Both peptides induced parasite cross-reactive antibodies alone and in combination and at all doses tested. Depending on the animal species one or two immunizations were enough to induce maximal IgG titers.

## Results

### Development of anti-peptide IgG responses in mice.

Mice were immunized three times with 10 $\mu$ g of virosomally formulated AMA49-CPE or UK-39 alone and in combination. Blood samples were taken pre-immune, 2 weeks after the first and second, and three weeks after the third immunization. High anti-peptide IgG titers could be detected after the second immunization with both vaccine components (Figure 1). After two immunizations titers were slightly lower in animals vaccinated with a combination of the two virosomally formulated peptides, but were boosted by the third immunization to levels comparable to titers in the single component immunization groups. No titer increase after the third vaccination was observed in the single immunization groups.

### Induction of parasite-binding antibodies in mice.

Sera from UK-39 immunized mice were tested for cross-reactivity with *P. falciparum* salivary gland sporozoites in IFA. One immunization was enough to induce parasite-binding antibodies in 19 out of 20 animals from the UK-39 single and double immunization group (Figure 2A). After the second immunization all UK-39 immunized animals had high parasite-binding IgG titers. Maximal titers were reached after three vaccinations for both groups, though the increase after the third vaccination in the single immunization group was small and statistically not significant ( $P=0.2500$ ). Parasite-binding properties of anti-AMA49-CPE IgG were tested in IFA with *in vitro* cultivated *P. falciparum* blood-stage parasites. 5 out of 20 animals developed a parasite cross-reactive IgG response already after the first immunization (Figure 2B). This number gradually increased to 16 out of 20 after the third immunization. The highest anti-peptide IgG titers were found for both peptides in the group receiving a combination of virosomally formulated UK-39 and AMA49-CPE.

After three immunizations with UK-39 all animals had IgG recognizing a double band typical for CSP in Western blot with lysate from *P. falciparum* salivary gland sporozoites. IgG endpoint titers were above 100 in Western blotting experiments (data not shown).

### Development of anti-peptide IgG responses in rabbits.

Rabbits were immunized with virosomally formulated UK-39 or AMA49-CPE at three different doses (10, 25 and 50 $\mu$ g peptide-PE conjugate) alone and in combination.

Already after one immunization all rabbits receiving UK-39 developed an anti-UK-39 IgG response. Maximal anti-UK-39 IgG titers were reached already after one immunization in all groups and no significant increase in titers was observed after subsequent immunizations. Vaccine formulation, e.g. dose and combination of peptides, had no marked effect on anti-UK-39 IgG titer development. Also all animals immunized with AMA49-CPE alone or in combination with UK-39 developed an anti-AMA49-CPE IgG response already after the first immunization regardless of the dose of peptide. A booster effect after the second immunization was observed in the low dose AMA49-CPE single and double immunization groups. A booster effect after the third immunization was not observed in any of the AMA49-CPE immunized animals. The highest anti-AMA49-CPE titers were measured after one vaccination with 50µg of AMA49-CPE in combination with UK-39. No peptide-specific IgG responses were found in control animals immunized with IRIVs alone.

### **Rabbit anti-UK-39 IgG cross-reactivity with sporozoites.**

Sera from all UK-39 immunized rabbits were cross-reactive with *P. falciparum* sporozoites isolated from mosquito salivary glands (Figure 4). IFA IgG titer development was closely following the observations made in ELISA, i.e. one immunization was enough to induce high parasite cross-reactive IgG titers, regardless of vaccine formulation. Booster immunizations did not lead to an increase in parasite-binding IgG. No induction of sporozoite cross-reactive antibodies was seen in rabbits immunized with AMA49-CPE alone (data not shown) or empty IRIVs (Figure 4). One animal in the IRIV control group had a titer of 100 after the first immunization. No boost of this response was seen after subsequent immunizations. Parasite-binding properties of anti-AMA49-CPE responses could not be assessed due to high unspecific cross-reactivity of rabbit sera with blood-stage parasites masking possible AMA-1 specific antibody stainings.

All sera from rabbits immunized with UK-39 recognized a characteristic double band corresponding to *P. falciparum* CSP in Western blot after two immunizations (Figure 5). All animals had Western blot titers above 500 and no dose-dependency or interference by combined delivery with AMA49-CPE was observed. No staining of CSP was seen with pre-immune sera and sera from animals immunized with AMA49-CPE alone or empty IRIVs (data not shown).

17 out of 18 AMA49-CPE immunized rabbits recognized an AMA-1 specific double band in Western blot after the third immunization (Figure 6). No dose-dependency was observed and combination with UK-39 did not interfere with the development of an AMA-1 cross-reactive response. Pre-immune sera and control sera from animals immunized with UK-39-IRIVs or IRIVs alone (data not shown) showed unspecific background staining but no reactivity with parasite derived AMA-1.

### Discussion

The immunogenicity of peptide vaccines is a matter of debate since many years. The main problems are poor immunogenicity and the lack of cross-reactivity of elicited antibodies with native target antigens. We circumvent these problem by using IRIVs, a safe and highly immunostimulatory adjuvant/delivery system, and by optimizing synthetic peptide antigens based on their potential to elicit parasite cross-reactive antibodies (Polti-Frank et al., 1999; Moreno et al., 2001; Mueller et al., 2003; Pfeiffer et al., 2003) (Okitsu et al. submitted). The goal of this study in mice and rabbits was to analyze the dependence of the immune response elicited by different doses of virosomally formulated UK-39 and AMA49-CPE. Additionally the influence of combined delivery of the two candidate antigens for a multivalent vaccine on immunogenicity was tested.

ELISA results showed that virosomally formulated UK-39 and AMA49-CPE are highly immunogenic in both animal species. A low dose of 10 $\mu$ g was enough to induce high titers of anti-UK-39 IgG after two immunizations in mice and after one immunization in rabbits. Higher doses of UK-39 tested in rabbits did not increase anti-UK-39 IgG titers. With AMA49-CPE a dose of 50 $\mu$ g was superior to lower doses of peptide in terms of antibody titers elicited. AMA49-CPE-specific IgG was detected after two immunizations in mice and after one immunization in rabbits. Combined delivery of separate IRIV preparations carrying the two peptides did not interfere with the immune response against one of the components. There was even a tendency for higher titers after combined delivery, which may be due to the doubling of the dose of IRIVs. The induction of high antibody titers upon two immunizations with relatively low doses of synthetic peptides demonstrates that the IRIV system is highly suitable for peptide antigen delivery in vaccine development. The possibility to combine different antigens makes it an ideal system for the development of a multivalent malaria vaccine, targeting antigens from different life-stages of *P. falciparum*.

The fact that all outbred rabbits immunized with UK-39 and AMA49-CPE mounted a strong peptide-specific immune response, including a T cell dependent immunoglobulin class-switch, shows the possibility to use the IRIV system in individuals with different immunogenetic backgrounds.

UK-39 induced IgG was cross-reactive with *P. falciparum* sporozoites in IFA and stained a band corresponding to CSP in Western blot analyses in mice and rabbits. IFA titers closely followed ELISA titers, indicating a close relationship between UK-39

and the natural conformation of the CSP repeats. This observation is supported by a comparison of NMR studies of UK-39 with the crystal structure of an NPNA repeat showing that both adopt a type-I b-turn (Ghasparian et al., 2006) (Okitsu et al. submitted). AMA49-CPE elicited IgG cross-reacting with *P. falciparum* blood-stage parasites. 95% of all AMA49-CPE immunized mice produced parasite-binding IgG, recognizing *P. falciparum* in IFA. Despite problems with high unspecific cross-reactivity of rabbit sera with blood-stage parasites in IFA we were able to show the induction of AMA-1-binding antibodies in Western blot.

Purified IgG preparations of UK-39 immunized rabbits have shown inhibitory activity in *in vitro* parasite inhibition assays (Okitsu et al. submitted). Reduced gliding motility and agglutination of sporozoites in the presence of anti-UK-39 antibodies indicate an interference with gliding motility of the parasite by disrupting the molecular machinery itself, sterical hindrance and/or cross-linking of CSP of neighboring parasites. It has been reported before that anti-CSP antibodies can immobilize sporozoites in the skin after injection by a mosquito (Vanderberg and Frevert, 2004).

The results presented in this report are confirmed by results from a phase I clinical trial in Caucasians (Genton et al. submitted), where the same vaccine proved to be safe and immunogenic. At an appropriate dose UK-39 and AMA49-CPE led to 100% seroconversion alone and in combination. The detailed immunogenicity results (Genton et al. submitted, Okitsu et al. manuscript in preparation) closely followed the animal results presented in this report, validating the mouse and rabbit model for immunogenicity studies of virosomally formulated vaccine candidates.

Taken together easy to synthesize peptides together with IRIVs, already registered for use in humans, are a very promising system for the swift development of a multivalent subunit malaria vaccine.

## Materials and Methods

**Mouse immunogenicity studies.** Peptide synthesis and vaccine formulation was done as described before (Mueller et al., 2003) (Okitsu et al. submitted). BALB/c mice were pre-immunized intramuscularly with 0.1 ml of the commercial whole virus influenza vaccine Inflexal Berna™ (Berna Biotech, Bern, Switzerland). Three weeks later they were immunized with 10µg peptide delivered on IRIVs in intervals of at least two weeks. Blood was collected before each immunization and three weeks after the final injection.

**Rabbit immunogenicity studies.** New Zealand rabbits were pre-immunized intramuscularly with 0.5 ml of the commercial whole virus influenza vaccine Inflexal Berna™ (Berna Biotech, Bern, Switzerland). Three weeks later they were immunized with peptide-loaded IRIVs (10, 25, and 50µg UK-39 or AMA49-CPE) in intervals of at least two weeks. For combined delivery of UK-39 and AMA49-CPE peptides were formulated separately and mixed before immunization. Blood was collected before each immunization and two weeks after the final injection.

**ELISA.** ELISA analyses with peptide-PE conjugates were performed essentially as described before (28). Briefly, Polysorp™ plates (Nunc, Fisher Scientific, Wohlen, Switzerland) were coated overnight at 4°C with 100 µl of a 10 µg/ml solution of UK-39 or AMA49-CPE in PBS (pH 7.2). After three washings, wells were blocked with 5% milk powder in PBS for 30 min at 37°C and washed three times again. Plates were then incubated with serial dilutions of anti-peptide mouse or rabbit sera in PBS containing 0.05% Tween-20 and 0.5% milk powder for 2 h at 37°C. After washing, plates were incubated with alkaline phosphatase-conjugated goat anti-mouse  $\gamma$ -heavy chain antibodies (Sigma, St. Louis, Mo) for 1 h at 37°C. After washing again, phosphatase substrate solution containing 1 mg/ml p-nitrophenyl phosphate (Sigma, St. Louis, Mo) was added and the plates were incubated until the colorimetric reaction had progressed sufficiently. The optical density was measured at 405 nm on a Titertek Multiscan MCC/340 reader (Labsystems, Helsinki, Finland).

For experiments with rabbit sera, horseradish peroxidase-conjugated goat anti-rabbit IgG heavy and light chain antibodies (Bio-Rad Laboratories, Hercules, CA) were used as secondary antibody. For experiments with horseradish peroxidase-

conjugated antibodies TMB microwell peroxidase substrate system (KPL, Gaithersburg, MD) was used according to the manufacturer and optical density was measured at 650 nm. After stopping the reaction by addition of 50  $\mu$ l 1N HCl per well OD was measured at 450nm.

**Indirect immunofluorescence assay (IFA) with *P. falciparum* sporozoites.** IFAs were essentially done as described before (Okitsu et al. submitted). Air-dried unfixed *P. falciparum* salivary gland sporozoites (strain NF54) attached to microscope glass slides were kindly provided by Hugues Matile (F. Hoffmann La Roche, Basel). After thawing, wells were incubated for 15 min at room temperature with 25  $\mu$ l blocking solution containing 1% fatty acid-free bovine serum albumin (BSA) in PBS. Immunostaining was performed by incubating the wells with 25  $\mu$ l of an appropriate serum dilution in blocking solution in a humid chamber for 1 h at room temperature. After five washes with blocking solution 25  $\mu$ l of 5  $\mu$ g/ml cyanine dye (Cy3)-conjugated affinity-pure F(ab')<sub>2</sub> fragment goat anti-mouse Ig  $\gamma$ -heavy chain antibodies (Jackson Immuno Research Laboratories, West Grove, Pa.), diluted in blocking solution containing 0.01 mg/ml Hoechst dye no. 33256 (Sigma, St. Louis, Mo) were added to the wells and incubated for 1 h at room temperature. Finally, wells were washed five times with PBS, mounted with mounting solution (90% [vol/vol] glycerol containing 0.1 M Tris-Cl, pH 8.0 and 2 mg/ml o-phenylenediamine) and covered with a coverslip. Antibody binding and DNA staining were assessed by fluorescence microscopy on a Leitz Dialux 20 fluorescence microscope and documented with a Leica DC200 digital camera system.

For IFAs with blood-stage parasites multiwell immunofluorescence microscopy slides (Flow Laboratories, Baar, Switzerland) were treated with 0.01% poly-L-lysine (Sigma) at room temperature for 30 min and washed five times with RPMI basal salts medium (Gibco-BRL, Basel, Switzerland). Erythrocytes from *in vitro* cultures (Matile and B.Pernis, 1990) of *P. falciparum* strain K1 with a parasitemia between 5 and 10% were washed twice in RPMI and resuspended in RPMI and 2 volumes of a solution containing 4% formaldehyde and 0.1% Triton X-100. From this cell suspension, 30  $\mu$ l was added to each well, incubated at room temperature for 30 min, and washed five times with PBS. Blocking and incubation with sera or mabs was done as described above for IFA with sporozoites.

For experiments with rabbit sera a Cy3-conjugated donkey anti-rabbit IgG heavy and light chain antibody (Jackson Immuno Research Laboratories, West Grove, PA) was used.

**SDS-PAGE and immunoblotting.** 100  $\mu$ l of *Anopheles stephensi* salivary gland lysate kindly provided by Hugues Matile (F. Hoffmann La Roche, Basel) containing about 100'000 *P. falciparum* sporozoites were thawed, diluted with an equal volume of 2x loading buffer (1.7ml, 0.5M Tris-HCl pH 6.8, 2 ml glycerol, 4.5 ml 10% SDS, 1 ml  $\beta$ -mercaptoethanol, 0.8 ml bromophenol blue (0.3% w/v)) and heated to 95°C for 10 minutes. Proteins were separated on a 10% SDS PAGE mini-gel. Separated proteins were electrophoretically transferred to a nitrocellulose filter (Protran® Nitrocellulose, BA85, Schleicher & Schuell) by semi-dry blotting. Blots were blocked with PBS containing 5% milk powder and 0.1% Tween-20 over night at 4°C. The filter was cut into strips and incubated with appropriate dilutions of immune sera in blocking buffer for 2 h at room temperature. Filter strips were then washed three times for 10 minutes in blocking buffer and incubated at room temperature for 1 h with alkaline peroxidase-conjugated goat anti-mouse  $\gamma$ -heavy chain antibodies (Sigma, St. Louis, Mo) diluted 1:30'000 in blocking buffer. After washing again, blots were finally developed using ECL™ Western blotting detection (Amersham Biosciences, Buckinghamshire, England) reagents to visualize bands. Blood stage parasite lysates were prepared essentially as described previously (Matile and B.Pernis, 1990) by saponin lysis of *P. falciparum* K1-infected erythrocytes. In brief, cultured parasites were washed once with PBS. Pelleted infected red blood cells were lysed by mixing with a large volume (adjusted to 5% hematocrit) of 0.015% (wt/vol) saponin in 150 mM NaCl and 15 mM sodium citrate (pH 7.0) and incubated on ice for 20 min. Finally, the pelleted parasites were resuspended in 3 volumes of PBS and stored at -80°C until further use. A total of 50  $\mu$ l of parasite lysate was solubilized in an equal volume of 2x loading buffer (1.7 ml of 0.5 M Tris-HCl [pH 6.8], 2 ml of glycerol, 4.5 ml of 10% sodium dodecyl sulfate, 1 ml of  $\beta$ -mercaptoethanol, 0.8 ml of bromophenol blue [0.3%, wt/vol]) and heated to 95°C for 10 min. Proteins were separated on an SDS-10% PAGE minigel and transferred to a nitrocellulose membrane as described above.

Experiments with rabbit sera were performed with horseradish peroxidase-conjugated goat anti-rabbit IgG heavy and light chain antibodies (Bio-Rad Laboratories, Hercules, CA) diluted 1:6'000 in blocking buffer.

### **Parasite culture**

*P. falciparum* strain K1 was cultured essentially as described previously (Matile and B.Pernis, 1990). The culture medium was supplemented with 0.5% AlbuMAX (Gibco, Paisley, Scotland) as a substitute for human serum (Dorn et al., 1995). Synchronization of cultures was achieved by sorbitol treatment as described before (Lambros and Vanderberg, 1979).

### **Statistical analysis**

All statistical analyses and graphs were made using GraphPad Prism version 4.03 for Windows, GraphPad Software, San Diego California USA, [www.graphpad.com](http://www.graphpad.com). Sera showing no reactivity in immunogenicity tests were assigned a titer of 1 instead of 0 to calculate geometric means. Statistical significance of boost effects in ELISA and IFA was tested using a Wilcoxon test to compare the geometric mean titers between different immunizations.

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### Figure legends

Figure 1. Development of anti-UK-39 IgG responses in mice. Mice were immunized three times with virosomally formulated UK-39 alone or in combination with AMA49-CPE. Sera were taken pre-immune (pi) and two weeks after the first, second, and three weeks after the third immunization. Shown are serial dilutions of sera in ELISA (means +/-SD of 10 mice per group and immunization).

Figure 2. Mouse anti-UK-39 IgG cross-reactivity with *P. falciparum* sporozoites. Sera of immunized mice (pre-immune and two weeks after the first, second, and three weeks after the third immunization) were tested for parasite binding in IFA with *P. falciparum* sporozoites from mosquito salivary glands. Shown are 10 animals, lines representing the geometric mean.

Figure 3. Anti-UK-39 IgG titer development in ELISA. Rabbits were immunized three times with different amounts of virosomally formulated UK-39 alone or in combination with AMA49-CPE. Blood samples were taken pre-immune and two weeks after the first, second and third immunization. Shown are serial dilutions of sera in ELISA (means +/- SD 3 rabbits per group and immunization).

Figure 4. Anti-UK-39 IgG cross-reactivity with sporozoite derived CSP. Shown are sera from three rabbits immunized with 10µg virosomally formulated UK-39. Sera were taken pre-immune and two weeks after the second immunization. Dilution for pre-immune sera is 1:100, for immune sera 1:2500, 1:5000, 1:10,000, and 1:20,000.

Table 1. Rabbit anti-UK-39 IgG cross-reactivity with *P. falciparum* sporozoites. Shown are the geometric mean IFA endpoint titers and 95% CI of three animals per group.

## Figures and Tables

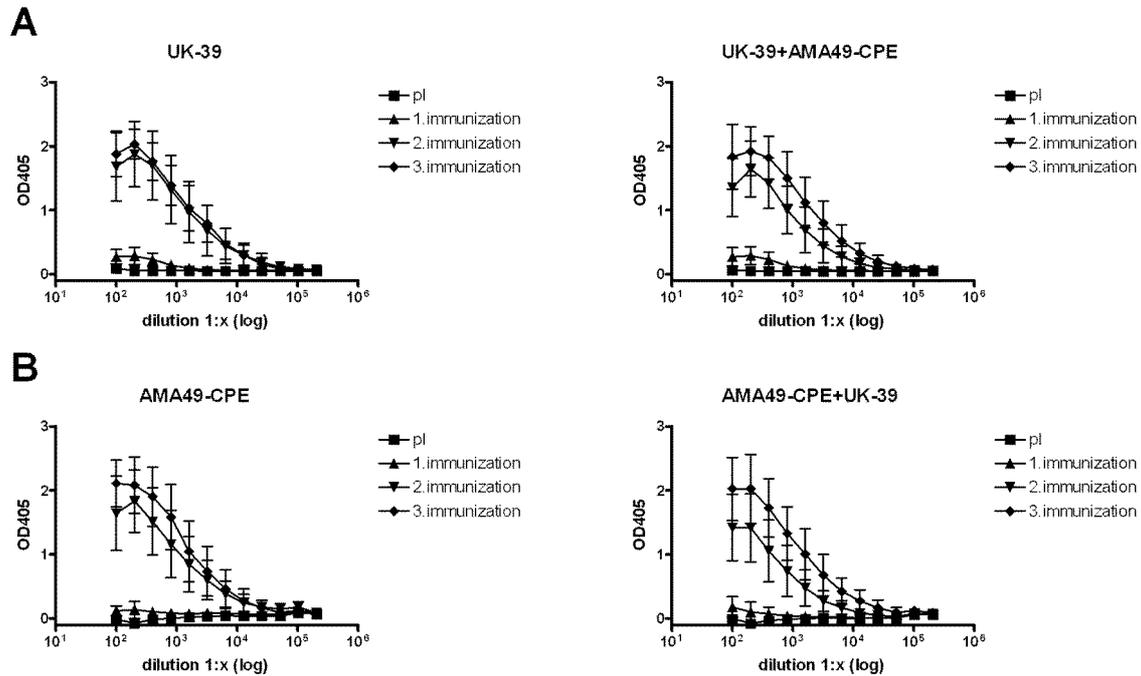


Figure 1. Development of anti-peptide IgG responses in mice. Mice were immunized three times with virosomally formulated UK-39 or AMA49-CPE alone and in combination. Sera were taken pre-immune (pi) and two weeks after the first, second, and three weeks after the third immunization. Shown are serial dilutions of sera in peptide-ELISA (means +/-SD of ten mice per group and immunization). A: Anti-UK-39 IgG response of animals immunized with UK-39 alone and in combination with AMA49-CPE. B: Anti-AMA49-CPE response of animals immunized with AMA49-CPE alone and in combination with UK-39.

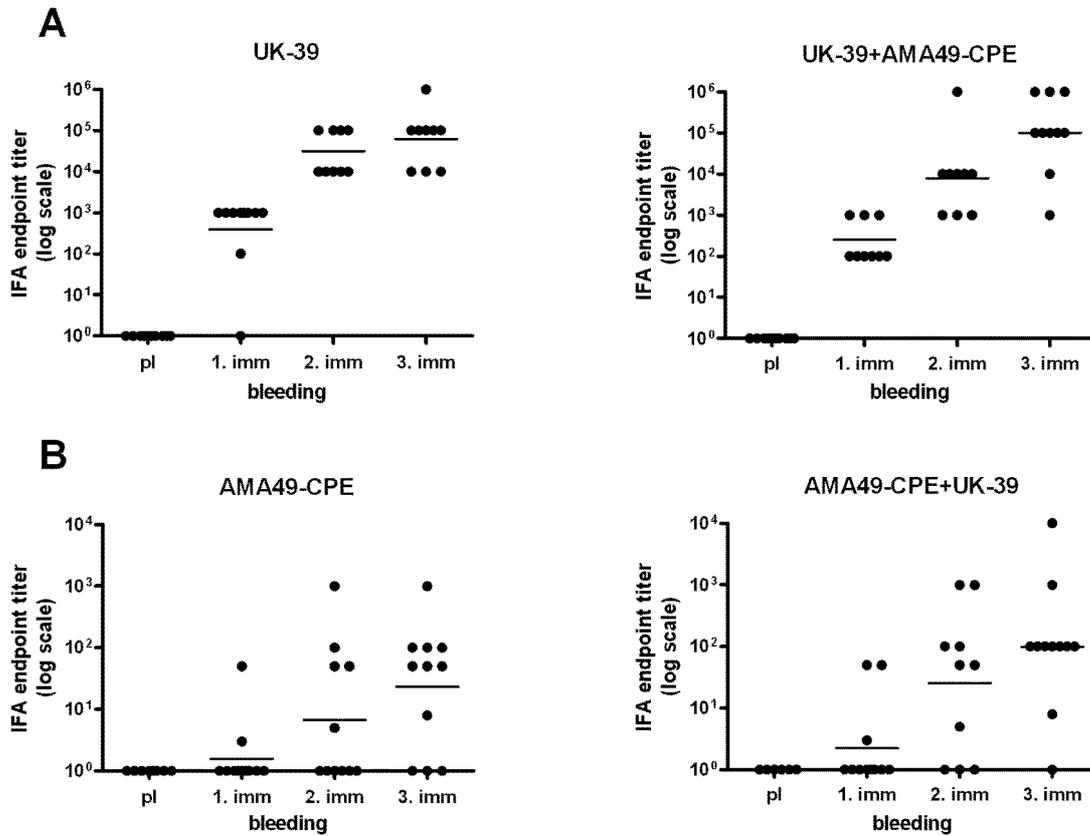
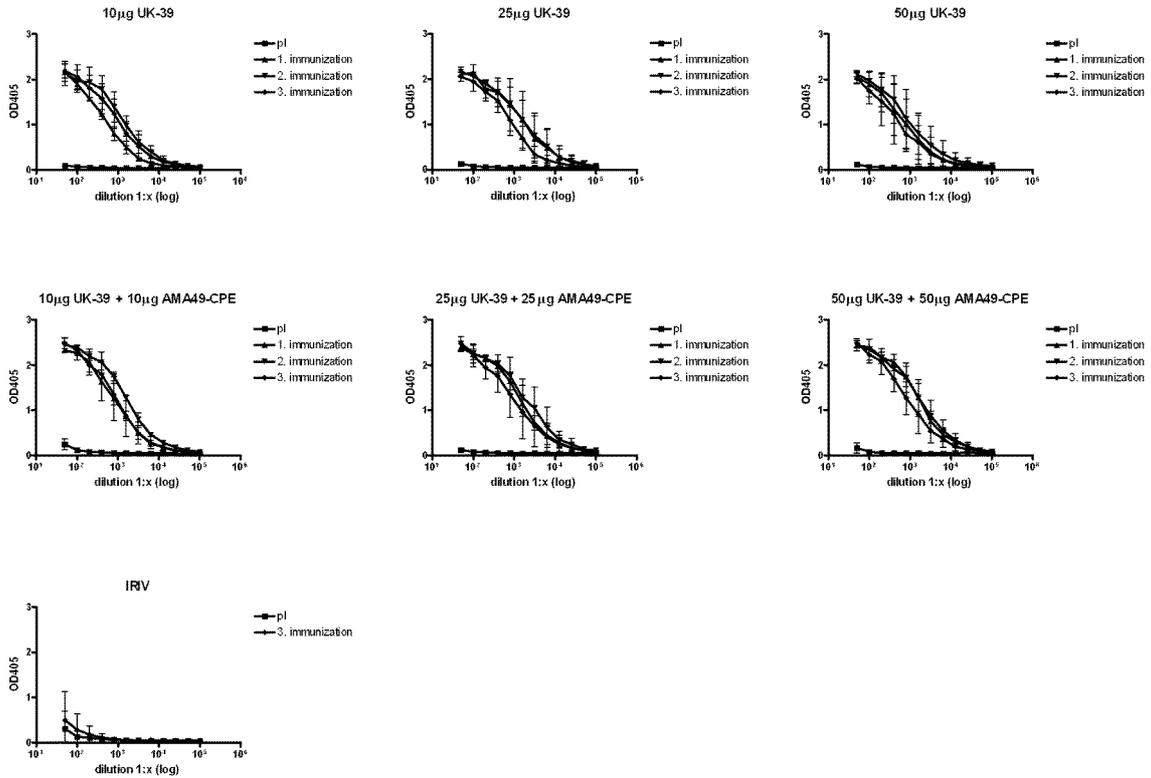


Figure 2. Induction of *P. falciparum* cross-reactive IgG after immunization of mice with UK-39 or AMA49-CPE alone and in combination. Sera of immunized mice were taken pre-immune (pl) and two weeks after the first, second, and three weeks after the third immunization. Shown are ten animals per group, lines representing the geometric mean. A: Parasite-binding properties of anti-UK-39 responses as measured in IFA with *P. falciparum* sporozoites isolated from mosquito salivary glands. B: Cross-reactivity of anti-AMA49-CPE IgG responses with cultured *P. falciparum* blood-stage parasites.

# Chapter 4: Preclinical Profiling of a Virosomal Vaccine Candidate

**A**



**B**

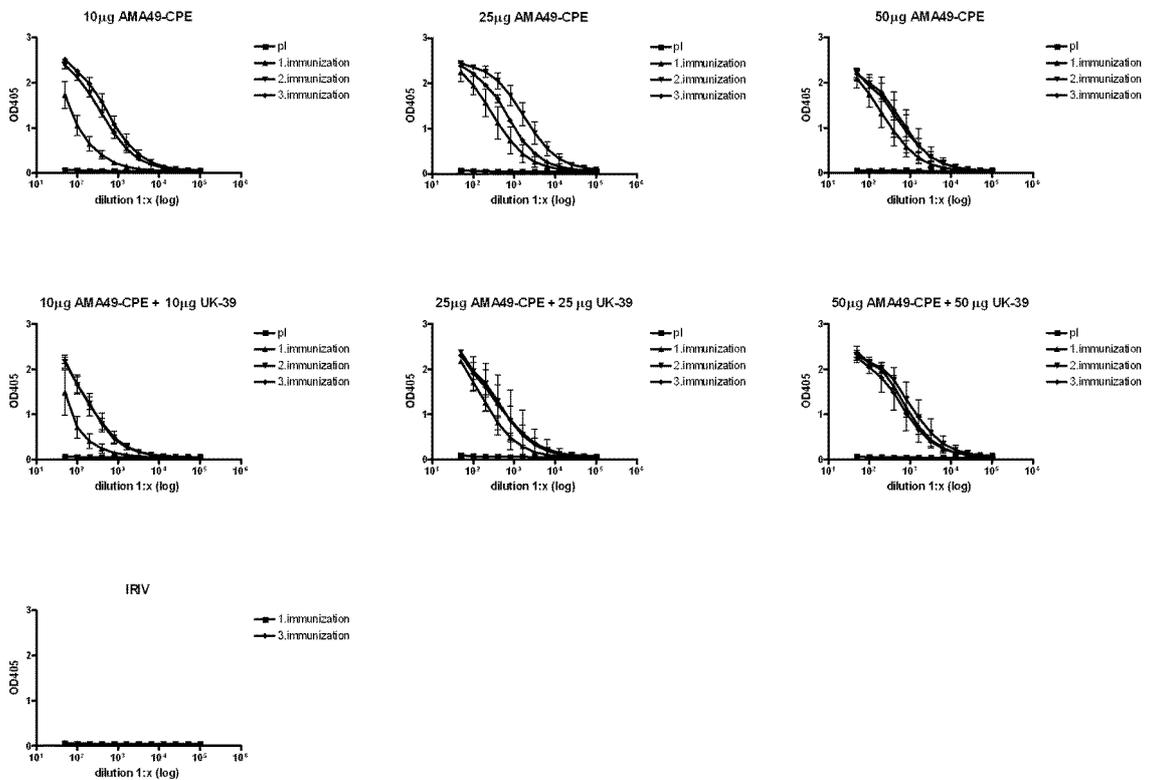


Fig 3. Anti-peptide IgG titer development in ELISA. Rabbits were immunized three times with virosomally formulated UK-39 or AMA49-CPE at three different doses of peptide (10, 25 and 50 $\mu$ g) alone and in combination. Blood samples were taken pre-immune (pI) and two weeks after the first, second and third immunization. Shown are serial dilutions of sera in ELISA (means  $\pm$  SD) of three rabbits per group and immunization). A: Development of anti-UK-39 IgG responses measured in UK-39 peptide ELISA. B: Development of anti AMA49-CPE IgG responses measured in AMA49-CPE peptide ELISA.

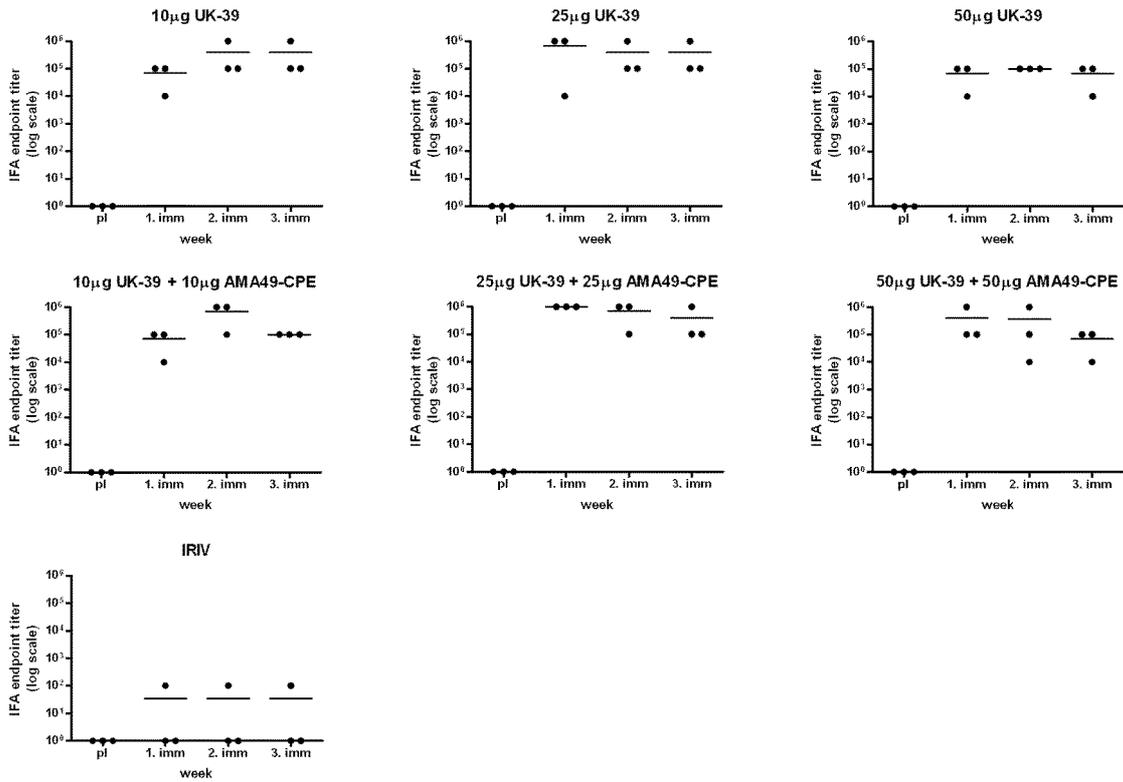


Figure 4. Cross-reactivity of anti-UK-39 responses measured in IFA with *P. falciparum* sporozoites from mosquito salivary glands. Sera of immunized rabbits were taken pre-immune (pl) and two weeks after the first, second and third immunization. Shown are three animals per group, lines represent the geometric mean.

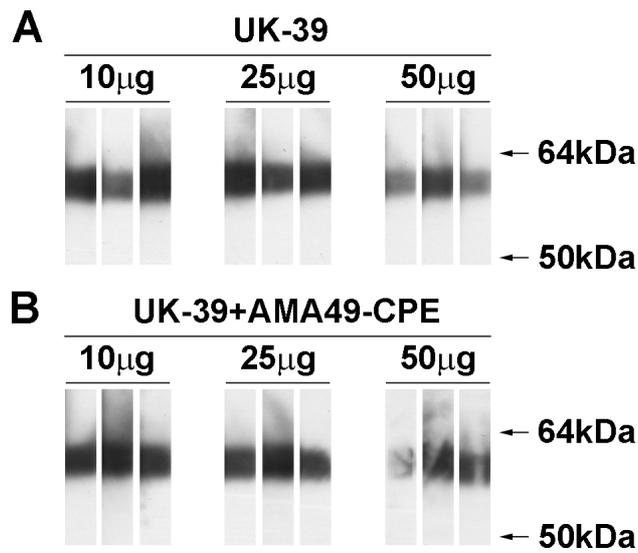


Figure 5. Anti-UK-39 IgG cross-reactivity with sporozoite derived CSP in Western blot analysis. Shown are results obtained with sera from rabbits immunized with virosomally formulated UK-39 alone (A) and in combination with AMA49-CPE (B). Sera were taken two weeks after the second immunization and used at a dilution of 1:500.

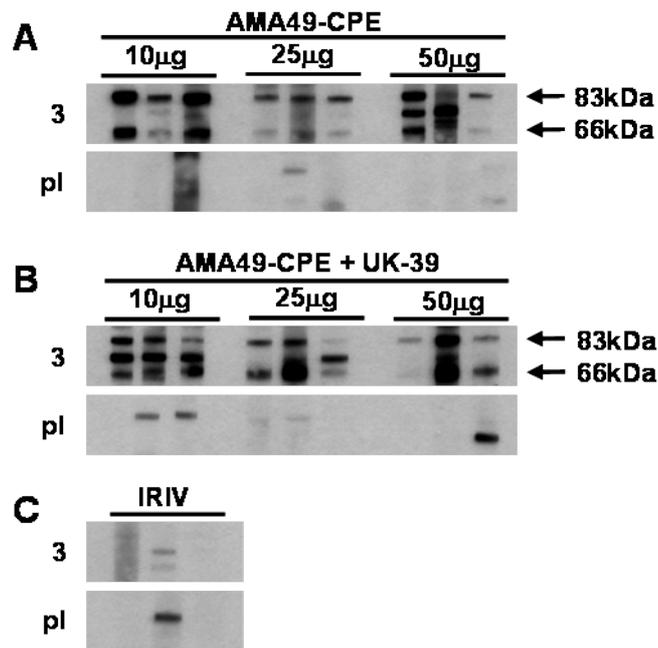


Figure 6. Western-Blot analysis of rabbit serum samples with *P. falciparum* blood stage parasite saponin lysate. Each lane represents a serum sample derived from one individual rabbit immunized either with AMA49 alone (A), a combination of AMA49 and UK-39 (B), or IRIVs alone (C). Upper rows represent serum samples taken two weeks after the third immunization (3), lower rows samples taken before the first immunization (pi). All serum samples were tested at a dilution of 1:1000. Arrows indicate full-length AMA-1 (83kDa) and its major processing product (66 kDa).



**Chapter 5: A Randomized Placebo-Controlled Phase Ia  
Malaria Vaccine Trial of Two Virosome-Formulated  
Synthetic Peptides in Healthy Adult Volunteers**

Submitted to Journal of Infectious Disease

**A randomized placebo-controlled Phase Ia malaria vaccine trial of two virosome-formulated synthetic peptides in healthy adult volunteers**

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Running head: Phase I trial of a virosome formulated synthetic peptide malaria vaccine

Key words: *malaria – vaccine – synthetic peptide – virosomes – phase I – safety – immunogenicity*

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## Abstract

### Background and objectives

Influenza virosomes represent an innovative human-compatible antigen delivery system that has already proven its suitability for subunit vaccine design. The aim of the study was to proof the concept that virosomes can also be used to elicit high titers of antibodies against synthetic peptides. The specific objective was to demonstrate the safety and immunogenicity of two virosome-formulated *P. falciparum* protein derived synthetic peptide antigens given in two different doses alone or in combination.

### Methodology/Principal findings

The design was a single blind, randomized, placebo controlled, dose-escalating study involving 46 healthy Caucasian volunteers aged 18-45 years. Five groups of 8 subjects received virosomal formulations containing 10 µg or 50 µg of AMA49-CPE, an apical membrane antigen-1 (AMA-1) derived synthetic phosphatidylethanolamine (PE)-peptide conjugate or 10 µg or 50 µg of UK-39, a circumsporozoite protein (CSP) derived synthetic PE-peptide conjugate or 50 µg of both antigens each. A control group of 6 subjects received unmodified virosomes. Virosomal formulations of the antigens (designated PEV301 and PEV302 for the AMA-1 and the CSP virosomal vaccine, respectively) or unmodified virosomes were injected i. m. on days 0, 60 and 180.

In terms of safety, no serious or severe adverse events (AEs) related to the vaccine was observed. 11/46 study participants reported 16 vaccine related local AEs. Of these 16 events, all being pain, 4 occurred after the 1<sup>st</sup>, 7 after the 2<sup>nd</sup> and 5 after the 3<sup>rd</sup> vaccination. 6 systemic AEs probably related to the study vaccine were reported after the 1<sup>st</sup> injection, 10 after the 2<sup>nd</sup> and 6 after the 3<sup>rd</sup>. Generally, no difference in the distribution of the systemic AEs between either the doses applied (10 respectively 50 µg) or the synthetic antigen vaccines (PEV301 and PEV302) used for immunization was found. In terms of immunogenicity, both PEV301 and PEV302 elicited already after two injections a synthetic peptide-specific antibody response in all volunteers immunized with the appropriate dose. In the case of PEV301 the 50 µg antigen dose was associated with a higher mean antibody titer and seroconversion

rate than the 10 µg dose. In contrast, for PEV302 mean titer and seroconversion rate were higher with the lower dose. Combined delivery of PEV301 and PEV302 did not interfere with the development of an antibody response to either of the two antigens. No relevant antibody responses against the two malaria antigens were observed in the control group receiving unmodified virosomes.

### **Conclusions**

The present study demonstrates that three immunizations with the virosomal malaria vaccine components PEV301 or/and PEV302 (containing 10 µg or 50 µg of antigen) are safe and well tolerated. At appropriate antigen doses seroconversion rates of 100% were achieved. Two injections may be sufficient for eliciting an appropriate immune response, at least in individuals with pre-existing anti-malarial immunity. These results justify further development of a final multi-stage virosomal vaccine formulation incorporating additional malaria antigens.

## Introduction

Apart from plans to develop a radiation-attenuated sporozoite vaccine [1], vaccine development against *Plasmodium falciparum* malaria is focusing largely on subunit vaccine technologies [2]. It is thought that an effective malaria subunit vaccine will have to incorporate antigens against several development stages of the parasite. A combination of effects against sporozoites, infected liver cells, merozoites and infected red blood cells may be required to achieve substantial protective activity [2]. Attempts to produce such a multi-stage subunit vaccine against malaria have so far met with limited success, indicating that new strategies both for the targeting of the immune response to suitable antigenic determinants of the parasite and for the safe and appropriate delivery of antigens are required. We are addressing these problems by developing synthetic peptide structures that elicit antibodies against the native conformation of the malaria antigens [3-5], and by displaying them as PE-peptide conjugates on the surface of immunopotentiating reconstituted influenza virosomes (IRIV) as carrier and adjuvant system [3;6].

IRIVs are spherical, unilamellar vesicles, prepared by detergent removal from a mixture of natural and synthetic phospholipids and influenza surface glycoproteins. The hemagglutinin of the influenza virus is a fusion-inducing membrane glycoprotein, which facilitates antigen delivery to immunocompetent cells. IRIVs represent a universal antigen-delivery system for multi-component subunit vaccines, since antigens can be either attached to their surface to elicit CD4 T cell and antibody responses or encapsulated in their lumen to elicit CD8 T cell responses. They have an excellent safety profile and are already registered for human use, as two virosomal vaccines, the influenza vaccine Inflexal V<sup>®</sup> and the hepatitis A vaccine Epaxal<sup>®</sup> are commercialized [7].

We have shown, that synthetic peptide vaccine antigens bypassing many of the problems associated with the production of stable recombinant protein-based formulations can be developed by iterative cycles of compound design, synthesis and immunological profiling [3-6]. Sequential rounds of optimization may lead to candidate antigens which elicit primarily antibodies that contribute to immune protection when displayed as PE-conjugates on the surface of IRIVs. Here we

describe the first clinical profiling of virosomal formulations of two optimized synthetic antigens, the CSP repeat region derived PE-peptide conjugate UK-39 and the AMA-1 derived PE-peptide conjugate AMA49-CPE [4]. CSP of *P. falciparum* is a leading sporozoite candidate antigen for inclusion in a malaria subunit vaccine. In a stepwise medicinal chemistry lead optimization process [3,8-10], we have developed the conformationally constrained synthetic compound UK-39, which has optimal structural and antigenic similarity to the immunodominant NPNA-repeat region of native CSP. PEV302, the virosomal formulation of UK-39, elicits high titers of sporozoite cross-reactive antibodies in mice and rabbits and these antibodies inhibit invasion of liver cells by *P. falciparum* sporozoites. AMA-1 of *P. falciparum* is a leading merozoite candidate antigen. Its ectodomain can be divided into three subdomains each with disulfide bond-stabilized structures. Since the majority of antibodies raised against the ectodomain appear to recognize epitopes in domain I that are strain-specific, we have developed a synthetic antigen based on the more conserved loop-I of domain III, which interacts with the erythrocyte membrane protein Kx [11]. PEV301, the virosomal formulation of the optimized loop-I derived cyclized peptide antigen AMA49-CPE, elicits high titers of blood stage parasite cross-reactive antibodies in mice and rabbits and these antibodies recognize primarily discontinuous epitopes comprising conserved sequence stretches of AMA-1 [4]. Monoclonal antibodies generated from PEV301 immunized mice have shown *P. falciparum* blood stage growth inhibitory activity *in vitro* [4].

The specific objectives of the Phase I trial were to demonstrate the safety, tolerability and immunogenicity of PEV301 and PEV302 incorporating two different amounts of PE-peptide conjugate (10 µg or 50 µg per dose) given alone or in combination (50 µg of each conjugate).

## Methods

### Overall Study Design and Plan

This prospective phase I, single blind, randomized, placebo controlled, dose-escalating study was conducted in 46 healthy adult volunteers at the Clinical Research Center, University Hospital, Basel, Switzerland. Eligible study participants were randomized into six groups: PEV301 10 µg (n = 8; group 1), PEV302 10 µg (n = 8; group 2), PEV301 50 µg (n = 8; group 3), PEV302 50 µg (n = 8; group 4), PEV301 50 µg + PEV302 50 µg (n = 8; group 5) or unmodified virosomes (IRIVs) serving as controls (n = 6; group 6). Vaccine formulations contained different amounts of PE-peptide conjugate, but throughout the same amounts of IRIV per dose.

### Subject Selection Criteria

Healthy volunteers of both sexes, aged between 18 and 45 years, with a BMI  $\geq$  18.5 and  $<$ 30 were included if they gave written informed consent. Outside usual criteria (chronic disease, pregnancy etc.), volunteers were excluded if they lived in the past in a malaria endemic area, had visited such an area in the last 12 months, or had a history of clinical malaria.

### Vaccine

Two virosome-formulated *P. falciparum* malaria vaccines PEV301 (incorporating the AMA-1 derived PE-peptide conjugate AMA49-CPE) and PEV302 (incorporating the CSP derived PE-peptide conjugate UK-39) were produced according to the rules of GMP. Tests for sterility, pyrogenicity, immunogenicity in animals, stability and chemical composition were performed on the vaccine lots used in this trial. Each dose was composed of 10 or 50 µg AMA49-CPE or UK-39, 10 µg *Influenza* hemagglutinin, 100 µg phospholipids and PBS ad 0.500 ml. Volunteers immunized with a combination of AMA49-CPE and UK-39 received a double dose of virosomes i.e. hemagglutinin and phospholipids.

During the course of the study, stability tests were performed. We identified an unusual curve profile of PEV301 analyzed by HPLC. Potency tests were not performed at this stage. This potential stability problem may have slightly impacted on the immunogenicity of this component, but has been solved since then, thanks to lyophilization of the vaccine candidate.

### **Randomization, assignments to treatment groups and trial procedures**

Sequence generation for the randomization was computer performed (SAS software). The first 18 volunteers were stratified into males and females and then randomized by two blocks of 9 (1 block = females, 1 block = males) in 3 groups: PEV301 10 µg (n= 8), PEV302 10 µg (n = 8), and IRIVs alone (control; n = 2). Five weeks later, another 18 volunteers followed the same procedure: PEV301 50 µg (n = 8), PEV302 50 µg (n = 8), and IRIVs (n = 2). Five weeks after the 2<sup>nd</sup> vaccination of previous groups, 10 volunteers were randomized in blocks of 5 to either the combination of PEV301 & PEV302 50 µg (n = 8) or IRIVs (n = 2).

Randomization concealment was done using sealed envelopes with numbers (1 to 46) corresponding to the sequence of assignment to the study. For implementation, the physician in charge of the screening and inclusion opened the envelopes sequentially, using one block for each sex.

The study was single blind (participant unaware of product applied). The trial vaccines were administered i.m. in the left arm on day 0, in the right one on day 56-65, and the left one on day 175-186.

### **Safety parameters**

Occurrence of local and systemic adverse events was assessed by the physician in charge for 30 minutes, and on days 2 (+1) and 7 (+2) after each vaccination, and by the subject him/herself on a diary card for 4 days. Intensity of local adverse events was graded as follows: for pain, 1= painful on touch, 2 painful when moved, 3 spontaneously painful; for redness and swelling, 1 = >5 mm, 2 = > 20mm, 3 = >50 mm. For all other adverse events, grading and reporting was done according to Common Terminology Criteria for Adverse Events v3.0 (CTCAE). Hematological and biochemical analysis was carried out at screening visit (baseline), 7 (+2) and 21 (+5) days after each vaccination.

### **Immunogenicity parameters**

Blood samples for antibody titer measurement by ELISA against AMA49-CPE and UK-39 were taken during the screening visit, at the days of the 2<sup>nd</sup> and 3<sup>rd</sup> vaccination, 21 days after each vaccination and one year after the third vaccination (groups 2, 3 and 5 only).

### **Laboratory methods**

ELISA polysorp microtiter plates (Nunc, Dr. Grogg, Stetten-Deiswill, Switzerland) were coated at 4°C overnight with 10 µg/ml AMA49-CPE (for PEV301) or UK-39 (for PEV302) in PBS, pH 7.2. Wells were then blocked with 5% milk powder in PBS for 2 h at 37°C followed by three washings with PBS containing 0.05% Tween-20. Plates were then incubated with two-fold serial dilutions of human serum starting with 1:50 in PBS containing 0.05% Tween-20 and 0.5% milk powder for 2 h at 37°C. After washing, the plates were incubated with horseradish-peroxidase-conjugated goat anti-human IgG antibodies (KPL, Socochim, Lausanne, Switzerland) (1:2000 in PBS containing 0.05% Tween-20) for 1 h at 37°C and then washed. Citrat-buffer containing 4 mg/ml 1,2-diaminobezene substrate (OPD; Fluka, Sigma, Buchs, Switzerland) and 0.01% H<sub>2</sub>O<sub>2</sub> was added and incubated at room temperature. After 10 minutes the reaction was stopped by addition of sulphuric acid (Merck, Darmstadt, Germany) to reach a final concentration of 0.5M. The optical density (OD) of the reaction product was recorded at 492 nm using a microplate reader (SpectraMax plus, Bucher Biotech, Basel, Switzerland). Titration curves were registered using Softmax PRO software. Endpoint titers were calculated by comparing the ELISA OD of the test serum with the ELISA OD of a negative serum pool. Endpoint titer is last serum dilution where the  $OD_{\text{test sera}} \geq 2 \times OD_{\text{negativ serum}}$ .

### **Data Quality Management**

The study was monitored by the Pharmaceutical Medicine Unit (PMU) of the Swiss Tropical Institute (STI) according to a monitoring plan.

### **Statistical Methods**

The *safety analysis population* included all subjects who received at least one injection, independently from protocol deviations. Data collected from drop-outs were used for the statistical analysis until the date of discontinuation. Safety of the injected study materials was determined as the incidence of adverse events and the occurrence of significant clinical, hematological and biochemical abnormalities during the procedure and at the intervals indicated in the schedule of assessments. The *safety analysis population* listings were made of the safety data collected at each time point.

The *Per Protocol (PP) immunogenicity analysis population* included all subjects, who received the three doses of the allocated product in the allowed intervals and timely attended all the scheduled blood sampling visits. The *Intention-to-treat (ITT) immunogenicity analysis population* included all subjects and all time points, independently from protocol deviations (which mainly consisted in a 1-2 day deviation from the allowed time interval). Data collected from drop-outs were included for *ITT* analysis until the date of discontinuation.

The criteria for the evaluation of immunogenicity of PEV301 and PEV302 were the results of antibody titers by Elisa against AMA49-CPE and UK-39. ELISA responders were defined as subjects with a minimum titer  $\geq 1: 500$  and a  $>2$  fold increase after immunization.

The geometric means and 95% confidence intervals of the antibody titers determined by ELISA (total IgG) for PEV 301 and PEV 302 in the four different groups were calculated separately for each time point and study group. The statistical significance of the vaccine component effects was tested using a Wilcoxon test to compare the median of the antibody titers between group 1 (PEV301 10  $\mu\text{g}$ ) and 3 (PEV301 50  $\mu\text{g}$ ), group 1 (PEV301 10  $\mu\text{g}$ ) and 5 (PEV301&302 50  $\mu\text{g}$ ) and group 3 (PEV301 50  $\mu\text{g}$ ) and 5 (PEV301&302 50  $\mu\text{g}$ ) at each study week assessment. Same was done for the respective groups of PEV302. The Fishers exact test was applied to compare the proportion of respondents between the groups above and at each time point with the exception weeks 9/10 and 26/27 where the titre could not be determined.

Indices of antibody response were expressed as the ratio of the antibody concentration obtained by Elisa at the 4, 12/14 and 29/30 weeks with reference the baseline measurement point. The geometric mean and 95% confidence limits were calculated and the Wilcoxon test was used to compare between groups and between the follow-up times at the 4, 12/14 and 29/30 week and the baseline.

### **Determination of Sample Size**

The sample size of this pilot study was determined by the requirement to determine safety of each formulation and dose. A sample size of 8 in each group constituted a reasonable sample size to estimate the incidence rate of frequent AEs with an acceptable accuracy, allowing for dropouts. The study was not powered to ensure that differences in safety or immunogenicity between regimens would be statistically significant.

**Ethics**

The protocol was approved by the Ethikkommission beider Basel (EKBB) and the study conducted in full compliance with the international ethical guidelines for biomedical research involving human subjects.

## RESULTS

The study was conducted from November 2003 to October 2005. 46 Caucasian subjects (half female, see study procedures) were enrolled as planned. All 46 received the 1<sup>st</sup> injection, 44 the 2<sup>nd</sup> and 43 the 3<sup>rd</sup>. Demographic and other baseline characteristics of the population enrolled in the study are listed in Table 1. Three subjects discontinued participation in the trial, all of them due to a systemic AE (one of them possibly related to the study vaccine) (see below and study flowchart on figure 1).

### Safety evaluation

No subject was excluded from the safety analysis. No serious or severe AE occurred after the 1<sup>st</sup>, 2<sup>nd</sup> or 3<sup>rd</sup> vaccination. Exclusively pain was reported as local AE, i.e. no subject experienced at any time redness and/or swelling at the injection site. In total, 16 local AEs were reported by 11 study participants (3 from PEV301 10 µg , 2 from PEV301 50 µg , 2 from PEV302 50 µg , 2 from PEV301 & 302 50 µg and 2 from IRIV) during the period of the trial. Of these 16 events, 4 occurred after the 1<sup>st</sup>, 7 after the 2<sup>nd</sup> and 5 after the 3<sup>rd</sup> vaccination. No volunteer from PEV302 10 µg suffered from local AEs. Ten events (62.5%) were of mild and 6 (37.5%) of moderate intensity. All listed local vaccine related AEs completely resolved without sequelae within maximal 3 days after injection and no intervention was required to treat any of the local AEs.

In total 69 systemic AEs (6 possibly related and 63 either unrelated, or unlikely related to the study vaccine) were reported by 31 subjects after the 1<sup>st</sup> vaccination, 44 systemic AEs (10 possibly related and 34 unrelated/unlikely related) by 22 subjects after the 2<sup>nd</sup> immunization, and 18 systemic AEs (6 possibly related and 12 unrelated/unlikely related) by 13 subjects after the 3<sup>rd</sup> injection. One serious, vaccine unrelated, adverse event was reported in the PEV302 10 µg group after the 1<sup>st</sup> immunization (hospitalisation for tooth abscess). This subject and two others voluntarily discontinued participation in the trial, the first two prior to the 2<sup>nd</sup> and the last prior to the 3<sup>rd</sup> vaccination. Only one was graded as possibly related by the investigator (dishydrosis).

The type and distribution of vaccine related systemic AEs is shown in Table 2. Generally, no difference in the distribution of the systemic AEs between either the

doses applied (10 respectively 50 µg) or the synthetic antigens (PEV301 and PEV302) was found. No possibly related systemic AE was experienced by volunteers from the IRIV group and less flu-like symptoms were reported in the IRIV group when compared to the verum groups.

Only one subject was measured with a body temperature  $\geq 38.0^{\circ}\text{C}$  ( $38.0^{\circ}\text{C}$  one day after the 3<sup>rd</sup> vaccination in the PEV301 50 µg group). Most of the AEs (related or unrelated), either observed by the physician in charge, or mentioned in the diary card were of mild (39%) or moderate (59%) intensity. Only 2% experienced severe AEs. Several laboratory values were reported to be outside the normal ranges during the trial. However, none of them was judged as clinically significant by the investigator. After pooling groups and doses, the parameters most often outside the normal ranges were: red blood cells count (45 values), ALAT (29 values), CRP (19 values), white blood cells count (15 values) and hemoglobin (14 values).

### **Immunogenicity evaluation**

The per protocol analysis population for the ELISA evaluation included 37 subjects (see study flowchart on figure 1 for details). ELISA was performed with blood samples taken during the screening visit, at the days of the 2nd and 3rd vaccination 21 days after each vaccination and one year after the third vaccination. Mean ELISA endpoint titers are given in Table 3 and the antibody titers of the optimal doses are shown in figure 2.

Indices of responses of the two PEV301 50 µg groups were significantly higher than those of the 10 µg group at all time points ( $p$  always  $< 0.004$ ), demonstrating that the dose of 50 µg AMA49-CPE was superior to that of 10 µg. While all volunteers in the 50 µg groups reached a titer  $> 1:500$  after the second and third immunization, none of the PEV301 10 µg group reached this titer (Table 3). Nevertheless, there was a significant difference between the response indices (shown in figure 3a) of PEV301 10 µg after the second and third immunization versus that of the IRIV (control group) (both  $p=0.004$ ). Differences in response indices between groups PEV301 50 µg and PEV301 & 302 50 µg were not statistically significant at weeks 4, 9/10, and 29/30 ( $p$  always  $\geq 0.1$ ), indicating that the combination with PEV302 did not interfere with the response to PEV301. These differences were borderline significant at weeks 12/14 ( $p=0.055$ ) and 26/27 (0.067). When comparing response indices after the first to the ones after the second immunization in the PEV301 50 µg groups, the difference was

borderline significant ( $p=0.09$ ) indicating that the initial response was boosted by the second administration. There was no additional benefit of having a third immunization ( $p=0.7$ ). All volunteers in the two 50  $\mu\text{g}$  groups had developed an IgG response after the second immunization, whereas 62.5% (5/8) of the volunteers in the 10  $\mu\text{g}$  group seroconverted. Importantly, antibody titers evaluated one year after the third immunization had remained high (Fig. 2 and Tab. 3).

With PEV302 the highest mean ELISA titers were observed after three injections, versus two for both PEV301 50  $\mu\text{g}$  groups (Table 3 and figure 2). In contrast to the results with PEV301, mean ELISA endpoint titers of the group receiving 10  $\mu\text{g}$  of the antigen were higher than those receiving 50  $\mu\text{g}$ . Furthermore, all volunteers in the PEV302 10  $\mu\text{g}$  group, but only 11/14 in the two PEV302 50  $\mu\text{g}$  groups had seroconverted after two injections. After the third immunization, all volunteers in the PEV302 10  $\mu\text{g}$  group, but only 11/14 in the two PEV302 50  $\mu\text{g}$  groups reached an ELISA titer  $>1:500$ . The percentage of volunteers reaching this titer increased with each immunization (Table 3). Response indices of the three groups receiving PEV302 were not significantly different (see figure 3b) ( $p$  always  $>0.2$ ), demonstrating that the combination of PEV302 with PEV301 did not interfere with the response to PEV302. However, the index of response obtained in the PEV301 & 302 50  $\mu\text{g}$  group was higher than that of PEV302 50  $\mu\text{g}$  group at all time points (although not statistically significant), suggesting that the combination with PEV301 may improve the response to PEV302. When comparing response indices after the first to the ones after the second immunization in the 10  $\mu\text{g}$  group, the difference was borderline significant ( $p=0.056$ ) indicating that the initial response was boosted by the second administration. There was no significant additional benefit of having a third immunization ( $p=0.2$ ). As for PEV301, antibody titers tested one year after the last immunization had remained high (Fig. 2 and Tab. 3).

In contrast to PEV301 and PEV302, no significant ELISA endpoint titers and response indices were observed against the malaria antigens in the control subjects receiving IRIVs only. The results after ITT analysis are perfectly superposable to those obtained after PP analysis (data not shown).

## Discussion

This is the first clinical trial that tested two new technology platforms in malaria vaccinology: synthetic peptides were optimized for eliciting parasite binding and inhibitory antibodies in iterative cycles of compound design, synthesis and immunological profiling and IRIV were tested as an immunopotentiating delivery system for PE-peptide conjugates. The results presented in this paper prove the relevance and the feasibility of the concept: i) GMP formulated material can be easily and quickly manufactured, ii) the vaccine formulation is extremely safe, and iii) the vaccine formulation is immunogenic leading to seroconversion of all volunteers after two injections with an appropriate antigen dose.

In terms of safety, the antigen delivery platform (IRIVs) used for the tested vaccines is already commercialized and has been given to ~20 million people, including children and infants [12-13]. This safety record is a major advance, since, the two malaria vaccines that have shown some efficacy in Phase IIb trials in endemic areas are formulated in adjuvants that have been given at best to only a few thousands of subjects so far [14-15]. Also, although probably safe, these new adjuvants are quite reactogenic [16-17]. As with other vaccine types, rare serious adverse events have been reported following immunizations with synthetic malaria peptides [18], but these may have occurred, because the doses of antigen were too high for the potent adjuvant used. Since the two synthetic antigens used in the present study were applied in lower doses and combined with the human approved IRIVs we expected an excellent safety profile. This was confirmed by our results that showed that the virosome-formulated PE-peptide conjugates AMA49-CPE and UK-39 as well as the combination of both are very safe. The local reactogenicity was minimal with only mild or moderate pain, and no redness or swelling. This contrasts with results obtained in Phase I trials with recombinant malaria proteins formulated with ASO2A or Montanide ISA 720, or DNA vaccines in viral vectors [28]. There was no difference in the incidence rate and intensity of AEs between the subjects given one or the other synthetic antigen, or both, and between the two doses, which proves the safety of the formulation.

In terms of immunogenicity, the ELISA results demonstrate that the formulation of synthetic peptides with virosomes elicited a response in all volunteers immunized with an appropriate dose. These results represent a proof of principle, i.e. they

demonstrate that virosomes are a suitable antigen delivery system for peptide antigens in humans and that immunogenetic restriction of the response does not represent a serious limitation for this approach. In the majority of volunteers immunized with PEV302, we observed cross-reactivity with the target proteins on the cell surface of *P. falciparum* sporozoites (data not shown). Also in the case of a minority of PEV301 immunized volunteers development of an AMA-1 specific staining pattern of malaria blood stage parasites was found in spite of variable background staining. These results demonstrate that the two synthetic antigens display the native structure of antigenic domains of the target antigens AMA-1 and CSP.

In the case of PEV301, the higher antigen dose (50 µg) was significantly superior to the lower dose (10 µg) with respect to mean antibody titers and seroconversion rate. For PEV302, the trend was reverse. These results may imply that additional antigens included in a multivalent vaccine will need a proper dose-finding assessment. In the case of PEV301 and PEV302 trends observed in animal immunogenicity studies and the clinical trial were comparable (Mueller et al. unpublished). Since the combined delivery of PEV301 and PEV302 did not interfere with the development of an immune response to either of the two components, it is hoped that the inclusion of additional antigens should not be detrimental to the overall immunogenicity. In addition, a persistent antibody titer was measured after one year. In the present trial a 100% seroconversion rate was achieved in malaria non-immune volunteers already after two injections with the appropriate dose of antigen. This is highly promising, since it is expected that the immunogenicity will be even higher in populations with a certain degree of pre-existing immunity. The present findings justify the ongoing efforts to develop a virosomal multi-valent multi-stage malaria vaccine. Our approach of developing optimized IRIV-formulated synthetic peptide vaccines should be generally applicable and amenable for other infectious and non-infectious diseases.

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## **Author contributions**

BG, GP, RZ, MT designed the study. LK was the clinical supervisor. SS was responsible for all clinical affairs of the study. GP, ARK, NW, SLO and MM performed the immunological tests. PV performed the statistical analysis. BG, GP and RZ wrote the paper. All authors revised the manuscript and agreed with the content.

## **Competing interests**

B Genton received a travel grant from Berna Biotech to attend an international meeting (unrelated to the present clinical trial). G Pluschke received research and travel grants from Pevion Biotech. A Kammer, N Westerfeld and R Zurbriggen are employees of Pevion Biotech.

**Figure legends:**

Figure 1: Study flowchart

Figure 2: Geometric mean anti-AMA49-CPE and anti-UK-39 IgG endpoint titers in ELISA of groups immunized with optimal doses of PEV301 (50µg) or PEV302 (10µg), or with 50µg each of both antigens. Sera analyzed were collected during the screening visit, at the days of the 2<sup>nd</sup> and 3<sup>rd</sup> vaccination, 21 days after each vaccination and one year after the third vaccination

Figure 3: Geometric means of the ratios of geometric mean antibody endpoint titers (log scale) for samples taken three weeks after each immunization with reference to pre-vaccination titers in sera taken at the screening visit (week -1).

a) anti-AMA49-CPE IgG titers (Group 1= PEV301 10 µg; Group 3= PEV301 50 µg; Group 5= PEV301&302 50 µg, Group 6=IRIV)

b) anti-UK-39 IgG titers (Group 2= PEV302 10 µg; Group 4= PEV302 50 µg; Group 5= PEV301&302 50 µg, Group 6=IRIV)

Figure 1: Study flowchart

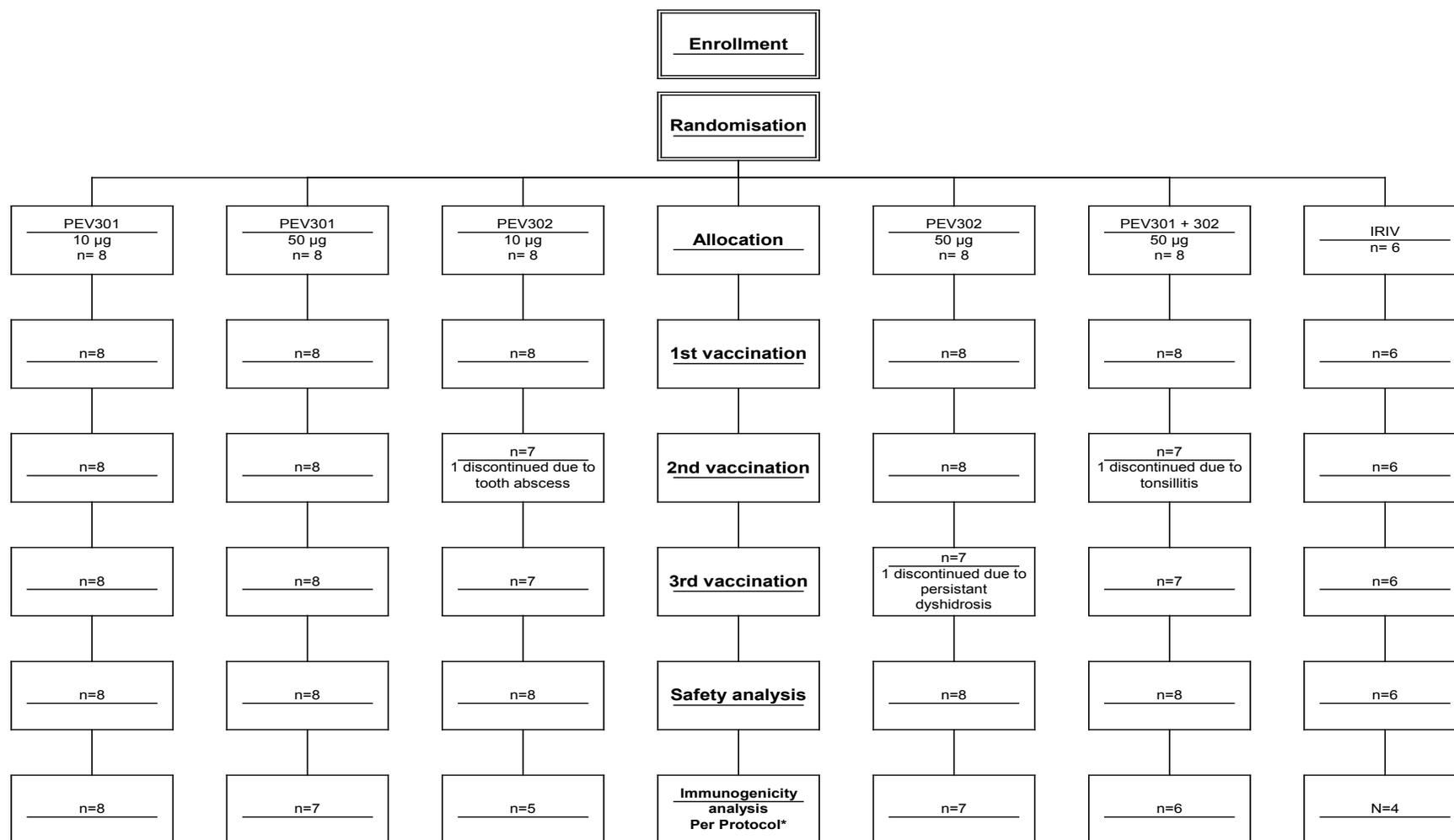


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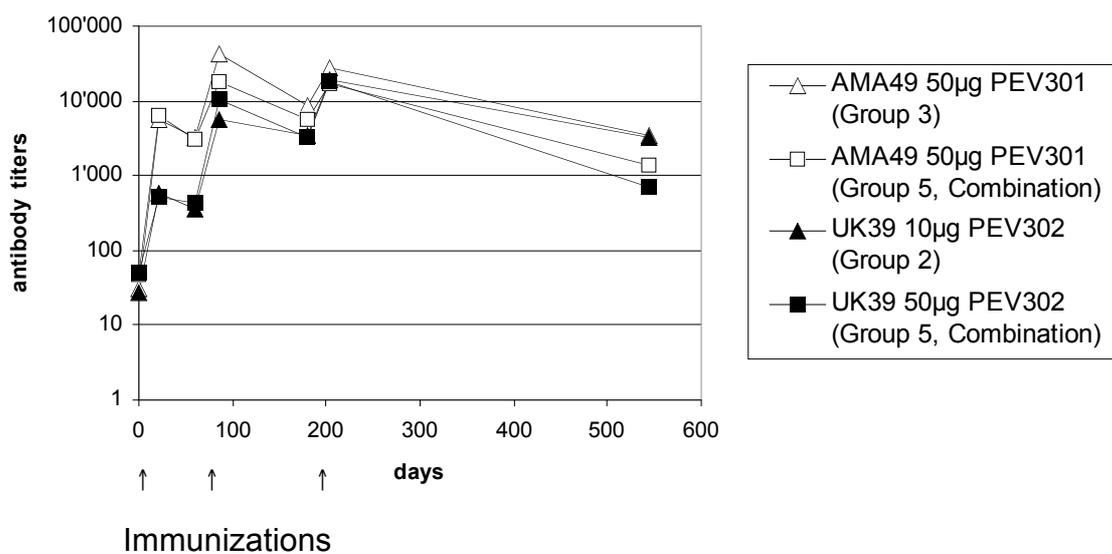
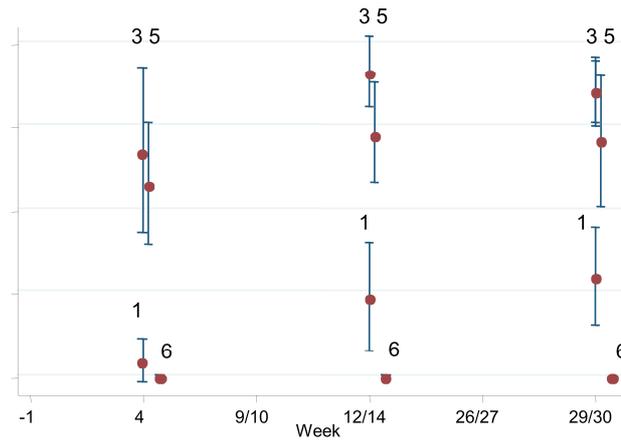


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b) anti-UK-39 IgG titres (Group 2= PEV302 10 µg; Group 4= PEV302 50 µg;  
Group 5= PEV301&302 50 µg, Group 6=IRIV)

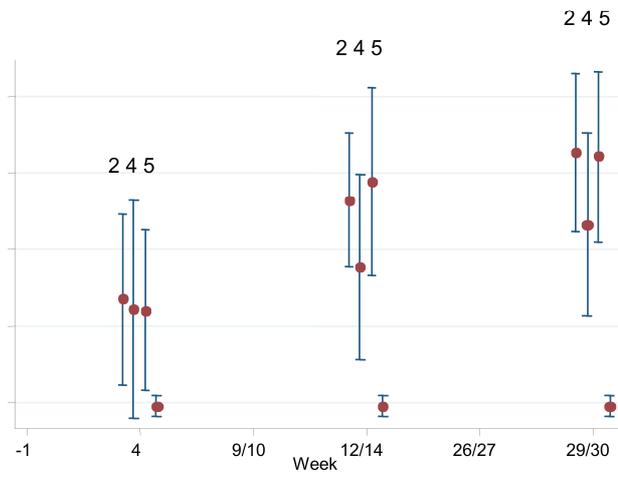


Table 1: Demographic characteristics at baseline.

	PEV301		PEV302		PEV301 + 302	IRIV
	10 µg N= 8	50 µg N= 8	10 µg N= 8	50 µg N= 8	50 µg of each N= 8	
<b>Age (years)</b>						
Mean	26	27	28	28	25	26
Min-Max	22-37	22.-40	21-42	20-40	20-30	20-42
<b>Weight (kg)</b>						
Mean	68.1	68.7	71.3	69.4	71.8	70.1
Min-Max	50.9-82.5	60.8-84.0	56.5-106.3	49.7-96.4	55.0-86.5	57.0-80.6
<b>BMI</b>						
Mean	22.6	22.9	23	22	23.8	23.7
Min-Max	19.6-25.5	20.4-28.7	19.3-29.8	19.2-26.2	20.4-29.8	20.0-27.4

Table 2: Vaccine-related systemic adverse events

Vaccine	PEV301				PEV302				PEV301 & 302		IRIV	
	10 µg		50 µg		10 µg		50 µg		50 µg each		n = 6	
Study group	n = 8		n = 8		n = 8		n = 8		n = 8		n = 6	
<b>After 1<sup>st</sup> immunization</b>												
Headache	–		<b>1</b>	12.5%	<b>2</b>	25.0%	–		–		–	
Nasopharyngitis	<b>1</b>	12.5%	–		–		–		–		–	
Rhinitis	–		<b>1</b>	12.5%	–		–		–		–	
Dyshidrosis	–		–		–		<b>1</b>	12.5%	–		–	
<b>Number of subjects with at least one AE</b>	1/8	12.5%	2/8	25.0%	2/8	25.0%	1/8	12.5%	0/8	0%	0/6	0%
<b>After 2<sup>nd</sup> immunization</b>												
Headache	–		<b>1</b>	12.5%	–		<b>1</b>	12.5%	–		–	
Pharyngolaryngeal pain	–		<b>2</b>	25.0%	–		–		–		–	
Ear pain	–		<b>1</b>	12.5%	–		–		–		–	
Rhinitis	–		<b>1</b>	12.5%	–		–		–		–	
Nausea	–		–		–		<b>1</b>	12.5%	–		–	
Vertigo	–		–		–		<b>1</b>	12.5%	–		–	
Fatigue	–		–		–		<b>1</b>	12.5%	–		–	
Malaise	–		–		–		<b>1</b>	12.5%	–		–	
<b>Number of subjects with at least one AE</b>	0/8	0%	2/8	25.0%	0/7	0%	2/8	25.0%	0/7	0%	0/6	0%
<b>After 3<sup>rd</sup> immunization</b>												
Pyrexia	–		<b>1</b>	14.3%	–		–		–		–	
Rhinitis	–		–		<b>1</b>	14.3%	–		–		–	
Asthenia	–		–		–		<b>1</b>	14.3%	–		–	
Vertigo	–		–		–		<b>1</b>	14.3%	–		–	
Vaginal infection	–		–		–		–		<b>1</b>	14.3%	–	
Headache	–		–		<b>1</b>	14.3%	–		–		–	
<b>Number of subjects with at least one AE</b>	0/8	0.0%	1/8	12.5%	2/7	28.6%	1/7	14.3%	1/7	14.3%	0/6	0.0%

Note: the values in **bold** indicate the number of AEs experienced.

Table 3. Geometric means of anti-AMA49 and anti-UK-39 IgG endpoint titers in ELISA and number of volunteers that seroconverted

Vaccine Dose Study group	PEV301		PEV302		PEV301 & PEV302		IRIV	
	10 µg Group 1	50 µg Group 3	10 µg Group 2	50 µg Group 4	50 µg Group 5		Group 6*	
<b>Target antigen in ELISA</b>	AMA49	AMA49	UK-39	UK-39	AMA49	UK-39	AMA49	UK-39
<b>Titers after 1st injection</b>	77	6893	425	488	5593	392	25	77
95% CI	21-289	2057-28893	40-4559	10-23019	1654-18911	24-6391	25-25	
<b>Titers after 2nd injection</b>	360	45614	5658	1449	18813	11404	25	77
95% CI	88-1479	20170-103156	1064-30083	61-34615	6849-51676	963-135025	25-25	
<b>Titers after 3rd injection</b>	582	29117	19993	4307	16600	21945	25	77
95% CI	248-1367	11850-71541	2734-146208	243-76258	6391-43112	2892-166548	25-25	
<b>Titers 1 year after 3rd</b>	n.d.	3342	3200	n.d.	1213	696	n.d.	n.d.
<b>Nb. responders after 1st</b>	2/8	6/8	4/7	3/7	7/7	3/7	0/6	1/6
<b>Nb. responders after 2nd</b>	5/8	8/8	7/7	5/7	7/7	6/7	0/6	1/6
<b>Nb. responders after 3rd</b>	5/8	8/8	7/7	6/7	7/7	7/7	0/6	1/6
<b>Nb. responders 1 year after 3rd</b>	n.d.	7/8	5/6	n.d.	5/5	3/5	n.d.	n.d.

\* Controls included simultaneously to Group 1 and 3 (301) or 2 and 4 (302)  
n.d.: not done



**Chapter 6: A Virosomally Formulated Malaria Peptide  
Candidate Vaccine Elicits a Long-Lasting *Plasmodium  
falciparum* Sporozoite-Inhibitory Antibody Response in  
Human Volunteers**

Submitted to Journal of Infectious Disease

**A virosomally formulated malaria peptide candidate vaccine elicits a long-lasting *Plasmodium falciparum* sporozoite-inhibitory antibody response in human volunteers**

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**Footnote page**

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## **Abstract**

### **Background**

The development of a malaria vaccine is an urgent need in tropical countries. Here we report a detailed immunological and functional analysis of the antibody response elicited by a virosomal malaria vaccine candidate.

### **Methods**

46 Healthy malaria-naïve adults were immunized with virosomal formulations of two peptides, derived from the NANP repeat region of *P. falciparum* circumsporozoite protein (designated UK-39), and from *P. falciparum* apical membrane antigen 1 (designated AMA49-CPE). The two peptides were delivered in two different concentrations alone and in combination.

### **Results**

Three vaccinations with a 10µg dose of UK-39 induced high titers of sporozoite-binding antibodies in all volunteers. This IgG response was affinity matured and long-lived. Co-administration of UK-39 and AMA49-CPE loaded virosomes did not interfere with the immunogenicity of UK-39. Purified total IgG from UK-39 immunized volunteers inhibited sporozoite migration and invasion of hepatocytes in vitro. Sporozoite inhibition closely correlated with titers measured in immunogenicity assays.

### **Conclusions**

Virosomal delivery of a short, conformationally constrained peptide induced a long-lived parasite-inhibitory antibody response in humans. Combination with a second virosomally-formulated peptide did not interfere with the immunogenicity of either peptide, demonstrating the suitability of the IRIV system for the development of multivalent subunit vaccines.

**Key words:**

Malaria, vaccine, virosomes, synthetic peptide, circumsporozoite protein, antibody affinity maturation, invasion inhibition

## Introduction

With more than 2 million deaths per year, malaria remains one of the most important infectious diseases in humans [1]. More than 30 years after the first successful protective vaccination of man with attenuated sporozoites, vaccine development against both *Plasmodium falciparum* and *P. vivax* is still ongoing [2, 3]. The most advanced experimental vaccine, RTS,S/AS02A gave 35% protection against the first episode of malaria and 49% protection against severe malaria for at least 18 months in a clinical trial in Mozambican children [4, 5]. Despite this success it is assumed that a malaria vaccine that is more effective and more cost effective than current malaria control tools, such as insecticide treated bed nets and drug treatment, will not be available in the next ten years [6-8]. It is thought that a successful malaria subunit vaccine will have to incorporate antigens against several developmental stages of the parasite. A combination of activities against sporozoites, infected liver cells, merozoites and infected red blood cells may be required to achieve substantial immune protection [9]. Vaccine development against malaria is focusing largely on subunit technologies [9], where the major obstacles include difficulties to retain the native conformation of key antibody epitopes and the need for an effective but safe human-compatible exogenous adjuvant [10]. A main advantage of the subunit approach is that the ideal vaccine will induce immune responses against only those determinants relevant to protection, thus minimizing the possibility of deleterious responses.

We are addressing the problem of protein subunit vaccine design by developing synthetic peptide structures and coupling them to the surface of immunopotentiating reconstituted influenza virosomes (IRIV) as a liposomal carrier system [11-14] (Okitsu et al. submitted). IRIVs are spherical, unilamellar vesicles, prepared by detergent removal from a mixture of natural and synthetic phospholipids and influenza surface glycoproteins. Hemagglutinin, a membrane glycoprotein of the influenza virus mediates binding to sialic acid on target cells and is a fusion-inducing component, facilitating antigen delivery to immunocompetent cells. IRIVs represent a universal antigen-delivery system for multivalent subunit vaccines, since antigens can be either attached to their surface to elicit antibody responses or encapsulated in their lumen to elicit CD8 T cell responses. They have an excellent safety profile and are already registered for human use [15].

We are optimizing synthetic peptides in an iterative selection process to develop vaccine components with a native-like conformation that elicit high titers of parasite cross-reactive antibodies [11-14, 16, 17] (Okitsu et al. submitted). Peptides are synthesized from antigens that (i) have a documented and essential role in parasite development, (ii) have secondary structure motifs suggesting surface exposition, (iii) have conserved sequence stretches, and (iv) induce parasite-inhibitory antibodies. Based on these criteria we try to choose protein domains containing protection-relevant epitopes, thus avoiding the induction of deleterious immune responses as observed during infection with *P. falciparum*. Lead peptides are optimized in a step-wise process. The key readout is the parasite-binding properties of antibodies elicited in mice after immunization with the corresponding peptide coupled to the surface of IRIVs. After preclinical profiling in experimental animals, two candidate peptides, i.e. AMA49-CPE, a cyclic peptide derived from loop I of domain III of *P. falciparum* apical membrane antigen 1 (AMA-1) [13], and UK-39, a conformationally constrained cyclic peptide derived from the central repeat region of *P. falciparum* circumsporozoite protein (CSP) (Okitsu et al. submitted) have been tested in a phase I clinical trial. Viroosomal formulations of AMA49-CPE (designated PEV301) and UK-39 (designated PEV302) were both safe and elicited anti-peptide IgG in all volunteers immunized with an appropriate peptide concentration (Genton et al. submitted). While ELISA titer development for the different vaccination groups has been described previously (Genton et al. submitted), we analyze and correlate here anti-peptide ELISA titers with titers of parasite cross-reactive antibodies and with parasite-inhibitory activities at the level of individual sera. Moreover, we show affinity maturation of UK-39-specific antibodies induced by vaccination.

## **Materials and Methods**

### **Overall Study Design and Plan**

This prospective phase I, single blind, randomized, placebo controlled, dose-escalating study was conducted in 46 healthy adult volunteers. Eligible study participants were randomized into six groups: 10 µg AMA49-CPE (group A, n = 8), 10 µg UK-39 (group B, n = 8), 50 µg AMA49-CPE (group C, n = 8), 50 µg UK-39 (group D, n = 8), 50 µg AMA49-CPE + 50 µg UK-39 (group E, n = 8) or unmodified virosomes serving as controls (group F, IRIV, n = 6). Vaccine formulations contained different amounts of PE-peptide conjugate, but throughout the same amounts of influenza proteins per dose. Volunteers immunized with a combination of virosomally formulated AMA49-CPE and UK-39 thus received a double dose of influenza proteins.

### **Vaccine**

Two virosome-formulated vaccines, PEV 301 (incorporating the AMA-1 derived PE-peptide conjugate AMA49-CPE) and PEV 302 (incorporating the CSP derived PE-peptide conjugate UK-39) were produced according to the rules of GMP. Tests for sterility, pyrogenicity, immunogenicity in animals, stability and chemical composition were performed on the vaccine lots used in this trial. Each dose was composed of 10 or 50 µg AMA49-CPE or UK-39, 10 µg Influenza hemagglutinin, 100 µg phospholipids and PBS ad 0.500 ml. The trial vaccines were administered i.m. in the left arm on day 0, in the right one on day 56-65, and in the left again on day 175-186.

### **Ethics**

The protocol was approved by the Ethikkommission beider Basel (EKBB) and the study conducted in full compliance with the international ethical guidelines for biomedical research involving human subjects. Informed consent was obtained from all volunteers.

### **Immunogenicity**

Blood sampling for antibody titer measurement by ELISA, IFA and Western blot was done at screening visit (baseline and 1<sup>st</sup> vaccination), at the days of the 2<sup>nd</sup> and 3<sup>rd</sup>

vaccination, 21 days after each vaccination and one year after the third vaccination (groups B and E only).

## **ELISA**

Standard ELISAs were performed as described before [13]. Briefly, Polysorp™ microtiter plates (Nunc, Dr. Grogg, Stetten-Deiswill, Switzerland) were coated with 10 µg/ml AMA49-CPE or UK-39 in PBS. After blocking, plates were incubated with two-fold serial dilutions of human serum. Horseradish-peroxidase-conjugated goat anti-human IgG antibodies (KPL, Socochim, Lausanne Switzerland) were used as secondary antibodies and 1,2-Diaminobezene substrate (OPD) (Fluka, Sigma. Buchs, Switzerland) in citrat-buffer (4 mg/ml OPD) + 0.01% H<sub>2</sub>O<sub>2</sub> for development.

The endpoint titer is the last serum dilution where the  $OD_{\text{test sera}} \geq 2 \times OD_{\text{negativ serum pool}}$ .

## **NH<sub>4</sub>SCN elution ELISA**

Avidity ELISA analyses with peptide-PE conjugates were performed essentially as described before [18]. After coating and blocking, as described for standard ELISA, serum samples were added in triplicates at constant dilutions (approx. halfmax titer). After a wash step, plates were incubated 15min with NH<sub>4</sub>SCN diluted in 0.1M NaH<sub>2</sub>PO<sub>4</sub> buffer (pH 6) at the following molarities: 5 M, 4 M, 3 M, 2 M, 1 M, 0.5 M, 0.25 M. Control wells were incubated with 0.1M NaH<sub>2</sub>PO<sub>4</sub> buffer without NH<sub>4</sub>SCN. After washing, plates were incubated with alkaline phosphatase-conjugated affinity-pure F(ab')<sub>2</sub> fragment goat anti-human IgG (Fc-specific) antibodies (Jackson Immuno Research Laboratories, West Grove, Pa.) and developed with phosphatase substrate solution.

The avidity index corresponds to the NH<sub>4</sub>SCN concentration (M) eluting 50% of the bound antibodies.

## **Indirect immunofluorescence assay (IFA)**

Air-dried unfixed *P. falciparum* (strain NF54) salivary gland sporozoites attached to microscope glass slides were incubated with serial dilutions of sera and detected with Cy<sup>TM</sup>3-conjugated affinity-pure F(ab')<sub>2</sub> fragment goat anti-human IgG (Fc-specific)

antibodies (Jackson Immuno Research Laboratories, West Grove, Pa.) diluted in blocking solution containing 0.01 mg/ml Hoechst dye no. 33256 (Sigma, St. Louis, Mo).

### **SDS-PAGE and immunoblotting**

*Anopheles stephensi* salivary gland lysate containing about 100'000 *P. falciparum* sporozoites was separated on a 10% SDS PAGE mini-gel and transferred to a nitrocellulose filter as described before [13]. Filter strips were incubated with serial dilutions of sera. Monoclonal antibody EP9 served as a positive control [14]. Antibodies were detected with alkaline peroxidase-conjugated affinity-pure F(ab')<sub>2</sub> fragment goat anti-human IgG (Fc-specific) antibodies (Jackson Immuno Research Laboratories, West Grove, Pa.) Blots were developed using ECL™ Western blotting detection (Amersham Biosciences, Buckinghamshire, England) reagents.

### ***P. falciparum* in vitro invasion inhibition assay**

Inhibition assays were performed as described before [19]. Briefly, triplicate primary human hepatocyte cultures were inoculated with *P. falciparum* sporozoites (1 x 10<sup>5</sup>/Lab-Tek well). After 3 h at 37 °C, cultures were washed, further incubated in fresh medium for 3 days, and fixed in methanol. Quantification of exoerythrocytic forms was done by immunofluorescence. To determine the effects of anti-UK-39 antibodies on sporozoite infectivity, sporozoites were incubated with hepatocytes in the presence of affinity purified polyclonal human IgG from immunized volunteers. Inhibition was determined in comparison to PBS control.

### ***P. falciparum* migration inhibition assay.**

Migration inhibition assays, were performed as described before [20]. Briefly, HepG2 cells were incubated with sporozoites in the presence of cell-impermeant tracer fluorescein isothiocyanate (FITC)-dextran. After 2 hours at 37°C cells were washed to remove extracellular FITC-dextran and analyzed by FACS. Inhibition was determined in comparison to PBS control.

### **Statistical analyses**

All statistical analyses and graphs were made using GraphPad Prism version 4.03 for Windows, GraphPad Software, San Diego California USA. Statistical significance of

boost effects by second and third immunization in IFA and Western blot was tested using a Wilcoxon test to compare geometric mean titers. Correlation of titers in immunogenicity assays was analyzed with nonparametric (Spearman) correlation. For correlation of invasion and migration inhibition a Pearson test for Gaussian distribution was used.

## Results

### Development of IgG responses against the synthetic antigen UK-39

Already after two immunizations, all volunteers receiving the virosomal formulation of 10 µg UK-39 alone (group B) or 50 µg UK-39 in combination with 50 µg of AMA49-CPE (group E) had developed an anti-UK-39 IgG response with an endpoint titer above 400 in ELISA (Figure 1), as shown before (Genton et al. submitted). In group D receiving 50 µg of UK-39 alone, 5 out of 7 volunteers had sero-converted after two immunizations with titers above 600. In all vaccination groups, a further increase in IgG titer after the third immunization was observed only in some volunteers. One year after the third immunization titers had dropped, but all volunteers of groups B and E remained positive (endpoint titer  $>10^2$ ) in ELISA. No increase in anti-UK-39 antibody titers after vaccination was observed in volunteers of the control group F receiving empty IRIVs (Figure 1). The overall highest endpoint titer ( $>10^6$ ) was observed after the first immunization in a volunteer (35/D) from the 50 µg UK-39 group, which had a pre-immunization ELISA endpoint titer of about  $10^3$  (Figure 1).

Antibody affinity maturation is a key event during memory B cell development. Therefore we measured the avidity of anti-UK-39 IgG in elution ELISAs with chaotropic salt. The mean avidity of the vaccine-induced IgG responses gradually increased by two-fold in all groups over the course of immunization (Figure 2). Volunteer 35/D who had pre-existing UK-39 cross-reactive antibodies had high-affinity anti-UK-39 IgG already after the first immunization.

### Sporozoite cross-reactivity of anti-UK-39 IgG responses

After three immunizations all volunteers (14/14) immunized with 10µg UK-39 or 50µg UK-39 combined with 50µg AMA49-CPE and 5 of 7 volunteers immunized with 50µg UK-39 alone had developed IgG responses that were cross-reactive (endpoint titers  $\geq 10^2$ ) with *P. falciparum* sporozoites in IFA and in Western blotting (Figure 3). Mean IFA and Western blotting endpoint titers increased with each of the three immunizations. One year after the third immunization 10 out of 11 volunteers in the 10µg UK-39 and the combination group were still positive (endpoint titers  $\geq 10^2$ ). IRIVs alone (group F) did not elicit parasite cross-reactive IgG; volunteer 12/F that had exhibited a persistent anti-UK-39 ELISA titer already present before the first vaccination also had an IFA and Western blotting titer.

Statistical analysis of ELISA, IFA and Western blotting titers revealed a close correlation between all assays (Table 1). For the three vaccination groups r-values were  $>0.819$  ( $P<0.0001$ ) for the comparison of titers in ELISA and IFA,  $>0.561$  ( $P=0.0019$ ) for the comparison of titers in ELISA and Western blot, and  $>0.550$  ( $P=0.0024$ ) for the comparison of titers in IFA and Western blot.

Volunteer 35/D, who had a pre-immune ELISA titer that strongly increased by just one immunization, also developed very high IFA and Western blotting titers ( $10^5$ ) after the first immunization (Figure 4A). While this volunteer had a pre-existing Western blotting (Figure 4B) and ELISA titer of  $10^3$ , no IFA staining was observed with the pre-immune serum (Figure 4C).

### **Sporozoite inhibitory activity**

Anti-sporozoite antibodies can interfere with critical steps of sporozoite development like migration through tissue [20, 21] and invasion of hepatocytes [22]. To test the impact of anti-UK-39 antibodies on sporozoite infectivity we used two different in vitro assays measuring sporozoite migration through hepatocytes (HepG2 cell line) and sporozoite invasion of hepatocytes in the presence of purified IgG from immunized volunteers.

Migration inhibition experiments were performed as described by Mota et al. [20]. Sporozoites were incubated with HepG2 cells in the presence of cell-impermeant FITC-dextran. Wounded cells take up FITC-dextran, which is trapped in the cell cytoplasm after wound resealing and can be detected by FACS analysis. Total IgG from nine volunteers from all immunization groups representing the whole range of observed immune responses (including controls) were purified. The presence of purified total IgG (final concentration 1mg/ml) from UK-39-immunized volunteers reduced sporozoite migration in a dose dependent manner. Vaccination-induced increases in migration inhibitory activity were observed in all UK-39-immunized volunteers (with the exception of volunteer 46/E) (Figure 5A). Dose of UK-39 and combination with AMA49-CPE had no influence on the inhibitory potential of the elicited antibodies. IgG preparations from control individuals, immunized with AMA49-CPE or IRIVs alone showed no vaccine-induced inhibition of sporozoite migration (data not shown).

The impact of antibodies on sporozoite invasion can be determined in invasion inhibition assays where the development of liver stage parasites after incubation of

primary human hepatocyte cultures with *P. falciparum* sporozoites is quantified. Vaccine-induced inhibition of sporozoite invasion was seen with purified IgG (final concentration 1mg/ml) from all volunteers immunized with UK-39 (with the exception of volunteer 46/E) irrespective of dose and formulation of the vaccine (Figure 5B). IgG preparations from controls immunized with AMA49-CPE or IRIVs did not inhibit sporozoite invasion (data not shown). Boosting of a pre-existing UK-39 cross-reactive antibody response in volunteer 35/D (Figure 4) induced IgG with high migration and invasion inhibitory potential showing no difference to IgG preparations of other UK-39-immunized volunteers (Figure 5A and B).

We found a strong correlation ( $r = 0.8648$ ,  $P < 0.0001$ ) between inhibition of invasion and inhibition of migration by IgG preparations (Figure 6). To determine a relation between this functional activity and the immunogenicity results we also compared IgG titers measured in ELISA, IFA and Western blotting with inhibition results. IgG titers measured in all three immunogenicity assays closely correlated with the migration and invasion inhibitory activity of the antibodies (data not shown for invasion inhibition) (Figure 6).

## Discussion

In this report we describe immunological and functional properties of antibodies elicited by the virosomally formulated synthetic peptide UK-39 in a phase I clinical trial. Since virosomally formulated UK-39 molecules represent repeat structures on the surface of IRIVs, they have the potential to amplify the antigen-specific B cell pool enormously, allowing a very early and efficient switch to a long-lived IgG response (Bachmann and Zinkernagel 1997; Huckriede et al. 2003). Two immunizations with 10µg UK-39 were indeed enough to induce high titers of peptide-specific IgG in all volunteers (Genton et al. submitted). A higher dose of 50µg peptide delivered with the same amount of IRIVs was not superior in terms of sero-conversion and mean antibody titers elicited. However, when co-administered with a second virosomal vaccine component, the 50µg dose of UK-39 was as immunogenic as the 10µg dose alone. This may be related to the doubling of the amount of influenza antigens administered with the combination vaccine and the adjuvant-effect associated with the viral components. One volunteer (35/D) of the 50µg UK-39 group needs special attention. Pre-immune serum of this volunteer already contained antibodies that were reactive with UK-39 in ELISA and with CSP in Western blotting. This cross-reactivity, which was not related to a known history of malaria exposure, was exceptionally boosted already by the first immunization, yielding the highest titers observed in the entire trial. Even though the observed pre-existing humoral response was not malaria-related, this suggests that IRIV-based vaccines have the potential to boost natural immunity. It is therefore highly likely, that two or even one vaccine dose may be sufficient to boost pre-existing immunity in vaccine recipients from endemic areas. We found a close correlation between IgG titers against the peptide UK-39 in ELISA and IgG titers in IFA and Western blotting with whole parasites and parasite-derived antigens, respectively. Moreover titers in these immunogenicity assays were closely correlating with in vitro inhibition of sporozoite migration and invasion of hepatocytes. This confirms the observation that the conformationally restrained peptide UK-39 is very close to the natural conformation of the NANP repeats, as already indicated by NMR analysis of UK-39 and the crystal structure of an NPNA repeat [23] (Okitsu et al. submitted).

Vaccine induced immunity has to include formation of immunological memory, the key to successful immune protection [24]. One crucial step of memory formation is antibody affinity maturation in germinal centers where high-affinity variants of

antigen-specific B cells are selected for entry into the long-lived memory B cell compartment [25]. We found an increase in anti-UK-39 antibody avidity over the course of immunization in all three vaccine groups indicating memory B cell formation. Due to particularly high avidity indices in two individuals (35/D and 36/D), the mean avidity was highest in group D receiving 50µg of UK-39. One year after the third immunization all volunteers receiving the 10µg dose of UK-39 had remained positive in ELISA, Western blotting analysis and IFA supporting the hypothesis that vaccination with virosomally formulated UK-39 has induced long-term IgG production by memory B cells.

One of the major problems associated with malaria vaccine development are missing in vitro and in vivo correlates of protection. Various in vitro assays and experimental animal models are available to test functional activity of induced responses, but the predictive value of these systems cannot be determined until the results are correlated with clinical efficacy of vaccine candidates [26]. No in-depth study comparing in vitro results, animal studies, artificial human challenge and vaccine efficacy in endemic areas has been reported to date.

Induction of antibodies inhibiting in vitro sporozoite migration, growth, and development was observed in a majority of UK-39-immunized volunteers tested, regardless of vaccine formulation. Inhibition was IgG concentration-dependent and confirmed data obtained with animal sera (Okitsu et al. submitted). Inhibition of sporozoite migration and invasion was observed at concentrations of total purified IgG from immunized volunteers that were more than ten times lower than the physiological IgG concentration of about 13mg/ml. Even though this inhibition might only be partial and would not prevent the development of blood-stage infection, previous experience with sporozoite vaccines and insecticide treated bednets has shown that reduction of merozoites released from the liver can reduce the occurrence of severe forms of disease [5, 27]. It will be crucial to follow-up these inhibition results in phase 2a challenge trials and efficacy studies in field trials to validate these in vitro inhibition assays as correlates for protection.

Invasion inhibition was positively correlated with migration inhibition, suggesting that anti-UK-39 antibodies interfered with parasite gliding motility, which is based on the same mechanism of redistribution of proteins along the sporozoite surface as host cell invasion [28]. Whether inhibition is due to a specific block of a component of the machinery necessary for gliding or by steric hindrance is difficult to elucidate. In vitro

sporozoite invasion inhibition with primary human hepatocytes is a very labor intensive and demanding test system. Based on the observed close correlation of results in invasion inhibition experiments and the far easier migration inhibition assays, the migration inhibition system might be a valuable second line method to measure the biological activity of anti-CSP antibodies. Further advantages of the migration inhibition assays are the reduced time needed, the option for quantitative analysis by flow cytometry and the possibility to perform the experiments with hepatocyte cell lines. Altogether, these properties make the migration inhibition assay an interesting tool for screening large numbers of serum samples in clinical trials.

Taken together these results demonstrate the suitability of IRIVs as a carrier/adjuvant system for the induction of potent humoral immune responses against synthetic peptide antigens. The humoral immune responses characterized in this report were very uniform, cross-reactive with *P. falciparum*, affinity matured, long-lived and parasite-inhibitory. Combination of two components had no negative effect on the elicited immune response against the single components, showing the potential of this universal antigen delivery platform for multivalent vaccine development.

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### Figure legends

Figure 1. ELISA endpoint titers. Shown are anti-UK-39 IgG ELISA endpoint titers for individual volunteers at baseline (0), 21 days after each vaccination and one year after the third vaccination. Arrows indicate days of immunization. Only the groups immunized with 10 $\mu$ g UK-39 or 50 $\mu$ g UK-39+50 $\mu$ g AMA49-CPE were followed up for one year.

Figure 2. Avidity indices. Mean avidity indices for the anti-UK-39 IgG responses in all vaccination groups receiving UK-39 three weeks after the first, second and third immunization. The avidity index is the NH<sub>4</sub>SCN concentration (M) where 50% of the bound antibodies are eluted. Shown are results obtained with individual sera and the geometric mean (line) for each time point. Wilcoxon t-test was used to calculate the statistical significance of the avidity increase after the third immunization compared to the first vaccination.

Figure 3. IFA and Western blotting endpoint titers. IFA (left panel) and Western blotting (right panel) endpoint titers and number of volunteers showing an increase in titer compared to pre-immune (pi) serum. Blood samples were taken pre-immune, three weeks after the first, second and third immunization and one year after the third immunization. Lines represent geometric means of all volunteers in the respective group. Arrows indicate volunteer 35/D that had a pre-existing anti-UK39 IgG ELISA titer of 10<sup>3</sup>. Only the groups immunized with 10 $\mu$ g UK-39 or 50 $\mu$ g UK-39+50 $\mu$ g AMA49-CPE were followed up for one year.

Figure 4. Boost of a pre-existing anti-UK-39 cross-reactive antibody response in volunteer 35/D. **A:** Comparison of ELISA, IFA and Western blot titers of pre-immune serum and sera taken three weeks after each immunization. **B:** Western blotting results obtained with serum taken before and three weeks after the first immunization. Dilutions are: 1, 1:200; 2, 1:400; 3, 1:800; 4, 1:3,000; 5, 1:9,000; 6, 1:27,000; 7, 1:81,000; 8, 1:243,000; 9, 1:729,000; 10, 1:2,187,000; 11, 1:6,561,000; 12, Positive control anti-NANP mAb EP9. **C:** IFA on *P. falciparum* sporozoites with serum taken before and three weeks after the first immunization. An overlay of DNA staining with Hoechst dye no. 33256 (blue) and Cy3-immunofluorescence staining (red) is shown.

Fig 5. Functional activity of anti-UK39 IgG responses. Sporozoite migration and invasion inhibition by IgG preparations (final concentration 1mg/ml) purified from sera taken before the first (pl) and three weeks after the third immunization (3.imm). Three volunteers (xy/B) immunized with 10 $\mu$ g UK-39, one volunteer (35/D) immunized with 50 $\mu$ g UK-39, and three volunteers (xy/E) immunized with 50 $\mu$ g UK-39+50 $\mu$ g AMA49-CPE were chosen. Shown are the means and SDs from at least two independent experiments. A: In vitro inhibition of sporozoite migration through human HepG2 cells. B: In vitro Inhibition of sporozoite invasion of primary human hepatocytes.

Figure 6. Correlation (r) of functional activity with antibody titers. Sera taken before the first and 21 days after the third immunization from nine volunteers analyzed in inhibition assays were included in the analysis. Correlation coefficients were calculated with a Pearson test (Gaussian distribution) for correlation of invasion and migration inhibition; nonparametric (Spearman) correlation was used for analysis with immunogenicity assays.

Table 1. Correlation (Spearman r) of IgG endpoint titers in immunogenicity assays.

Figure 1

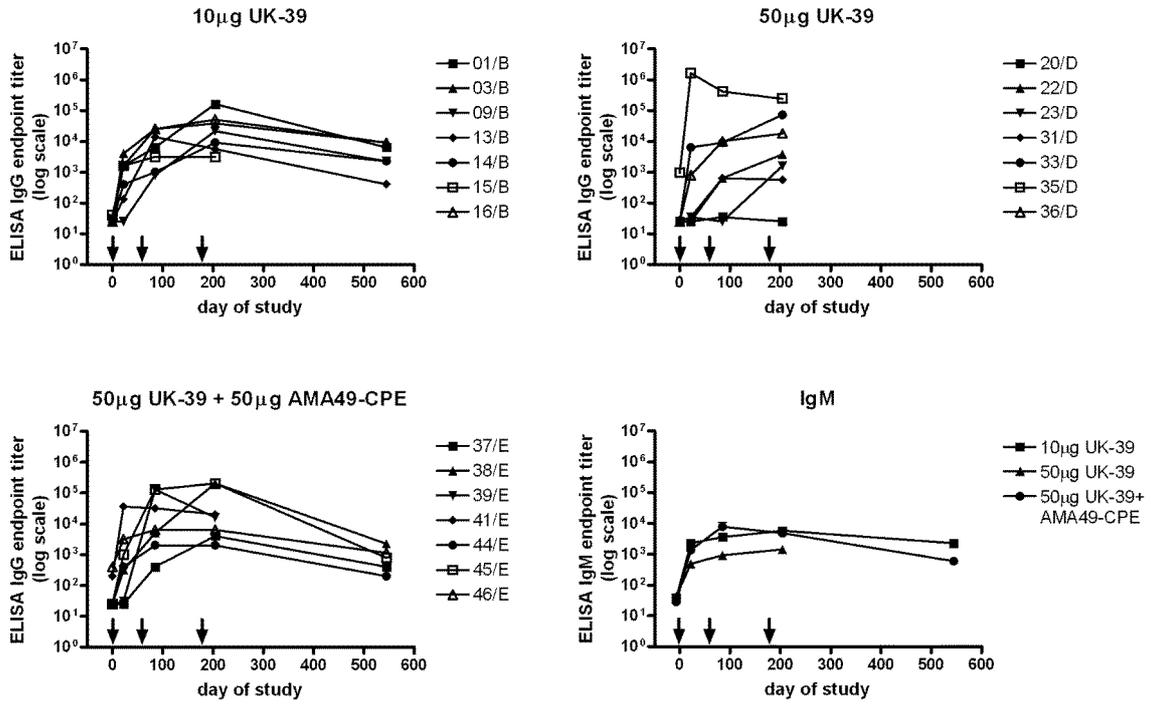


Figure 2

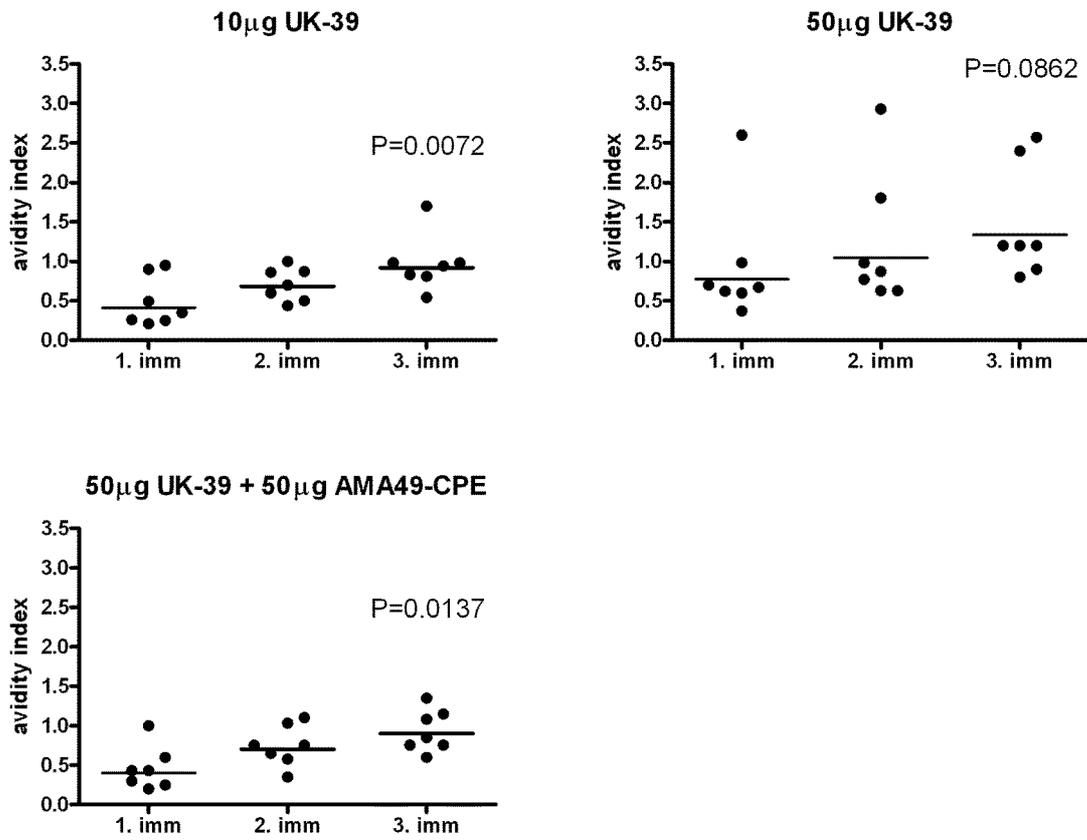


Figure 3

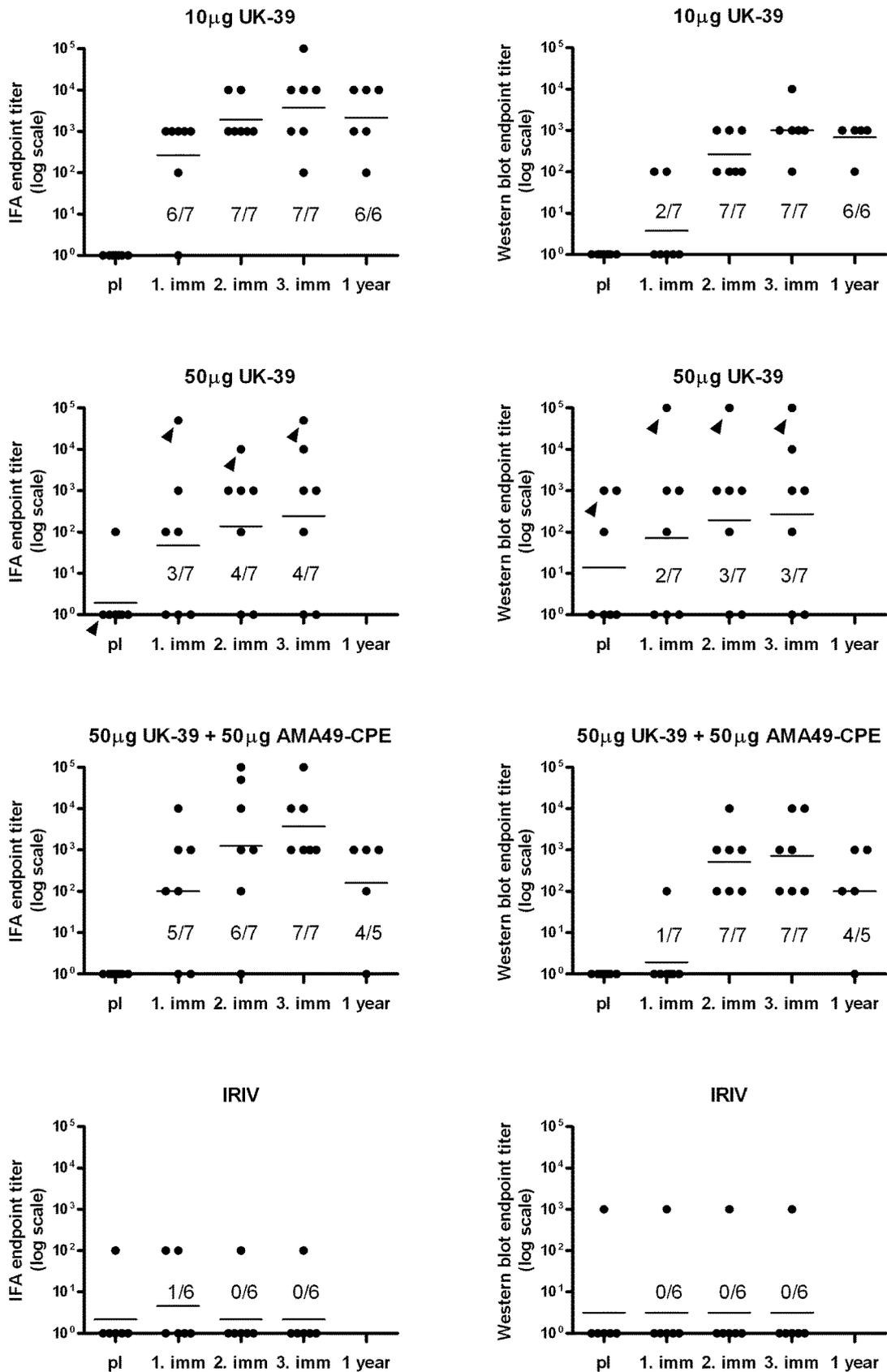


Figure 4

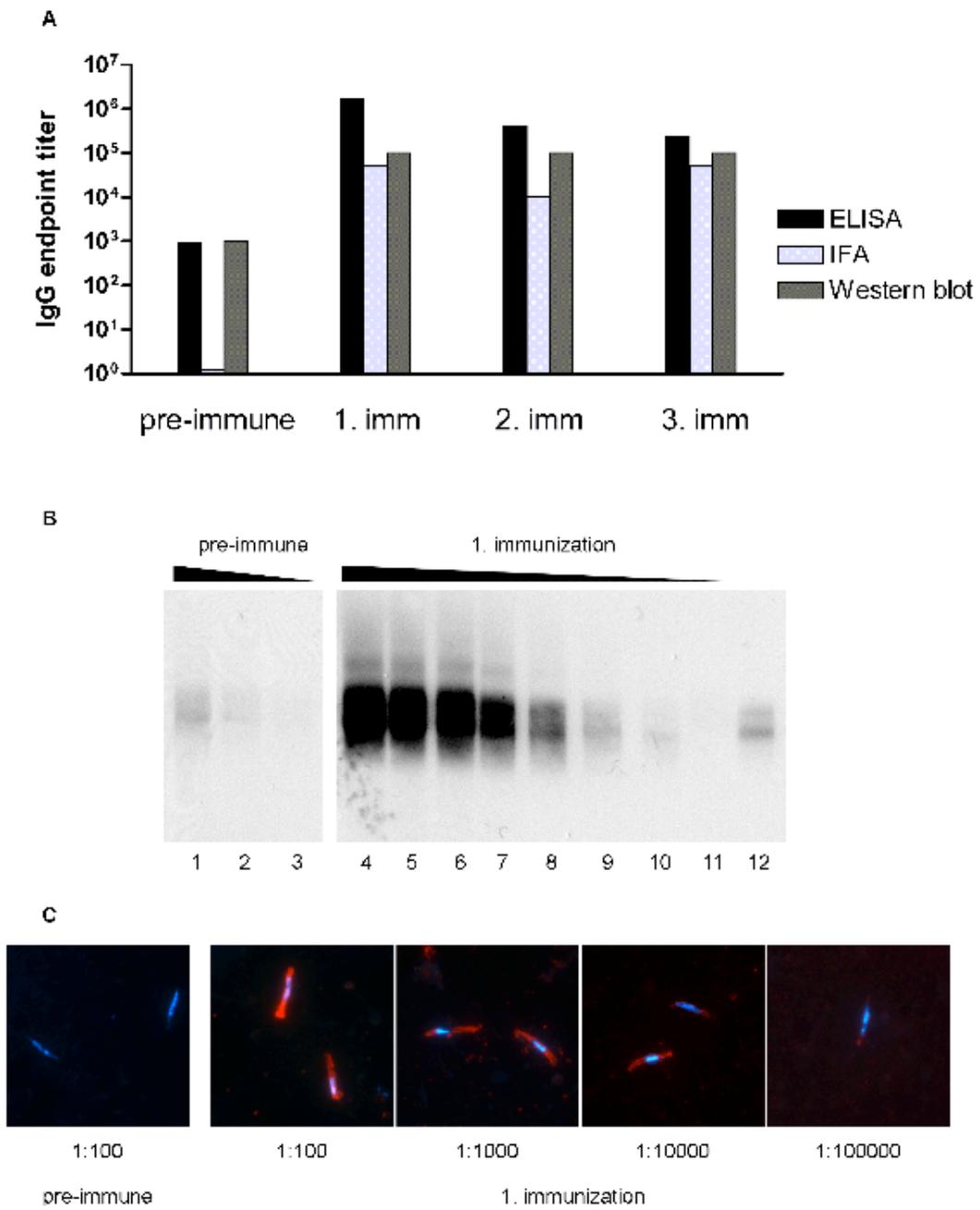


Figure 5

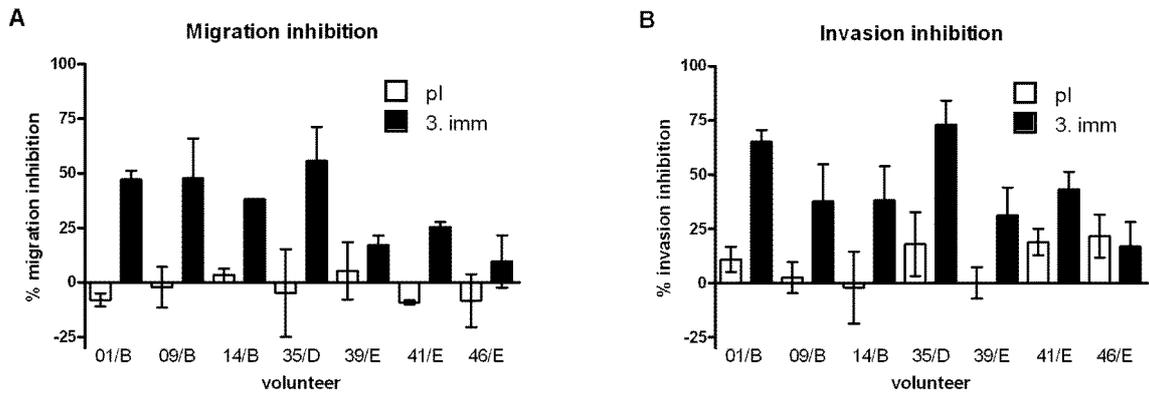


Figure 6

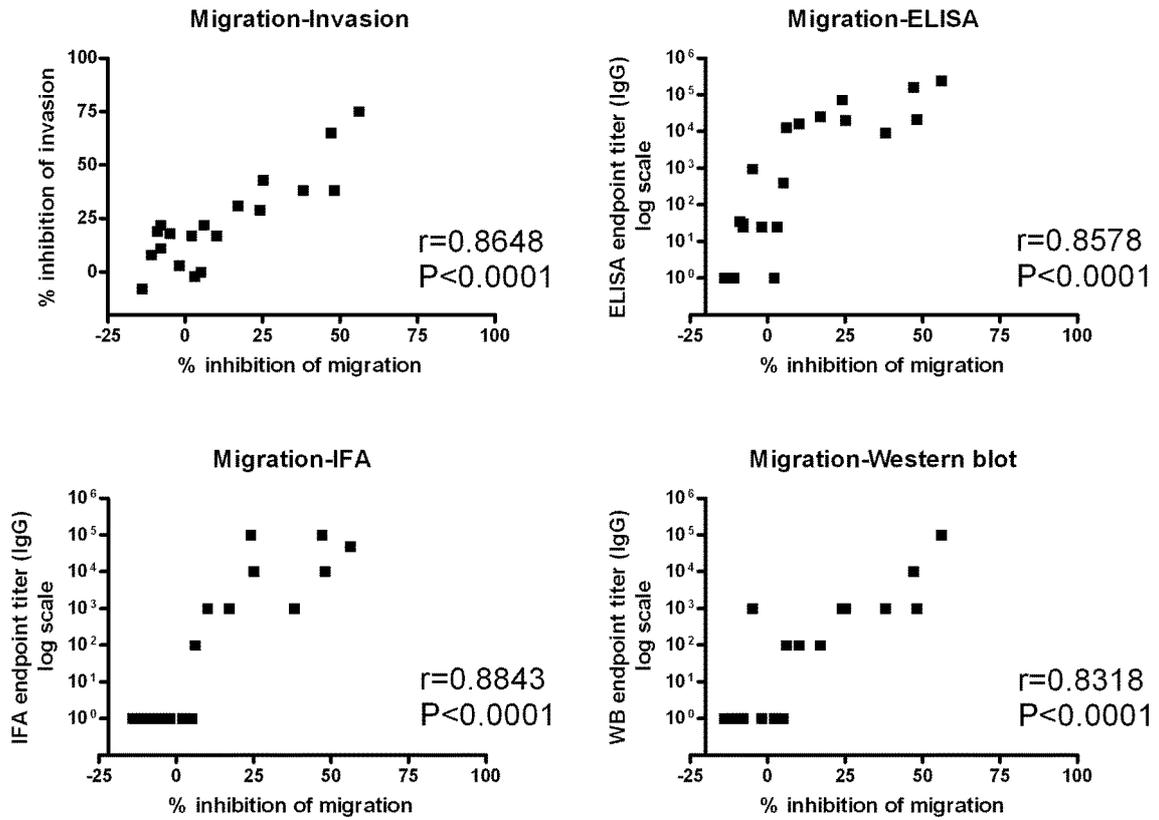


Table 1. Correlation (Spearman r) of IgG endpoint titers in immunogenicity assays.

Comparison	10µg UK-39	50µg UK-39	50µg combination
ELISA-IFA	0.9233 (P<0.0001)	0.8192 (P<0.0001)	0.8401 (P<0.0001)
ELISA-Western blot	0.8563 (P<0.0001)	0.5609 (P=0.0019)	0.7505 (P<0.0001)
IFA-Western blot	0.7928 (P<0.0001)	0.5499 (P=0.0024)	0.6691 (P<0.0001)

**Chapter 7: A Virosomally-Formulated Synthetic Peptide  
Derived from *Plasmodium falciparum* Apical Membrane  
Antigen 1 Elicits Long-Lasting Parasite-Binding  
Antibody Response in Malaria-Naïve Volunteers**

**A virosomally-formulated synthetic peptide derived from *P. falciparum* apical membrane antigen 1 elicits a long-lasting parasite-binding antibody response in malaria-naïve volunteers**

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Running title: Phase 1 clinical trial of an AMA-1 peptide based malaria vaccine component

Key words: malaria vaccine, virosomes, synthetic peptide, apical membrane antigen 1, antibody affinity maturation

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**Abstract**

*Plasmodium falciparum* apical membrane antigen 1 (AMA-1) is one of the leading blood stage candidate antigens for inclusion in a malaria subunit vaccine. The 83kDa protein consists of three domains. Loop 1 of domain III binds to the erythrocyte protein Kx, underlining an important role of AMA-1 during invasion of host cells. We have previously described the development and characterization of a virosomally formulated synthetic peptide vaccine component (designated PEV301) which is based on the sequence of loop 1. Monoclonal antibodies against this peptide inhibited parasite growth *in vitro*. PEV301 was safe and immunogenic in a phase 1 clinical trial in malaria-naïve adult volunteers. Two vaccinations were sufficient to induce high titers of peptide-specific IgG in all volunteers immunized with 50µg of virosomally formulated AMA-1 peptide-phospholipid conjugate. Co-administration with a second peptide-based vaccine component did not interfere with the development of an AMA-1 peptide specific response. In this report cross-reactivity of the IgG response elicited by PEV301 with parasite derived AMA-1 is described. After the third immunization half of the volunteers were positive for *P. falciparum* blood stage parasite-expressed AMA-1 in IFA and/or Western blotting analysis. With one exception, all peptide induced IgG responses increased in affinity over the course of the immunizations and AMA-1 peptide-reactive IgG was still detectable one year after the third immunization, indicating formation of long-lived memory B cells. Taken together results demonstrate, that the virosome-based universal antigen delivery platform is suitable to elicit long-lived and parasite cross-reactive IgG responses against suitable synthetic peptide-phospholipid conjugates anchored on the surface of the virosomes.

## Introduction

With more than 2 million deaths per year (23) malaria remains one of the most threatening human diseases. Current methods of control include the use of antimalarial drugs to treat the disease, as well as preventive tools like insecticide treated bednets (ITN), indoor spraying with residual insecticides (IRS), and environmental changes. As emerging resistance of parasites against drugs and mosquitoes against insecticides pose a problem to these control measures, vaccine development is as urgent as never before. A lot of progress has been done in the development of a malaria vaccine and RTS,S/AS02A was the first malaria vaccine to show efficacy against severe disease in an endemic area (1). Despite this success a cheap and effective vaccine might still be years away and faces many problems like production of recombinant material and missing human compatible adjuvant and delivery systems (15).

We try to circumvent these problems by delivering easy to synthesize peptides corresponding to sequence stretches of *P. falciparum* antigens on the surface of immunopotentiating reconstituted influenza virosomes (IRIVs). IRIVs are a liposomal carrier system characterized as spherical, unilaminar vesicles with a mean diameter of approximately 150 nm. They consist of coat proteins of H1N1 influenza virus (A/Singapore/6/86) and phospholipids. Like fusion-inducing viruses, virosomes enter cells by receptor-mediated endocytosis and subsequently fuse with the endosomal membrane. The strong immune response induced by antigens presented on the surface of virosomes is predominantly humoral, whereas a supplementary T-helper immune response is evoked by virosomal influenza-derived glycoproteins. Antigens encapsulated in the virosome are delivered to the host cell cytosol and enter the MHC I pathway, inducing cytotoxic T cell responses. Two virosome-based vaccines are registered for human use in more than 38 countries, and have been administered to more than 20 million individuals (24).

One of the leading candidate antigens for inclusion in a malaria vaccine is apical membrane antigen 1 (AMA-1), a blood-stage protein of *P. falciparum*. This 83kDa protein is synthesized in mature stages of the parasite and is first localized in the neck of the rhoptries (5, 20). It is rapidly processed after synthesis giving rise to a 83- and a 66-kDa fragment initially localized at the merozoite apex. While the full-length 83-kDa molecule remains in the apical region following merozoite release, the processed 66-kDa form spreads around the merozoite surface (19, 20). Several

passive and active immunization studies have indicated that AMA-1 is involved in eliciting protective immune responses (2-7, 12, 18, 25) and serves as a target for invasion-blocking antibodies (6, 7, 9, 12, 13). The ectodomain of AMA-1 is divided into three subdomains, each with disulfide bond-stabilized structures. Domain III of AMA-1 binds to the erythrocyte membrane protein, Kx, and the invasion rate of Kx<sub>null</sub> erythrocytes is reduced, indicating a significant role of AMA-1 and Kx in parasite invasion of erythrocytes (11). We have developed a synthetic peptide designated AMA49-CPE, which is mimicking the semi-conserved loop I of domain III (residues 446 to 490) and elicits parasite growth-inhibitory antibodies in mice. A virosomal formulation of this peptide was tested in a phase I clinical trial in combination with a peptide derived from *P. falciparum* circumsporozoite protein (CSP) and proved to be safe and immunogenic in malaria naïve Caucasians (Genton et al. submitted). Here we describe the individual anti-peptide IgG responses and compare anti-peptide IgG responses with the generation of parasite cross-reactive antibodies.

## Results

### Development of IgG responses against the synthetic antigen AMA49-CPE

One immunization with 50µg of virosomally formulated AMA49-CPE alone (group C) or in combination with 50µg UK-39 (group E) was enough to induce anti-AMA49-CPE IgG titers above 250 in all volunteers (Figure 1). A second immunization with the same vaccine formulations increased the titers to more than 10,000 with the exception of one volunteer (37/E) who had a titer of 4525. A third immunization did not lead to a further titer increase in most of the volunteers. Combined delivery of AMA49-CPE together with UK-39 did not interfere with the development of anti-AMA49-CPE antibody responses. One year after the third immunization 12 out of 13 volunteers immunized with the 50µg dose still had anti-AMA49-CPE IgG titers above 800. No volunteer of the control group (group F) receiving IRIVs alone developed an anti-AMA49-CPE IgG response. One volunteer in this group (34/F) had a pre-existing anti-AMA49-CPE IgG titer of 200, which decreased over the course of the immunizations.

In volunteers receiving 10µg of virosomally formulated AMA49-CPE (group A), humoral immune responses were weaker. Only 3/8 volunteers had developed anti-AMA49-CPE IgG titers after one immunization (Figure 1) and 100% sero-conversion in ELISA was only observed after the third immunization with a relatively low mean anti-peptide titer of 582.

An AMA49-CPE-specific IgM titer of ~1000 was found after the first immunization in the two groups receiving 50µg AMA49-CPE (Figure 2). An increase in titer to 10,000 after the second immunization was only observed in the combination group. Virtually no anti-AMA49-CPE IgM was found in the 10µg AMA49-CPE group (max geomean titer ~65). No peptide-specific IgM was found in controls immunized with IRIVs.

The mean avidity index, a measure for antibody affinity maturation, increased with one exception in all individuals of group C and E, receiving the 50µg AMA49-CPE dose (Figure 3). A three-fold increase was observed in the group receiving AMA49-CPE alone after two immunizations, whereas a more gradual two-fold increase over all three immunizations was observed after combined immunization with AMA49-CPE and UK-39. Avidity indices were not assessed for group 1 immunized with 10µg virosomally formulated AMA49-CPE.

### **Cross-reactivity of anti-AMA49-CPE IgG responses with *P. falciparum* blood-stage parasites**

None of the volunteers immunized with empty IRIVs or with 10µg AMA49-CPE developed a sufficiently high titer of AMA-1 cross-reactive IgG to be detectable in IFA or Western blotting analysis. In contrast, 50% (8/16) of volunteers receiving 50µg AMA49-CPE alone or in combination with UK-39 sero-converted in IFA and/or Western blotting analysis (Table 1) in spite of a considerable background staining observed with many of the pre-immune sera.

In Western blotting analysis with complete *P. falciparum* blood stage parasite lysate, most of the pre-immune sera yielded some background staining, which was highly variable both with respect to intensity and the molecular mass of the stained proteins (Figure 4). Nevertheless, development of IgG, reactive with the characteristic AMA-1 66/83kDa double bands (Figure 4), was clearly detectable with immune sera from 8/16 volunteers receiving 50µg AMA49-CPE. While none of the volunteers had sero-converted after the first immunization, 7/8 Western blotting sero-converters were already positive after the second immunization (Table 1). One year after the third immunization 2/13 volunteers still had an anti-AMA-1 Western blotting IgG titer of 100.

As expected from the Western blotting results, many of the pre-immune sera yielded variable background staining in IFA with *P. falciparum* blood stage parasites (Figure 5). It is likely, that this often compromised detectability of a potential vaccine-induced AMA-1 cross-reactive IgG response. Nevertheless, development of a punctuate staining pattern characteristic for AMA-1 (Figure 5) was observed with immune sera from 5/15 volunteers receiving 50µg AMA49-CPE (endpoint titer  $\geq 1000$  after the third immunization). Parasite cross-reactive responses were first detected after the second immunization and both titers and the proportion of identified responders increased after the third immunization (Table 1). One year after the third immunization only one of the volunteers still had a titer of parasite cross-reactive IgG that was above the detection threshold of 100.

## Discussion

The difficulties of producing a vaccine with attenuated sporozoites and problems with recombinant expression of full-length parasite proteins make the subunit approach very interesting for the development of a malaria vaccine. We have shown before the development of synthetic peptides for inclusion in a multivalent virosomal subunit vaccine (10, 16, 17, 21, 22) (Okitsu et al. submitted). Two peptides derived from CSP and AMA-1 respectively were safe and immunogenic in a phase 1 clinical trial (Genton et al. submitted). This report presents a detailed immunological analysis of the humoral response in this phase 1 trial. We found that immunization with 50µg of virosomally formulated AMA49-CPE induced high anti-peptide titers in all volunteers already after one immunization. After the second immunization the induced titers were very homogeneous, highlighting the potential of IRIVs as a powerful delivery and adjuvant system for synthetic peptides. In group A receiving 10µg AMA49-CPE both sero-conversion rates and titers were low. Also in rabbit immunization studies the 50µg dose of AMA49-CPE was superior to the 10µg dose (manuscript in preparation).

Measurement of IgG cross-reactivity in IFA with blood-stage parasites was difficult, due to high background staining already observed with pre-immune sera. Individual-specific cross-reactivity with a range of protein bands was also found Western blotting analysis. In particular in IFA negative results may be the result of a masking of an AMA-1 specific staining by high background reactivity. Western blot staining patterns were easier to evaluate with respect to AMA-1 reactivity and confirmed the presence of a pre-existing anti-parasite response not related to vaccination.

Taken together we have shown that it is possible to induce parasite-binding antibodies in malaria-naïve volunteers by immunization with an optimized peptide of AMA-1 in a virosomal formulation. The good immunogenicity, the possibility to combine various antigens, and the excellent safety profile make the IRIV approach highly suitable for the development of a multivalent malaria subunit vaccine.

<b>10µg AMA49-CPE</b>	<b>IFA</b>		<b>Western blotting</b>	
	titer	sero-conv	titer	sero-conv
<b>pl</b>	-	0/0	-	0/0
<b>1. Imm</b>	-	0/0	-	0/0
<b>2. Imm</b>	-	0/0	-	0/0
<b>3. Imm</b>	-	0/0	-	0/0
<b>1y after 3. Imm</b>	nd	nd	nd	nd

<b>50µg AMA49-CPE</b>	<b>IFA</b>		<b>Western blotting</b>	
	titer	sero-conv	titer	sero-conv
<b>pl</b>	-	0/0	-	0/0
<b>1. Imm</b>	-	0/0	-	0/0
<b>2. Imm</b>	1000	1/8	8409	4/8
<b>3. Imm</b>	1710	3/8	7071	4/8
<b>1y after 3. Imm</b>	-	0/0	25	2/8

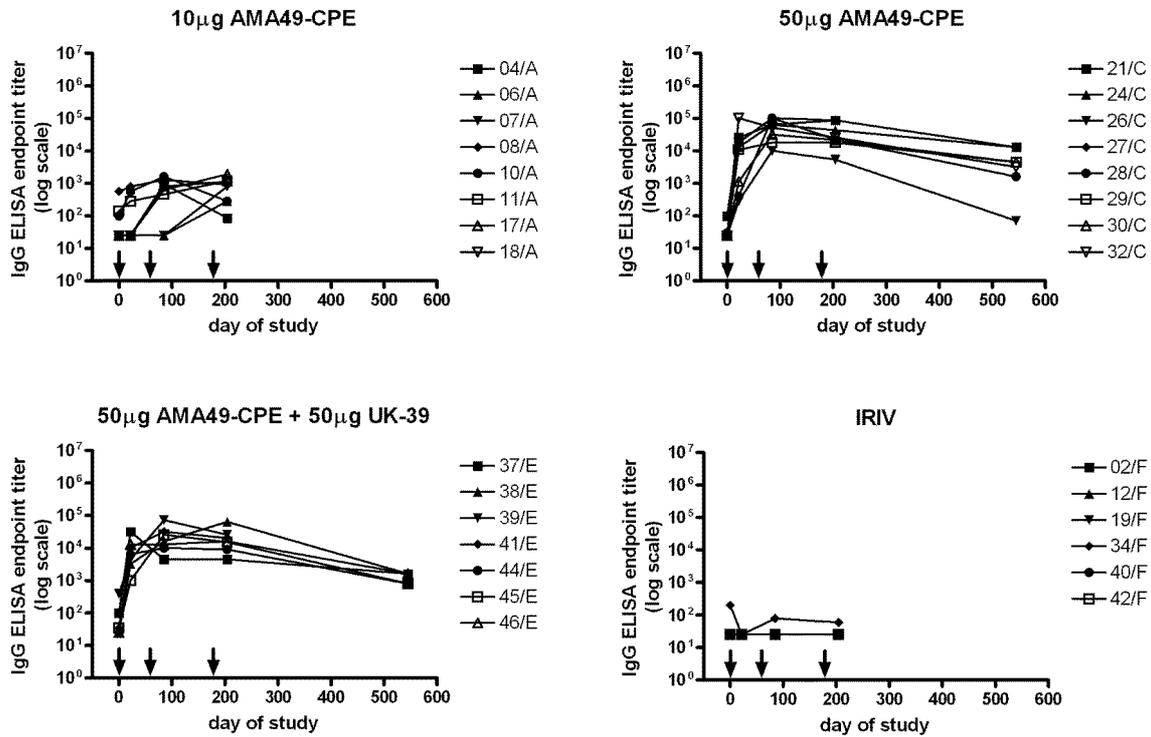
  

<b>50µg combi</b>	<b>IFA</b>		<b>Western blotting</b>	
	titer	sero-conv	titer	sero-conv
<b>pl</b>	-	0/0	-	0/0
<b>1. Imm</b>	-	0/0	-	0/0
<b>2. Imm</b>	707	2/7	4642	3/8
<b>3. Imm</b>	1000	2/7	8409	4/8
<b>1y after 3. Imm</b>	100	1/7	-	0/8

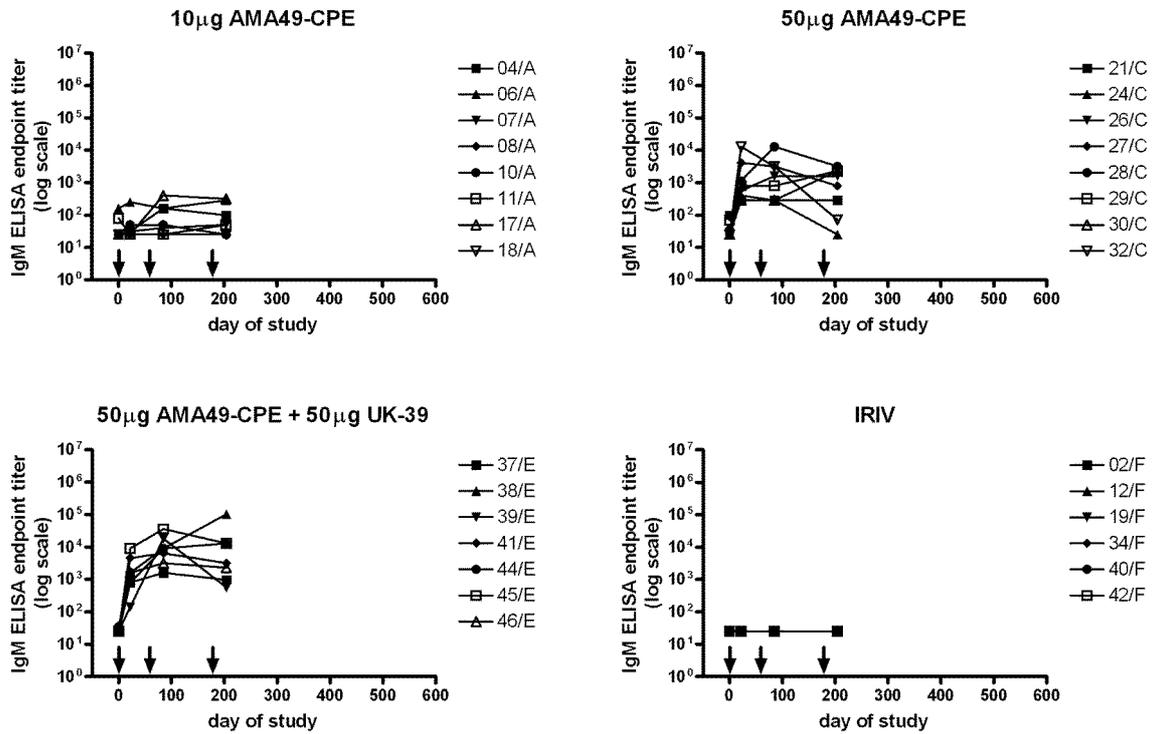
  

<b>IRIV</b>	<b>IFA</b>		<b>Western blotting</b>	
	titer	sero-conv	titer	sero-conv
<b>pl</b>	-	0/0	-	0/0
<b>1. Imm</b>	-	0/0	-	0/0
<b>2. Imm</b>	-	0/0	-	0/0
<b>3. Imm</b>	-	0/0	-	0/0
<b>1y after 3. Imm</b>	nd	nd	nd	nd

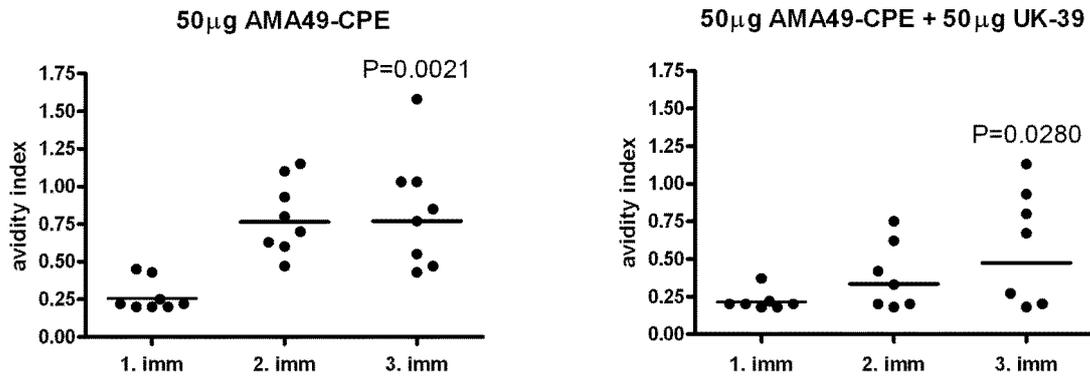
**Table 1.** Parasite cross-reactivity of anti-AMA49-CPE IgG responses. Shown are geometric mean IgG titers of responders and rates of sero-conversion for IFA and Western blotting with sera from immunized volunteers taken pre-immune, three weeks after every immunization and one year after the third immunization.



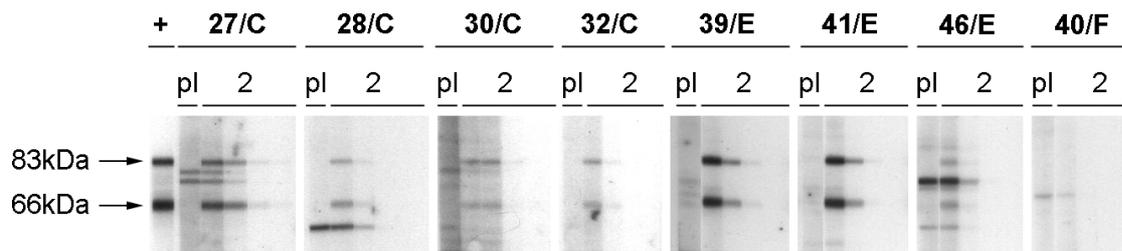
**Figure 1.** Development of anti-AMA49-CPE IgG responses. Shown are anti-AMA49-CPE IgG ELISA endpoint titers for individual volunteers at baseline (0), 21 days after each vaccination and one year after the third vaccination. Arrows indicate days of immunization. One year samples were only taken for groups immunized with 50µg AMA49-CPE.



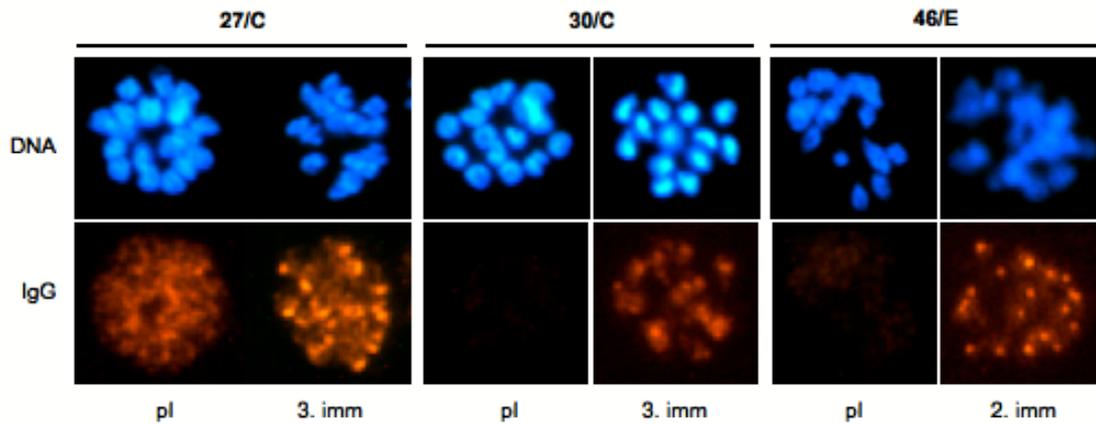
**Figure 2.** Development of anti-AMA49-CPE IgM responses. Shown are anti-AMA49-CPE IgM ELISA endpoint titers for individual volunteers at baseline (0), and 21 days after each vaccination. Arrows indicate days of immunization.



**Figure 3.** Affinity maturation of anti-AMA49-CPE IgG. Mean avidity indices for the anti-AMA49-CPE IgG responses in the 50µg AMA49-CPE (C), and the 50µg AMA49-CPE+50µg UK-39 (E) groups three weeks after the first, second and third immunization. Shown are results obtained with individual sera and the geometric mean (line) for each time point. Wilcoxon t-test was used to calculate the statistical significance of the avidity increase after the third immunization compared to the first vaccination.



**Figure 4.** Development of AMA-1 cross-reactive IgG in Western blotting analysis with total lysate from *P. falciparum* schizonts. Serum samples taken pre-immune (pl) and three weeks after the second immunization (2) from volunteers immunized with either 50µg of AMA49-CPE (group C), 50µg AMA49-CPE plus 50µg UK-39 (group E), or IRIVs alone (group F; negative control) were tested at a dilution of 1:100 (pl sera) or 1:100, 1:500, 1:2500 and 1:5000 (2 sera), respectively. Full-length AMA-1 (83kDa) and its 66kDa processing fragment are indicated by arrows. AMA-1 fragments were identified by staining with an anti-AMA-1 monoclonal antibody (lane +).



**Figure 5.** Development of AMA-1 cross-reactive IgG in IFA with *P. falciparum* schizonts. Serum samples were taken from human volunteers immunized with 50µg AMA49-CPE (27/C and 30/C) or 50µg AMA49-CPE plus 50µg UK-39 (46/E) pre-immune (pl), three weeks after the second (2. imm) or three weeks after the third immunization (3. imm). Sera were diluted 1:1000 (pre-immune and after the second immunization) or 1:2000 (after the third immunization). The upper panel shows DNA staining with Hoechst dye no. 33256 (blue), the lower panel Cy3-immunofluorescence staining (red). Representative examples demonstrating the variation in background staining by pre-immune sera are shown.

## Materials and Methods

### Overall Study Design and Plan

This prospective phase I, single blind, randomized, placebo controlled, dose-escalating study was conducted in 46 healthy adult volunteers. Eligible study participants were randomized into six groups: PEV 301 (virosomally formulated AMA49-CPE) 10 µg (group A, n = 8), PEV 302 (virosomally formulated UK-39) 10 µg (group B, n = 8), PEV 301 50 µg (group C, n = 8), PEV 302 50 µg (group D, n = 8), PEV 301 50 µg + PEV 302 50 µg (group E, n = 8) or unmodified virosomes serving as controls (group F, IRIV, n = 6). Vaccine formulations contained different amounts of PE-peptide conjugate, but throughout the same amounts of IRIV per dose. Volunteers immunized with a combination of virosomally-formulated AMA49-CPE and UK-39 received a double dose of IRIVs.

### Vaccine

Two virosome-formulated vaccines, PEV 301 (incorporating the AMA-1 derived PE-peptide conjugate AMA49-CPE) and PEV 302 (incorporating the CSP derived PE-peptide conjugate UK-39) were produced according to the rules of GMP. Tests for sterility, pyrogenicity, immunogenicity in animals, stability and chemical composition were performed on the vaccine lots used in this trial. Each dose was composed of 10 or 50 µg AMA49-CPE or UK-39, 10 µg *Influenza* hemagglutinin, 100 µg phospholipids and PBS ad 0.500 ml. The trial vaccines were administered i.m. in the left arm on day 0, in the right one on day 56-65, and in the left again on day 175-186.

### Ethics

The protocol was approved by the Ethikkommission beider Basel (EKBB) and the study conducted in full compliance with the international ethical guidelines for biomedical research involving human subjects.

### Immunogenicity

Blood sampling for antibody titer measurement by ELISA, IFA and Western blot was done at screening visit (baseline and 1<sup>st</sup> vaccination), at the days of the 2<sup>nd</sup> and 3<sup>rd</sup> vaccination, 21 days after each vaccination and one year after the third vaccination (groups B, C and E only).

## **ELISA**

ELISA Polysorp™ microtiter plates (Nunc, Dr. Grogg, Stetten-Deiswill, Switzerland) were coated at 4°C overnight with 10 µg/ml AMA49-CPE in PBS, pH 7.2. Wells were then blocked with 5% milk powder in PBS for 2 h at 37°C followed by three washings with PBS containing 0.05% Tween-20. Plates were then incubated with two-fold serial dilutions of human serum starting with 1:50 in PBS containing 0.05% Tween-20 and 0.5% milk powder for 2 h at 37°C. After washing, the plates were incubated with horseradish-peroxidase-conjugated goat anti-human IgG antibodies (KPL, Socochim, Lausanne Switzerland) (1:2000 in PBS containing 0.05% Tween-20) for 1 h at 37°C and then washed. 1,2-Diaminobezene substrate (OPD) (20 mg/tablet (Fluka, Sigma. Buchs, Switzerland)) in citrat-buffer (4 mg/ml OPD) + 0.01% H<sub>2</sub>O<sub>2</sub> was added and incubated at room temperature. After 10 minutes the reaction was stopped by addition of sulphuric acid (final concentration 0.5M (Merck, Darmstadt, Germany)). The optical density (OD) of the reaction product was measured at 492 nm using a microplate reader (SpectraMax plus, Bucher Biotech, Basel, Switzerland). Titration curves were registered using Softmax PRO software.

Antibody titer measurement by ELISA against AMA49-CPE was done at screening visit (baseline), 7 (±2) days and 21 (±5) days after first, second and third vaccination. An additional sample was taken one year after the third immunization for the 10µg UK-39, the 50µgAMA49-CPE, and the 50µg AMA49-CPE+UK-39 groups. Endpoint titers were calculated by comparing the ELISA OD of the test serum with the ELISA OD of a negative serum pool. The endpoint titer is the last serum dilution where the  $OD_{\text{test sera}} \geq 2 \times OD_{\text{negativ serum}}$ .

## **NH<sub>4</sub>SCN elution ELISA**

Avidity ELISA analyses with peptide-PE conjugates were performed essentially as described before (8). Briefly, Polysorp™ plates (Nunc, Fisher Scientific, Wohlen, Switzerland) were coated overnight at RT with 50 µl of a 10µg/ml solution of AMA49-CPE in PBS (pH 7.2). After three washings with PBS containing 0.05% Tween-20 wells were blocked with 5% milk powder in PBS for 30 min at RT and washed two times. Serum samples were added in triplicates at constant dilutions (approx. halfmax titer) in PBS containing 0.05% Tween-20 and 0.5% milk powder for 2 h at RT. After antibody incubation and an additional wash step, NH<sub>4</sub>SCN (Sigma, St. Louis, MO) diluted in 0.1M NaH<sub>2</sub>PO<sub>4</sub> buffer (pH 6) was incubated for 15 min at room

temperature (100 µl/well) at the following molarities: 5 M, 4 M, 3 M, 2 M, 1 M, 0.5 M, 0.25 M. Control wells were incubated with 0.1M NaH<sub>2</sub>PO<sub>4</sub> buffer without NH<sub>4</sub>SCN. After a standard washing procedure with PBS-Tween-20, plates were incubated with alkaline phosphatase-conjugated affinity-pure F(ab')<sub>2</sub> fragment goat anti-human IgG (Fc-specific) antibodies (Jackson Immuno Research Laboratories, West Grove, Pa.) for 1 h at RT. After washing, phosphatase substrate solution (1 mg/ml p-nitrophenyl phosphate (Sigma, St. Louis, Mo) in a pH 9.8 buffer solution containing 10% [vol/vol] diethanolamine and 0.02% MgCl<sub>2</sub>) was added and the plates were incubated in the dark at RT until the colorimetric reaction had progressed sufficiently. The optical density was measured at 405 nm on a Titertek Multiscan MCC/340 reader (Labsystems, Helsinki, Finland).

### **Indirect immunofluorescence assay (IFA)**

Erythrocytes from *in vitro* cultures (14) of *P. falciparum* strain K1 with a parasitemia between 5 and 10% were washed twice in RPMI and resuspended in RPMI and 2 volumes of a solution containing 4% formaldehyde and 0.1% Triton X-100. From this cell suspension, 30 µl were added to each well of a multiwell diagnostic microscope slide (Erie Scientific Company, Portsmouth, NH), incubated at room temperature for 30 min, and washed five times with PBS. Slides were then incubated for 15 min at room temperature with 25 µl blocking solution containing 1% fatty acid-free bovine serum albumin (BSA) in PBS. Immunostaining was performed by incubating the wells with 25 µl of an appropriate serum dilution in blocking solution in a humid chamber for 1 h at room temperature. After five washes with blocking solution 25 µl of 5 µg/ml Cy<sup>TM</sup>3-conjugated affinity-pure F(ab')<sub>2</sub> fragment goat anti-human IgG (Fc-specific) antibodies (Jackson Immuno Research Laboratories, West Grove, Pa.) diluted in blocking solution containing 0.01 mg/ml Hoechst dye no. 33256 (Sigma, St. Louis, Mo) were added to the wells and incubated for 1 h at room temperature. Finally, wells were washed five times with PBS, mounted with mounting solution (90% [vol/vol] glycerol containing 0.1 M Tris-Cl, pH 8.0 and 2 mg/ml o-phenylenediamine) and covered with a coverslip. Antibody binding and DNA staining were assessed by fluorescence microscopy on a Leitz Dialux 20 fluorescence microscope and documented with a Leica DC200 digital camera system.

### **SDS-PAGE and immunoblotting**

Blood stage parasite lysates were prepared essentially as described previously (14) by saponin lysis of *P. falciparum* K1-infected erythrocytes. In brief, cultured parasites were washed once with PBS. Pelleted infected red blood cells were lysed by mixing with a large volume (adjusted to 5% hematocrit) of 0.015% (wt/vol) saponin in 150 mM NaCl and 15 mM sodium citrate (pH 7.0) and incubated on ice for 20 min. Finally, the pelleted parasites were resuspended in 3 volumes of PBS and stored at -80°C until further use. A total of 50 µl of parasite lysate was solubilized in an equal volume of 2x loading buffer (1.7 ml of 0.5 M Tris-HCl [pH 6.8], 2 ml of glycerol, 4.5 ml of 10% sodium dodecyl sulfate, 1 ml of β-mercaptoethanol, 0.8 ml of bromophenol blue [0.3%, wt/vol]) and heated to 95°C for 10 min. Proteins were separated on a 10% SDS PAGE mini-gel. Separated proteins were electrophoretically transferred to a nitrocellulose filter (Protran® Nitrocellulose, BA85, Schleicher & Schuell) by semi-dry blotting. Blots were blocked with PBS containing 5% milk powder and 0.1% Tween-20 over night at 4°C. The filter was cut into strips and incubated with appropriate dilutions of immune sera in blocking buffer for 2 h at room temperature. An anti-AMA49-CPE monoclonal antibody served as a positive control (17). Filter strips were then washed three times for 10 minutes in blocking buffer and incubated at room temperature for 1 h with alkaline peroxidase-conjugated affinity-pure F(ab')<sub>2</sub> fragment goat anti-human IgG (Fc-specific) antibodies (Jackson Immuno Research Laboratories, West Grove, Pa.) diluted 1:10'000 in blocking buffer. After washing again with PBS/0.1% Tween-20, blots were finally developed using ECL™ Western blotting detection (Amersham Biosciences, Buckinghamshire, England) reagents to visualize bands.

### **Statistical analysis**

All statistical analyses and graphs were made using GraphPad Prism version 4.03 for Windows, GraphPad Software, San Diego California USA, [www.graphpad.com](http://www.graphpad.com). Statistical significance of increase in antibody avidity was tested using a Wilcoxon test to compare the geometric mean avidity indices of different immunizations.

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**Chapter 8: Antibodies elicited by a virosomally formulated  
*Plasmodium falciparum* serine repeat antigen-5 derived  
peptide detect the processed 47KDa fragment both in  
sporozoites and merozoites**

Prepared to submit to Infection&Immunity

**Antibodies elicited by a virosomally formulated *Plasmodium falciparum* serine repeat antigen-5 derived peptide detect the processed 47KDa fragment both in sporozoites and merozoites**

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Running title: Synthetic peptide malaria candidate vaccine

Key words: malaria vaccine, virosomes, synthetic peptides, serine repeat antigen 5

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**Abstract**

Serine repeat antigen 5 (SERA5) is a candidate antigen for inclusion into a malaria subunit vaccine. During merozoite release and reinvasion the 120 KDa SERA5 precursor protein (P120) is processed and a complex consisting of a N-terminal 47 KDa (P47) and a C-terminal 18 KDa (P18) processing product associates with the surface of merozoites. This complex is thought to be involved in merozoite invasion of and/or egress from host erythrocytes. Here we describe the synthesis and immunogenic properties of virosomally formulated synthetic phosphatidylethanolamine (PE)-peptide conjugates incorporating amino acid sequence stretches from the N-terminus of *Plasmodium falciparum* SERA5. Choice of an appropriate sequence was crucial for the development of a peptide that elicited high titers of parasite cross-reactive antibodies in mice. Monoclonal antibodies (mAbs) raised against the optimized peptide FB-23 incorporating amino acids 57 to 94 of SERA5 bound both to P120 and to P47. Western blotting analysis demonstrated the presence of P120 in erythrocytic forms and of P47 both in merozoites and in sporozoites. These analyses formally prove for the first time the presence of P47 SERA5 in sporozoites. In immunofluorescence assays the mAbs stained the parasitophorous vacuole in trophozoites and the parasite cell membrane in merozoites. The virosomal formulation of the peptide FB-23 is suitable for use in humans and represents a candidate component for a multi-valent malaria subunit vaccine that is targeting both sporozoites and blood stage parasites.

## Introduction

Vaccine development in the past two hundred years since Jenner has been for a long time an empirical approach. However, it appears that development of vaccines against chronic infectious diseases, such as malaria, tuberculosis and HIV/AIDS will only be possible, if more rational approaches based on the growing body of knowledge in immunology and parasite biology are applied. In particular for the design of subunit vaccines new antigen production and delivery technologies are required. Although a broad spectrum of approaches is being evaluated for subunit vaccine development against *P. falciparum* malaria (34), progress towards development of an effective vaccine is still limited (14). To circumvent problems associated with the large scale production of correctly folded recombinant proteins and of highly immunogenic and stable formulations, we are developing a universal technology platform for multi-valent subunit vaccine design (25, 27, 31) Okitsu et al. submitted, James et al. accepted), which is based on synthetic peptides delivered on the surface of immunopotentiating reconstituted influenza virosomes (IRIVs). IRIVs are spherical, unilamellar vesicles, prepared by detergent removal from a mixture of natural and synthetic phospholipids and influenza surface glycoproteins. The influenza membrane glycoprotein hemagglutinin is a fusion-inducing component, inducing antigen uptake by immunocompetent cells via receptor-mediated endocytosis. IRIVs are a universal antigen-delivery system for multi-valent subunit vaccines, since antigens can be either attached to their surface to elicit CD4 T cell and antibody responses or encapsulated in their lumen to elicit CD8 T cell responses. They have an excellent safety profile and are already registered for human use (36).

One of the target antigens for inclusion into a malaria vaccine is the serine repeat antigen 5 (SERA5). SERA5 belongs to a gene family comprising eight members (SERA1 to 8) on chromosome 2 and one (SERA9) on chromosome 9 in *P. falciparum* (13, 15). SERA5 expression is abundant and found in the trophozoite and schizont stages of parasite blood stage development, as well as in salivary gland sporozoites (1, 10, 21). Accompanied by the removal of a 22-amino acid signal sequence, the SERA5 precursor protein (120 kDa) is secreted into the parasitophorous vacuole (PV) space (4). SERA5 is the major PV protein during the schizont stage and is also found on the surface of the developing merozoites (2, 6, 10, 19, 22, 29). During merozoite release and reinvasion a fraction of SERA5 is proteolytically processed

into a 47 kDa N-terminal (P47), a 56 kDa central (P56), and an 18 kDa C-terminal domain (P18) (3-5, 7). P56 is further processed into two fragments with a size of 50 kDa and 6 kDa (P50 and P6) (4). A complex of P47 and P18 formed through disulfide bonds associates with the surface of merozoites, whereas P50 is released into the culture medium after merozoite release (22). The central domain of recombinantly expressed SERA5 (P50) has been shown to possess proteolytic activity, which may represent a functional activity of SERA5 involved in merozoites invasion of and/or egress from host erythrocytes (16). Antibodies against P47 have been associated with protection against severe malaria in children under five years (28) and a recombinantly expressed fragment of the same sequence has been shown to induce protective immune responses in Aotus monkeys (9, 17, 18).

In this report we describe the immunological characterization of synthetic peptides derived from the P47 sequence of SERA5. Mice were immunized with virosomal formulations of the peptides to determine their immunogenicity and in particular their capacity to elicit *P. falciparum* parasite cross-reactive antibodies. Comparative immunological analysis led to the design of the optimized peptide FB-23, which induces high titers of parasite-binding antibodies in a virosomal vaccine formulation.

## Results

### Design and synthesis of peptides

Previous studies by Fox et al. (11) indicated that a parasite inhibitory epitope exists between residues 17-73 of SERA5, possibly in the region 57-73. Based on these results, we first prepared the lipopeptide FB-15 (Figure 1), which contains residues 17-73 linked through its N-terminus and a succinate linker to an isomer of phosphatidylethanolamine (PE). The shorter mimetic DB-12 contains only the residues 48-73, again linked to the phospholipid.

This region of SERA5 contains several proline residues within motifs that appear to have a significant propensity to form turn-like conformations. At present no 3D structural information is available for the protein. Nevertheless, a small library of constrained peptidomimetics was synthesized, in an attempt to stabilize turn structures and so improve the ability of the mimetics to elicit antibodies that cross-react with the native protein. These constrained mimetics (DB-11, -14, -16 and -20 (Figure-1)) contain an amide link between the C-terminus and the 4-amino group of (2S,4S)-4-aminoproline, which was introduced into the sequence in place of Pro.

Other studies by Fox et al., (12) also suggested that inhibitory epitopes might be located in the fragment 17-165. In order to extend the mimetics in the C-terminal direction, we first prepared the mimetic FB-19. Since, however, this molecule was poorly water soluble, we also prepared mimetic FB-23, which showed much improved solubility.

### Immunogenicity of SERA5-derived peptides

BALB/c mice were immunized with 10 $\mu$ g doses of virosomally formulated synthetic peptides comprising aa 48-73, aa 17-73 or aa 57-94 of *P. falciparum* SERA5 (Figure 1).

Sera taken prior to the first and two weeks after the third immunization were analyzed for the development of (i) peptide specific IgG in peptide ELISA, (ii) SERA5-binding IgG in Western blotting with *P. falciparum* blood stage lysate and (iii) parasite cross-reactive IgG in IFA with *in vitro* cultivated blood stage parasites. All peptides tested were immunogenic, but most of them elicited only in part of the animals low titers of IgG that cross-reacted with schizonts in IFA or Western blotting (Table 1). Only the C-terminally extended peptide FB-19 (aa 57-94) elicited IFA and Western blotting titers  $\geq$ 100 in all immunized mice. Since solubility of FB-19 was low, it was modified

by addition of four lysine residues to yield a better soluble derivative. This easier to produce and to formulate optimized peptide, designated FB-23, had immunological properties comparable to those of FB-19. Sera from all ten FB-23 immunized mice were parasite cross-reactive both in IFA and Western blotting analysis (Table 1). The mean IFA titer ( $2512 \pm 4846$ ) was 10 to 100 fold higher than titers elicited by immunization with the peptides incorporating aa 47-73 or aa 17-73 of SERA5.

### **Generation and characterization of anti-FB-23 mAbs**

MAbs against the optimized peptide FB-23 were generated for a more detailed analysis of the specificity of the elicited antibody response. Five cell lines producing mAbs (designated SO10.1 to SO10.5) binding to FB-23 in ELISA were obtained from a FB-23 immunized BALB/c mouse. While all five mAbs were of the IgG1 isotype and bound to the two peptides (FB-19 and FB-23) that comprised aa 57-94, they differed in cross-reactivity with FB-15 (aa 17-73) in ELISA (Table 2). mAb SO10.3 showed strong, mAbs SO10.1 and SO10.4 weak and mAbs SO10.2 and SO10.5 no cross-reactivity with peptide FB-15. None of the mAbs was positive in ELISA with the peptides comprising aa 48-73 of SERA5.

### **Detection of SERA5 in *P. falciparum* parasites**

To test whether the mAbs recognized full length (120 KDa) SERA5, as well as its N-terminal 47KDa processing product, we performed Western blotting analyses with lysates of synchronized *in vitro* cultivated *P. falciparum* blood stage parasites. Samples were taken at different time-points from the synchronized cultures, thus representing ring, trophozoite or schizont stages. In addition, free merozoites isolated by MACS sorting were analysed. Full length SERA5 was stained by four of the five mAbs in Western blotting (Table 2). While ring stages were negative, first traces of full length SERA5 became detectable in the lysates corresponding to the late ring/early trophozoite stages taken from the cultures at 18 and 24 hours (Figure 2). Only in the merozoite lysate both the full length 120KDa and the processed 47kDa fragment were detectable by all four Western blotting positive mAbs (Figure 2). In Western blotting analysis with a sporozoite lysate only the processed P47 domain, but no full-length SERA5 was detected with SO10.1 and SO10.4 (Figure 3). The other three mAbs did not detect any of the SERA5 associated bands.

Changes in sub-cellular localisation of SERA5 during parasite blood stage development was analysed by IFA with the same synchronized parasite cultures as used for preparation of lysates for the Western blotting experiments. All five mAbs, including the Western blotting negative mAb SO10.2, yielded comparable results. In trophozoites a ring around the parasites was stained, providing further evidence for the proposed localization of SERA5 in the PV during this developmental stage (Figure 4A) (7). In contrast, a staining pattern characteristic for antigens associated with the cell membrane of the parasite was observed with segmented schizonts (Figure 4B) and free merozoites (Figure 4C). No SERA5-specific staining of sporozoites was obtained in IFA.

## Discussion

For our approach to develop a synthetic peptide-based multi-valent malaria vaccine (25, 27, 30), we employ an iterative selection process to design peptide structures with optimal immunogenic properties. Virosoomally formulated candidate antigens are used to immunize mice and a biologically relevant test system, semi-quantitative detection of parasite-binding antibodies by IFA, is used to guide step-wise selection of suitable structures (Ref). As described here for all SERA 5 derived peptides tested, virosoomally formulated peptide formulations are practically always immunogenic and elicit peptide-reactive IgG responses in ELISA. However, only a minority of parasite antigen derived peptides are able to consistently induce parasite cross-reactive antibodies. In some cases conformational stabilisation of peptide chains by intramolecular cross-linking is required for the design of compounds that structurally resemble surface loops of the native target protein response (25, 27, 30). In other cases, as demonstrated here for SERA 5 derived peptides, choice of an appropriate sequence stretch may be sufficient. Evidence that virosoomally formulated linear peptides can elicit antibodies against conformational epitopes (25, 27, 30) indicates, that linear peptides may be able to acquire a native-like conformation, in particular, when they are delivered structurally undisturbed on the surface of virosomes in the context of a phospholipid bilayer. In contrast, delivery of peptide antigens adsorbed to alum or other adjuvants, may disturb the structure even of conformationally restricted peptides profoundly (25).

Generation of sets of mAbs against a peptide antigen is a valuable tool to evaluate the quality of vaccine candidate components (Mueller, Moreno, Okitsu). One important quality criterion is the proportion of parasite binding mAbs within the population of peptide reactive antibodies elicited. In the case of the SERA 5 derived peptide FB-23 all five mAbs analysed bound to blood stage parasites in IFA with staining patterns expected for this antigen, i.e. staining of the PV in trophozoites and surface staining in schizonts and merozoites. While both the full-length SERA 5 (P120) and its processed fragment P47 were recognized by four of the five mAbs, one mAb was generally negative in Western blot analysis. Further evidence for diversity of the fine specificity of the mAbs came from the analysis of their cross-reactivity with the set of peptides evaluated for immunogenicity. Three of the mAbs reacted to a variable degree with the peptide comprising aa 17-73. This indicates that the sequence stretch aa 57-73, that overlaps with the FB23 sequence (aa 57-94)

comprises at least part of their epitope. Conformational aspects may be responsible for lack of binding to the C-terminally truncated peptide comprising only aa 48-73. Attempts to improve the anti-parasite cross-reactivity of responses against the sequence stretch aa 48-73 by introduction of intramolecular disulfide bonds failed and also none of the anti- FB-23 mAbs bound to the disulfide bond containing variants.

Western blotting results reconfirmed that processing in the blood-stage parasites is a late event as P47 was only found in merozoites and not in schizonts. For the first time we could formally prove the expression of SERA 5 at the protein level in salivary gland sporozoites. In these the protein was completely processed and only P47 was detectable. The function of SERA5 in sporozoites remains to be elucidated, but it is tempting to speculate that that this protein is involved in invasion and/or egress not only in the blood stage, but also in other life stages of the parasite. While the N-terminus of SERA5 is highly polymorphic, the sequence stretch represented by FB-23 (aa 57-94) is highly conserved (12). An alignment of 62 SERA5 sequences from African, Asian and South American *P. falciparum* isolates (10, 23, 26, 32) revealed only four isolates with polymorphisms (one or two amino acid exchanges at the N-terminal end) in the FB-23 sequence.

Taken together we were able to induce parasite cross-reactive antibodies with a human compatible formulation of a synthetic SERA5 peptide in mice. In a similar way we have developed virosomal formulations of peptides derived from *P. falciparum* circumsporozoite protein (CSP), apical membrane antigen 1 (AMA-1) and merozoite surface protein 1 (MSP-1) for inclusion into an IRIV-based multi-component malaria vaccine (27) (Okitsu et al, submitted, James et al. accepted). After successful testing of the CSP and the AMA-1 components in a phase I clinical trial (Genton et al., submitted), the SERA5 peptide is now considered as candidate for clinical profiling.

## Materials and methods

### Synthesis of peptides

*Mimetic FB-15.* The peptide was synthesized on Rink Amide MBHA resin (0.66 mmol/g, *Novabiochem*, Switzerland) using an ABI 433A peptide synthesizer and Fmoc chemistry. Couplings were performed using 4-fold excess (1 mmol) of each standard protected amino acid with extended coupling cycles. The synthesis was started with 380 mg (0.25 mmol) of resin. Following chain assembly on the resin, PE-Succ (see below) was coupled to the free N-terminus of the peptide on the resin, using a solution of PE-Succ-OH (3 eq.), HATU (3 eq.), HOAt (3 eq.) and  $i\text{Pr}_2\text{EtN}$  (8 eq.) in DMF, with agitation overnight at rt. The resin was then filtered and washed with DMF (3 x 5 ml),  $\text{CH}_2\text{Cl}_2$  (3 x 5 ml), and MeOH (3 x 5 ml). The mimetic was then cleaved from the resin and deprotected by treatment with  $\text{CF}_3\text{COOH}/i\text{-Pr}_3\text{SiH}/\text{H}_2\text{O}$  (95:2.5:2.5) for 3 h at rt. After filtration and concentration in vacuo, the peptide was precipitated with cold  $\text{Et}_2\text{O}$ , and washed 3 times with cold  $\text{Et}_2\text{O}$ . Purification of the crude product by semi-preparative reverse phase-HPLC ( $\text{C}_4$  column: Vydac 214TP1010;  $10\mu\text{m}$ ,  $300\text{ \AA}$ ,  $10 \times 250\text{ mm}$ ; gradient from 20-100% EtOH + 0.1% TFA in 15 min.) gave *FB-15*. MALDI-TOF-MS ( $m/z \pm 0.1\%$ ): 6048  $[\text{M}+\text{H}]^+$ , 3028  $[\text{M}+2\text{H}]^{2+}$  (calc. MW: 6045.5 g/mol).

*Mimetic FB-19.* This was synthesized in the same way as for *FB-15*, except that two pseudoproline ( $\Psi\text{Pro}$ ) building blocks were used (*Novabiochem*, Switzerland), Fmoc-ValSer( $\Psi^{\text{Me,Me}}\text{Pro}$ )-OH at positions Val(19)-Ser(20) and Val(24)-Ser(25). ESI-MS ( $m/z \pm 0.01\%$ ): 1526.8  $[\text{M}+3\text{H}]^{3+}$ , 1145.4  $[\text{M}+4\text{H}]^{4+}$  (calc. MW: 4578.0 g/mol).

*Mimetic FB-23.* This was synthesized in the same way as for *FB-15*, except that five pseudoproline ( $\Psi\text{Pro}$ ) building blocks were used (*Novabiochem*, Switzerland); Fmoc-ValSer( $\Psi^{\text{Me,Me}}\text{Pro}$ )-OH at positions Val(17)-Ser(18), Val(23)-Ser(24) and Val(28)-Ser(29), and Fmoc-SerSer( $\Psi^{\text{Me,Me}}\text{Pro}$ )-OH at Ser(10)-Ser(11) and Ser(34)-Ser(35). MALDI-TOF-MS ( $m/z \pm 0.1\%$ ): 5089.4  $[\text{M}+\text{H}]^+$  (calc. MW: 5090.7 g/mol).

*Mimetic DB-12.* This was synthesized in the same way as for *FB-15*. ESI-MS ( $m/z \pm 0.01\%$ ): 1563.8  $[\text{M}+2\text{H}]^{2+}$ , 3126.7  $[\text{M}+\text{H}]^+$  (calc. MW: 3125.4 g/mol).

*Cyclic mimetics DB-11, -14, -16 and -20.* These mimetics were synthesized in the following 7 step procedure:

Step-1. The linear peptide sequence was assembled by solid phase peptide synthesis, using Fmoc chemistry and chlorotriyl chloride resin, with an initial loading

of 0.2-0.3 mmol/g. N( $\alpha$ )-Fmoc(2S,4S)-4-(Alloc)aminoproline ((2S,4S)-allyloxycarbonyl-4-amino-1-Fmoc-pyrrolidine-2-carboxylic acid) was incorporated in place of the desired proline. At the end of the chain assembly, the N-terminal Fmoc protecting group was not removed.

Step-2. The Alloc protecting group was removed by stirring the resin-bound peptide with Pd(PPh<sub>3</sub>)<sub>4</sub> and PhSiH<sub>3</sub> in CH<sub>2</sub>Cl<sub>2</sub> for 3 h at rt under Ar. The resin was then filtered and washed four times with DMF and 3 times with CH<sub>2</sub>Cl<sub>2</sub>.

Step-3. The peptide chain was cleaved from the resin by stirring in a solution of 0.6% CF<sub>3</sub>COOH in CH<sub>2</sub>Cl<sub>2</sub> for 1 h at rt. The solution was washed with brine and then evaporated to dryness.

Step-4. For cyclization, the peptide chain from above was treated with HATU and HOAt (each 6 eq.) and iPr<sub>2</sub>EtN (12 eq.) in dry DMF (peptide concentration 1 mg/ml) for 72 h at rt. The DMF was then removed under vacuo, and the residue was redissolved in CH<sub>2</sub>Cl<sub>2</sub> and washed twice with brine. The solution was then dried over Na<sub>2</sub>SO<sub>4</sub> and dried under vacuo.

Step-5. To remove the Fmoc-group, the peptide from above was dissolved in a solution of piperidine (20%) in DMF and stirred for 1 h at rt. The solution was then dried under vacuo and the desired product was purified by preparative reverse phase HPLC.

Step-6. For coupling to the phospholipid, PE-Succ-OH was made by reacting 1,3-dipalmitoyl-glycero-2-phospho-ethanolamine (*Bachem*, Switzerland) (1 g, 1.45 mmol) in CHCl<sub>3</sub> / MeOH (9:1, 100 ml) with succinic anhydride (203 mg, 2.03 mmol, 1.4 eq.) in CH<sub>2</sub>Cl<sub>2</sub> (100 ml) and Et<sub>3</sub>N (600 ml, 4.31 mmol, 3 eq.). The solution was stirred for 5 h at rt. Then CHCl<sub>3</sub> (100 ml) and 0.02 M citric acid/sodium phosphate buffer (100 ml, pH 5.5) were added and the aqueous phase was extracted with CHCl<sub>3</sub>. The combined organic phases were separated, dried over Na<sub>2</sub>SO<sub>4</sub> and solvent evaporated to give the product PE-Succ-OH (1.12 g, 98% yield). ESI-MS (m/z  $\pm$  0.01%): 790.5[M+H]<sup>+</sup>, 394.7[M+2H]<sup>2+</sup>. The cyclic peptide was then coupled to PE-Succ-OH in DMF with HATU (3 eq.) HOAt (3 eq.) and Et<sub>3</sub>N (6 eq.). After stirring at rt for 72 h, the DMF was removed under vacuo, the residue was dissolved in CH<sub>2</sub>Cl<sub>2</sub> and washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub> and evaporated to dryness.

Step-7. For deprotection, the product from above was dissolved in CF<sub>3</sub>COOH containing H<sub>2</sub>O (2.5 v/v%) and iPr<sub>3</sub>SiH (2.5%) and stirred at rt for 1 h. The solution was then evaporated to dryness under vacuo, and the product was purified by

preparative reverse phase HPLC. The product was obtained in >95% purity and was characterized by mass spectrometry. *DB-11*, ESI-MS ( $m/z \pm 0.01\%$ ): 1562.7  $[M+2H]^{2+}$ , 1042.3  $[M+3H]^{3+}$  (calc. MW: 3123.4 g/mol); *DB-14*, ESI-ES ( $m/z \pm 0.01\%$ ): 3125.1  $[M+H]^+$ , 1563  $[M+2H]^{2+}$ , 1042  $[M+3H]^{3+}$  (calc. MW: 3123.4 g/mol); *DB-16*, ESI-ES ( $m/z \pm 0.01\%$ ): 3124.4  $[M+H]^+$ , 1563  $[M+2H]^{2+}$ , 1042  $[M+3H]^{3+}$  (calc. MW: 3123.4 g/mol); *DB-20*, ESI-ES ( $m/z \pm 0.01\%$ ): 3124.6  $[M+H]^+$ , 1562.8  $[M+2H]^{2+}$ , 1042.2  $[M+3H]^{3+}$  (calc. MW: 3123.4 g/mol).

Sequence from *P. falciparum* isolate UF-5 (Indonesia); accession BAA78509; (23)

### **Preparation of peptide-loaded virosomes**

For the preparation of IRIV loaded with phosphatidylethanolamine (PE)-peptide conjugates, a solution of purified Influenza A/Singapore hemagglutinin (4 mg) in phosphate buffered saline (PBS) was centrifuged for 30 min at 100 000 g and the pellet was dissolved in PBS (1.33 ml) containing 100 mM octaethyleneglycolmonodecylether (PBS-OEG). Peptide-PE conjugates (4 mg), phosphatidylcholine (32 mg; Lipoid, Ludwigshafen, Germany) and PE (6 mg) were dissolved in a total volume of 2.66 ml of PBS-OEG. The phospholipid and the hemagglutinin solutions were mixed and sonicated for 1 min. This solution was then centrifuged for 1 hour at 100 000 g and the supernatant was filtered (0.22  $\mu\text{m}$ ) under sterile conditions. Virosomes were then formed by detergent removal using BioRad SM BioBeads (BioRad, Glattbrugg, Switzerland).

### **Animal studies**

All procedures involving living animals were performed in accordance with the Rules and Regulations for the Protection of Animal Rights (Tierschutzverordnung) of the Swiss Bundesamt für Veterinärwesen.

### **Mouse immunogenicity studies**

BALB/c mice were pre-immunized intramuscularly with 0.1 ml of the commercial whole virus influenza vaccine Inflexal Berna<sup>TM</sup> (Berna Biotech, Bern, Switzerland). At least three weeks later they were immunized with PE-peptide conjugate-loaded IRIVs (containing 10 $\mu\text{g}$  PE-peptide) in intervals of at least two weeks. Blood was collected before each immunization and two weeks after the final injection.

### **Generation of hybridomas and production of mAbs**

Hybridomas were generated from spleen cells of mice three days after an intravenous booster immunization with FB-23 loaded IRIVs using PAI mouse myeloma cells as a fusion partner. Hybrids were selected in HAT medium and cells that secreted anti-BP65 mAbs were identified by ELISA. For large-scale mAb production, hybridoma cell lines were cultured in 175 cm<sup>2</sup> flasks and mAbs were purified by protein A or G affinity chromatography (Protein A Sepharose<sup>™</sup> CL4B or HiTrap<sup>™</sup> Protein G HP, Piscataway, NJ). Purified mAbs were dialyzed against PBS, aliquoted and stored at -80°C. Generation of anti-*P. falciparum* sporozoite mAbs has been described previously (33).

### **ELISA**

ELISA analyses with peptide-PE conjugates were performed essentially as described before (27). Briefly, Polysorp<sup>™</sup> plates (Nunc, Fisher Scientific, Wohlen, Switzerland) were coated overnight at 4°C with 100 µl of a 10 µg/ml solution of UK39 in PBS (pH 7.2). After three washings with PBS containing 0.05% Tween-20 wells were blocked with 5% milk powder in PBS for 30 min at 37°C and washed three times again. Plates were then incubated with serial dilutions of anti-peptide mouse or anti-peptide mAbs in PBS containing 0.05% Tween-20 and 0.5% milk powder for 2 h at 37°C. After washing, plates were incubated with alkaline phosphatase-conjugated goat anti-mouse IgG (Fc-specific) antibodies (Sigma, St. Louis, Mo) for 1 h at 37°C. After washing again, phosphatase substrate solution (1 mg/ml p-nitrophenyl phosphate (Sigma, St. Louis, Mo) in a pH 9.8 buffer solution containing 10% [vol/vol] diethanolamine and 0.02% MgCl<sub>2</sub>) was added and the plates were incubated in the dark at room temperature until the colorimetric reaction had progressed sufficiently. The optical density was measured at 405 nm on a Titertek Multiscan MCC/340 reader (LAbSystems, Helsinki, Finland).

### **Indirect immunofluorescence assay (IFA)**

Air-dried unfixed *P. falciparum* (strain NF54) salivary gland sporozoites attached to microscope glass slides were incubated for 15 min at room temperature with 25 µl blocking solution containing 1% fatty acid-free bovine serum albumin (BSA) in PBS. Immunostaining was performed by incubating the wells with 25 µl of an appropriate serum dilution in blocking solution in a humid chamber for 1 h at room temperature.

After five washes with blocking solution 25  $\mu$ l of 5  $\mu$ g/ml cyanine dye (Cy3)-conjugated affinity-pure F(ab')<sub>2</sub> fragment goat anti-mouse IgG (Fc-specific) antibodies (Jackson Immuno Research Laboratories, West Grove, Pa.) or Cy3-conjugated donkey anti-rabbit IgG heavy and light chain antibody (Jackson Immuno Research Laboratories, West Grove, PA), diluted in blocking solution containing 0.01 mg/ml Hoechst dye no. 33256 (Sigma, St. Louis, Mo) were added to the wells and incubated for 1 h at room temperature. Finally, wells were washed five times with PBS, mounted with mounting solution (90% [vol/vol] glycerol containing 0.1 M Tris-Cl, pH 8.0 and 2 mg/ml *o*-phenylenediamine) and covered with a coverslip. Antibody binding and DNA staining were assessed by fluorescence microscopy on a Leitz Dialux 20 or a Leica DM5000 fluorescence microscope and documented with a Leica DC200 digital camera system. For IFAs with blood stage parasites 5  $\mu$ l infected erythrocytes from an *in vitro* culture (24) were used to prepare thin smears. These were fixed in 50/50 acetone/methanol (vol/vol) for 2 min at -20°C. Alternatively, multiwell immunofluorescence microscopy slides (Flow Laboratories, Baar, Switzerland) were treated with 0.01% poly-L-lysine (Sigma) at room temperature for 30 min and washed five times with RPMI basal salts medium (Gibco-BRL, Basel, Switzerland). Erythrocytes from *in vitro* cultures (24) of *P. falciparum* strain K1 with a parasitemia between 5 and 10% were washed twice in RPMI and resuspended in RPMI and 2 volumes of a solution containing 4% formaldehyde and 0.1% Triton X-100. From this cell suspension, 30  $\mu$ l was added to each well, incubated at room temperature for 30 min, and washed five times with PBS. Blocking and incubation with sera or mAbs was done as described above.

### **SDS-PAGE and immunoblotting**

100  $\mu$ l of an *Anopheles stephensi* salivary gland lysate containing about 60000 *P. falciparum* sporozoites were diluted with an equal volume of 2x loading buffer (1.7ml, 0.5M Tris-HCl pH 6.8, 2 ml glycerol, 4.5 ml 10% SDS, 1 ml  $\beta$ -mercaptoethanol, 0.8 ml 0.3% w/v bromophenol blue) and heated to 95°C for 10 minutes. Proteins were separated on a 10% SDS PAGE mini-gel. Separated proteins were electrophoretically transferred to a nitrocellulose filter (Protran® Nitrocellulose, BA85, Schleicher & Schuell) by semi-dry blotting. Blots were blocked with PBS containing 5% milk powder and 0.1% Tween-20 over night at 4°C. The filter was cut into strips

and incubated with appropriate dilutions of immune sera in blocking buffer for 2 h at room temperature. Filter strips were then washed three times for 10 minutes in blocking buffer and incubated at room temperature for 1 h with alkaline peroxidase-conjugated goat anti-mouse IgG (Fc-specific) antibodies (Sigma, St. Louis, Mo) diluted 1:30'000 in blocking buffer or horseradish peroxidase-conjugated goat anti-rabbit IgG heavy and light chain antibodies (Bio-Rad Laboratories, Hercules, CA) diluted 1:6'000 in blocking buffer. After washing again, blots were finally developed using ECL™ Western blotting detection (Amersham Biosciences, Buckinghamshire, England) reagents to visualize bands.

Blood stage parasite lysates were prepared essentially as described previously (24) by saponin lysis of *P. falciparum* K1-infected erythrocytes. In brief, cultured parasites were washed once with PBS. Pelleted infected red blood cells were lysed by mixing with a large volume (adjusted to 5% hematocrit) of 0.015% (wt/vol) saponin in 150 mM NaCl and 15 mM sodium citrate (pH 7.0) and incubated on ice for 20 min. Finally, the pelleted parasites were resuspended in 3 volumes of PBS and stored at -80°C until further use. A total of 50 µl of parasite lysate was solubilized in an equal volume of 2x loading buffer (1.7 ml of 0.5 M Tris-HCl [pH 6.8], 2 ml of glycerol, 4.5 ml of 10% sodium dodecyl sulfate, 1 ml of β-mercaptoethanol, 0.8 ml of bromophenol blue [0.3%, wt/vol]) and heated to 95°C for 10 min. Proteins were separated on an SDS-10% PAGE minigel and transferred to a nitrocellulose membrane as described above.

### **Parasite culture**

*P. falciparum* strain K1 was cultured essentially as described previously (24). The culture medium was supplemented with 0.5% AlbuMAX (Gibco, Paisley, Scotland) as a substitute for human serum (8). Synchronization of cultures was achieved by sorbitol treatment as described previously (20). Merozoites were isolated after parasite synchronization using MACS sorting (35) and continued parasite culture until schizont rupture. After rupture parasites were again passed through a MACS column and the flow through containing merozoites was collected. Serogroup A erythrocytes for passages were obtained from the Swiss Red Cross (Basel, Switzerland).

### **Blood-stage parasite synchronization.**

Parasites were synchronized using D-sorbitol, as described before (20). Merozoites were isolated after parasite synchronization using MACS sorting (35) and continued

parasite culture until schizont rupture. After rupture parasites were again passed through a MACS column and the flow through containing merozoites collected.

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## Figure legends

Figure 1. SERA5 processing products and SERA5-derived synthetic peptides. A: Proteolytic processing of SERA5. The shaded box indicates the sequence stretch (amino acids 17-94) comprising the synthetic peptides evaluated in this study and the filled box the signal peptide. Ser = serine repeat region. B: Structures of the peptidomimetics prepared in this work. The cyclic peptides contain a covalent amide link between the C-terminus (His<sup>73</sup>) and the 4-amino group in (2S,4S)-4-aminoproline (denoted X in the sequence).

Figure 2. Western blotting analysis with anti-FB-23 mAb SO10.3 using parasite lysates taken every 6 hours from synchronized *P. falciparum* blood stage cultures (0-42 hours). Merozoites (M) were purified using a MACS column. The anti-GAPDH mAb 1.10d served as a loading control. Comparable results were obtained with the other three Western blotting positive mAbs (Table 2).

Figure 3. Western blotting with a lysate of salivary glands of infected mosquitoes (lane 1: mAb SO10.1; 2: mAb SO10.2; 3: mAb SO10.3; 4: mAb SO10.4; 5: mAb SO10.5) or with a blood stage merozoite lysate (lane 6: mAb SO10.3).

Figure 4. Parasites were stained with anti-FB-23 mAb SO10.3 and a secondary antibody conjugated to Cy3. Nuclei were stained using Hoechst dye no. 33256. A: Localization of SERA5 in the parasitophorous vacuole in trophozoites. B: association of SERA5 with the parasite membrane of segmented schizonts. C: association of SERA5 with the membrane of free merozoites (mAb SO10.1).

Table 1. Sera obtained prior to the first and after the third immunization were compared. Peptide ELISA: the mean halfmaximal IgG titer  $\pm$ SD of immune sera and the proportion of animals that seroconverted are shown; IFA: the proportion of animals that had developed blood stage parasite cross-reactive IgG at a serum dilution of 1:50 is shown; Western blotting analysis: the proportion of immune sera that showed reactivity with a band of appropriate size (120 KDa) at a serum dilution of 1:100 is shown. None of the pre-immune sera was positive in IFA or Western blotting analysis. \* see Fig. 1 for type of cyclization.

Table 2. Cross-reactivity of anti FB-23 mAbs with SERA5-derived peptides in ELISA and with *P. falciparum* sporozoites and blood-stage parasites in IFA and Western blotting analysis. \*P120: detection of full length SERA5; P47: detection of processed SERA5 P47 fragment.

## Figures and Tables

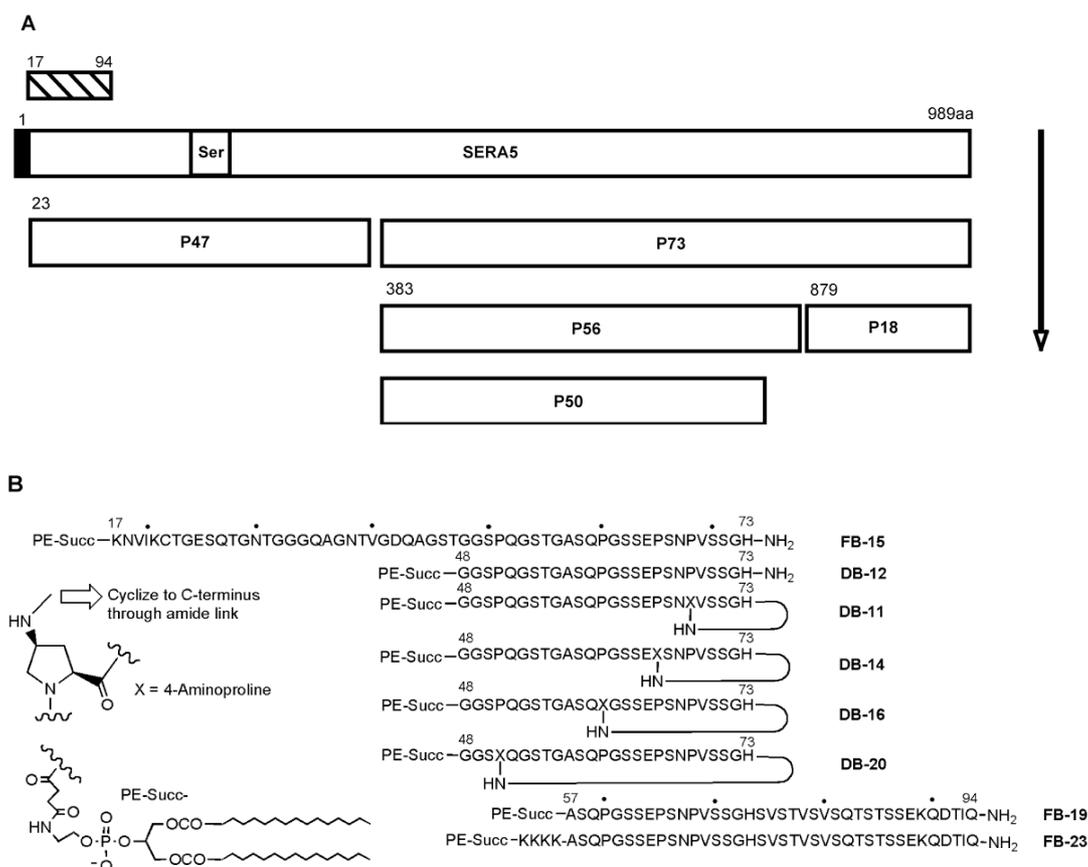


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Peptide	DB-012	DB-011	DB-014	DB-016	DB-020	FB-15	FB-19	FB-23
SERA5 aa sequence	48-73	48-73	48-73	48-73	48-73	17-73	57-94	57-94 + 4 lysines
Description	linear	cyclized*	cyclized*	cyclized*	cyclized*	linear	linear	linear
Mean ELISA titer	100±147	141±155	1006±379	157±103	346±58	276±428	1056±438	633±599
Responders in ELISA	5/5	4/4	4/4	4/4	4/4	5/5	5/5	10/10
Responders in IFA	4/5	0/4	2/4	3/4	2/4	4/5	5/5	10/10
Responders in Western blot analysis	1/5	1/4	0/4	0/4	0/4	3/5	5/5	10/10

Table 1. Immune responses elicited by SERA5 derived peptides. Sera obtained prior to the first and after the third immunization were compared. Peptide ELISA: the mean halfmaximal IgG titer  $\pm$ SD of immune sera and the proportion of animals that seroconverted are shown; IFA: the proportion of animals that had developed blood stage parasite cross-reactive IgG at a serum dilution of 1:50 is shown; Western blotting analysis: the proportion of immune sera that showed reactivity with a band of appropriate size (120 KDa) at a serum dilution of 1:100 is shown. None of the pre-immune sera was positive in IFA or Western blotting analysis. \* see Fig. 1 for type of cyclization.

peptide									schizonts		sporozoites			
	DB-011	DB-012	DB-014	DB-016	DB-020	FB-15	FB-19	FB-23	IFA	Western blot	IFA	Western blot		
	48-73	48-73	48-73	48-73	48-73	17-73	57-94	57-94		P120*	P47*	P120*	P47*	
SO10.1	-	-	-	-	-	(+)	+	+	+	+	+	-	-	+
SO10.2	-	-	-	-	-	-	+	+	+	-	-	-	-	-
SO10.3	-	-	-	-	-	+	+	+	+	+	+	-	-	-
SO10.4	-	-	-	-	-	(+)	+	+	+	+	+	-	-	+
SO10.5	-	-	-	-	-	-	+	+	+	+	+	-	-	-

Table 2. Cross-reactivity of anti FB-23 mAbs with SERA5-derived peptides in ELISA and with *P. falciparum* sporozoites and blood-stage parasites in IFA and Western blotting analysis. \*P120: detection of full length SERA5; P47: detection of processed SERA5 P47 fragment.

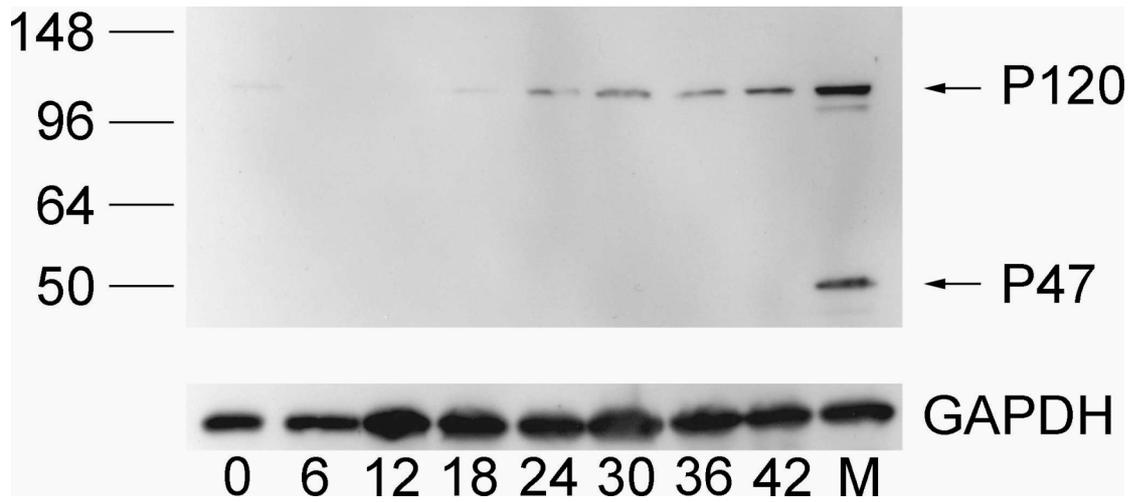


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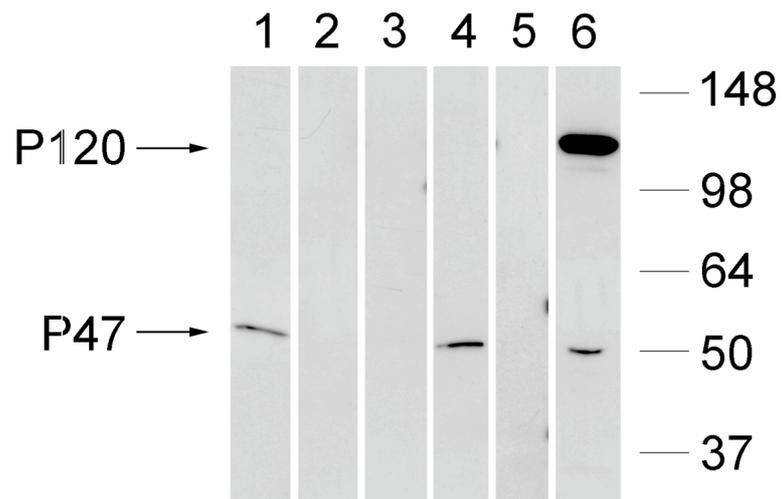


Figure 3. SERA5 expression in sporozoites. Western blotting with a lysate of salivary glands of infected mosquitoes (lane 1: mAb SO10.1; 2: mAb SO10.2; 3: mAb SO10.3; 4: mAb SO10.4; 5: mAb SO10.5) or with a blood stage merozoite lysate (lane 6: mAb SO10.3).

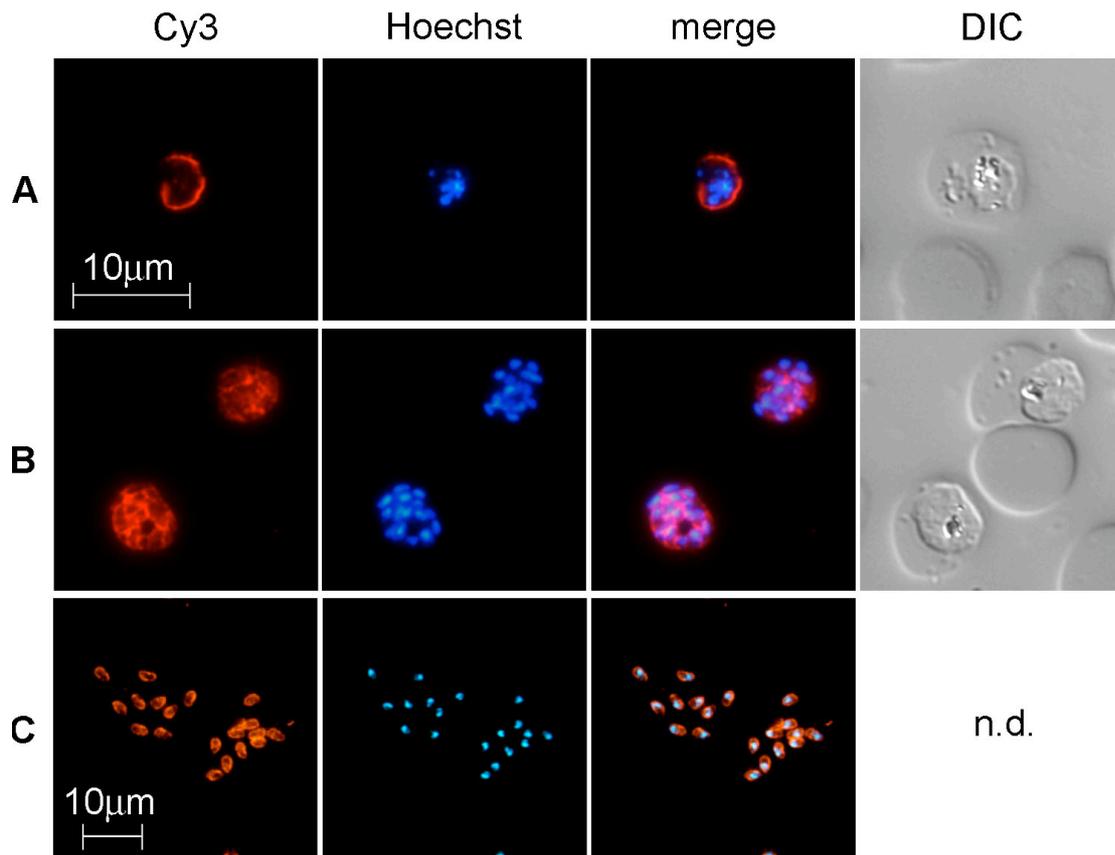


Figure 4. IFA with *P. falciparum* blood stage parasites. Parasites were stained with anti-FB-23 mAb SO10.3 and a secondary antibody conjugated to Cy3. Nuclei were stained using Hoechst dye no. 33256. A: Localization of SERA5 in the parasitophorous vacuole in trophozoites. B: association of SERA5 with the parasite membrane of segmented schizonts. C: association of SERA5 with the membrane of free merozoites (mAb SO10.1).



## **Chapter 9: General Discussion and Conclusions**

## 9.1 General Discussion and Conclusions

It is now over 30 years since it has been shown that man can be protected against malaria by vaccination<sup>1</sup>. In the meantime, many important advances in malaria vaccine development have taken place and a promising candidate, RTS,S/AS02A, is the first pre-erythrocytic vaccine to show clear protection against natural *P. falciparum* infection<sup>2-5</sup>. However, a safe, effective and affordable vaccine, that provides sustained protection to the residents of malaria endemic areas, is still many years away.

History shows that vaccines are most easily developed for those organisms that induce natural immunity after a single infection. For malaria, partial anti-parasite immunity develops only after several years of endemic exposure<sup>6</sup>. This inefficient induction of immunity is partly a result of antigenic polymorphism, poor immunogenicity of individual antigens, the ability of the parasite to interfere with the development of immune responses and to cause apoptosis of effector and memory T and B cells, and the interaction of maternal and neonatal immunity (reviewed in Bolad and Berzins, 2000; Good et al., 2004)<sup>7,8</sup>. Vaccine development is widely recognized as one of the most cost-effective ways to improve public health and to protect humans against infectious diseases. According to the Jordan Report<sup>9</sup> 75% of all vaccine candidates in Phase 3 trials are attempting to induce full immunity using either recombinant or synthetic subunits. It is difficult to induce immunity with subunits and, of the vaccines currently in general use, only 25% are subunit-based. One appeal of subunit vaccine technology is that, for some pathogens, it is difficult or impossible to grow whole native organisms *in vitro*, leaving little alternative to a subunit approach. A further appeal is that the ideal subunit vaccine will induce immune responses against only those determinants relevant to protection, thus minimizing the possibility of deleterious responses.

This thesis describes the detailed immunological characterization of synthetic peptide components for inclusion into a multivalent subunit malaria vaccine based on virosomal technology from preclinical development and profiling to a phase 1 safety and immunogenicity study in Caucasians.

### **9.1.1 A technology platform for the stepwise development and evaluation of synthetic peptide vaccine components**

*P. falciparum* candidate proteins were chosen on the basis of a known essential function in the host cell invasion process. Criteria for epitope selection include known induction of parasite inhibitory antibodies, sequence conservation and secondary structure motifs indicating surface exposition. Based on these criteria we started with the design of a series of peptides derived from *P. falciparum* antigens. The peptides were short in order to focus the immune response on protection-relevant epitopes. *P. falciparum* is known for various immune evasion mechanisms, like the presentation of irrelevant but immunodominant epitopes, inducing a non-protective immune response of the host. The epitope-focussed approach with short peptides should avoid such a useless response and induce only parasite-inhibitory antibodies. In this thesis we describe the development, evaluation and characterization process for CSP, AMA-1, and SERA5 derived peptides. All candidate peptides were hooked to virosomes via a phosphatidylethanolamine (PE) anchor for non-covalent linking to the membrane of virosomes. The key readout system for the evaluation of peptide quality and immunogenicity were parasite-binding properties of the elicited antibodies in IFA with either *P. falciparum* sporozoites or blood-stage parasites. The peptide sequences and/or conformations were optimized in cycles starting from a lead structure (Figure 1). Candidate peptides with satisfactory immunological properties were then ready to enter preclinical profiling and clinical testing.

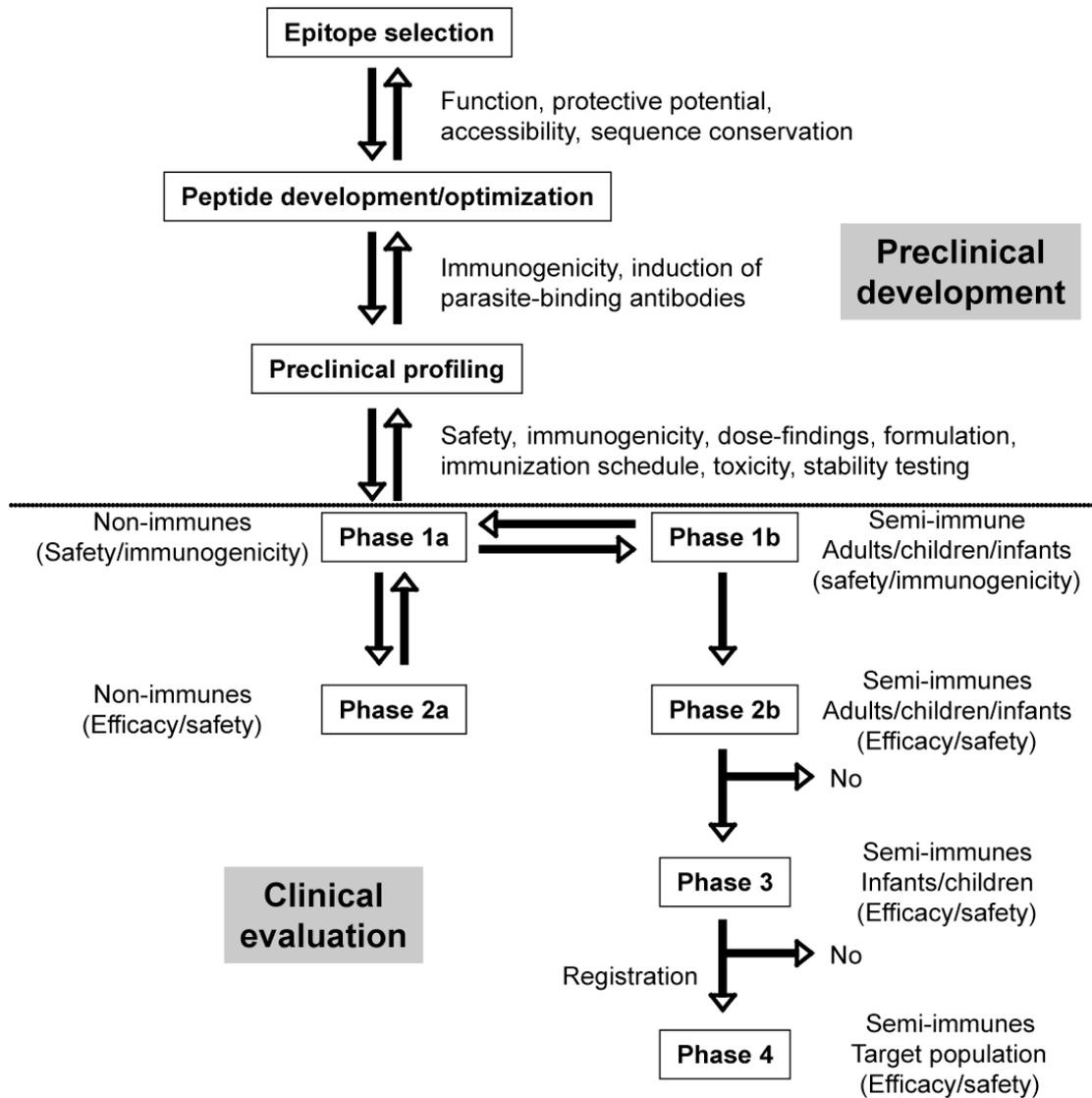


Figure 1. IRIV-based malaria vaccine development: from peptide antigen development to clinical evaluation.

In the case of CSP derived peptides we have shown that optimal conformational restriction of the peptide was crucial for the induction of parasite-binding antibodies in mice. First generation peptides with single or double loops of NANP repeats hooked to a rigid template were highly immunogenic but only low titers of parasite cross-reactive IgG were induced<sup>10</sup>. Alum-adjuvanted formulations of the same peptides induced higher titers of IgG than IRIV formulations but were inferior in terms of cross-reactivity of the induced antibodies with sporozoites, indicating interference of Alum with the peptide conformation<sup>10</sup>. Only internally cyclized and more flexible NANP peptides BP65<sup>11</sup> and UK-39 were good at eliciting parasite-binding antibodies. This more flexible type of conformation is close to the natural conformation of the NANP

repeats found on the surface of sporozoites, as indicated by NMR and crystallization studies. Another example of a conformationally restricted peptide is based on *P. falciparum* merozoite surface protein 1 (MSP-1). The MSP-1 19 kDa fragment is a rare example of a *P. falciparum* protein where the detailed structure has been elucidated by crystallization studies<sup>12</sup>. We have shown recently that peptides of the N-terminal epidermal growth factor (EGF)-like domain can be chemically synthesized and efficiently refolded to a native-like state (James et al. manuscript accepted). Only correctly folded peptides with all three disulfide bonds in place induced parasite-binding antibodies but not peptides with blocked cysteines. Moreover antibody binding to *P. falciparum* blood-stage parasites was abolished by competition with natively folded peptide but not with a linear one. Results with these two antigenic structures requiring conformational restriction of peptidomimetics are in contrast to results with peptides derived from the N-terminal region of SERA5 presented in this thesis. Optimal immunogenicity and induction of parasite-binding antibodies was depending on the right sequence stretch used for immunization. Cyclization of peptides via amide bonds had no positive influence on the parasite-binding properties of the antibodies induced after immunization of mice. SERA5 is a protein for which no structural information is available, therefore we cannot conclude whether the optimized peptide FB-23 has a conformation close to the natural N-terminal domain of the protein or not. Another example of a peptide where restriction of conformation was not needed is AMA49-CPE<sup>13</sup>. The optimized peptide AMA49-CPE seems to have enough internal driving force to adopt a conformation to elicit parasite cross-reactive antibodies, which recognize a discontinuous epitope. The cyclized form of this peptide had comparable immunogenic properties as the linear form<sup>13</sup>. AMA-1 is an example of an antigen, where induction of parasite inhibitory antibodies has been reported without a detailed characterization and localization of the epitope. In such cases peptide libraries covering the whole protein sequence can help selecting an epitope inducing inhibitory antibodies.

Taken together these examples show the importance of a detailed preclinical profiling and optimization of every candidate antigen for inclusion into a malaria vaccine. Synthetic peptide chemistry strongly facilitates this approach, allowing rational stepwise peptide optimization based on structure-activity observations. Moreover peptide delivery with IRIVs does not interfere with peptide conformation as it was observed for Alum. Our technology and evaluation platform, which was established

during the last nine years, is now available for the swift design, evaluation and optimization of new candidate peptides for inclusion into a multi-component subunit malaria vaccine.

### 9.1.2 Comparison of animal models and human studies

In the phase 1 study conducted with UK-39 and AMA49-CPE the same immunological evaluations were performed as in the preclinical profiling studies in animals. Peptide-ELISA to show immunogenicity of virosomally formulated peptides and to study the development of an anti-peptide response, IFA with *P. falciparum* parasites as key readout system to estimate the proportion of responders producing parasite-binding antibodies, and Western blot to confirm specific recognition of the parasite-derived antigen. In an attempt to gain indirect evidence for the induction of long-lived antigen-specific memory B cells upon vaccination we performed competition ELISAs with chaotropic salt to measure avidity of peptide-specific IgG. Parasite inhibition assays were done to assess a biological function of the induced antibody response and additionally ELISPOT and T cell proliferation assays were used to investigate the cellular immune response to the candidate vaccine (data not shown). Virosomal formulations of both UK-39 and AMA49-CPE were safe and immunogenic. Vaccine-induced anti-UK-39 IgG was parasite-binding and growth-inhibitory in a dose-dependent manner. Some of the AMA49-CPE positive sera were also parasite cross-reactive but not growth-inhibitory (data not shown). Antibody avidity was increasing over the course of immunization for both peptides, indicating formation of long-lived memory B cells. The presence of anti-peptide IgG titers one year after the third immunization supports this hypothesis.

Generally, the humoral response induced in humans by vaccination with virosomally formulated UK-39 and AMA49-CPE was comparable to the one in animal models. Two immunizations with a 10 $\mu$ g dose of UK-39 and with a 50 $\mu$ g dose of AMA49-CPE were enough to induce high anti-peptide titers in mice, rabbits and humans. After immunization with an appropriate dose of UK-39, sera were always cross-reactive with *P. falciparum* sporozoites and inhibited sporozoite invasion and growth in a dose dependent manner. Partial cross-reactivity of the antibody response after immunization with AMA49-CPE and problems with pre-existing vaccine-unrelated

blood-stage parasite cross-reactive IgG in humans closely resembled the results obtained in the rabbit model. The main difference observed between animal models and humans was a higher variability of the induced immune responses in humans, which is probably due to more immunogenetic variability in human volunteers.

Overall these findings confirm the suitability of the used animal models for immunogenicity, safety and dose-finding studies during design, optimization and pre-clinical profiling of virosomally-formulated candidate peptides.

### 9.1.3 Missing correlates of protection

After preclinical profiling and a phase 1a safety and immunogenicity study the question remains open whether our vaccine candidate components show any efficacy in protection from malaria. Conventional immunological tests provide information on dose and vaccination schedule required but they cannot predict whether a vaccine will be successful in protecting against malaria. The only available *in vitro* tests to measure the biological activity of elicited antibodies are parasite growth inhibition assays (GIAs). GIAs compare invasion, growth and development of parasites in the absence and presence of sera or purified antibodies. GIAs can be performed with *P. falciparum* sporozoites isolated from mosquito salivary glands cultured in primary human hepatocytes or with blood-stage parasites cultured in human red blood cells. Inhibition assays are very fragile test systems and results have to be interpreted carefully. Reproducibility of results is often difficult, especially with low rates of inhibition. These are not necessarily mediated by antibodies but can also be seen with non-Ig components of human sera<sup>14</sup>. Correlations between GIA results, antibody titers and protection from disease were found after immunization with recombinant MSP-1 in the *Aotus nancymai* model but similar studies of *in vitro* correlates of protection in human trials are still missing<sup>15</sup>. Despite all these constraints parasite growth-inhibitory antibody responses can be identified with GIAs and give a first idea about a possible functional activity of a vaccine induced antibody response. As long as no other correlates of protection are available, GIAs will remain the gold standard for *in vitro* activity of anti-parasite antibody responses and are often used as selection criterion for competing vaccine candidates during preclinical development.

Small (rodent) animal models are not available for infection with *P. falciparum*. *Plasmodium* species infecting rodents are available. However since most laboratory strains of *Plasmodium* are not natural pathogens of the laboratory mouse, and since the different parasite-mouse strain combinations can differ in virulence of the pathogen and in mechanisms of elimination by the host, these models have to be used with care (summarized in Langhorne et al., 2004)<sup>16</sup>. Another complication is the fact that many of the *P. falciparum* antigen candidates are either not present or antigenically different in those *Plasmodium* species. Transgenic *P. berghei* strains carrying hybrid *P. falciparum* proteins of CSP<sup>17</sup> and MSP-1<sup>18</sup> have been generated for murine studies but been of limited use in vaccine development up to now. Mice and rabbits are in many cases good models to study the safety and immunogenicity of candidate vaccines but fail to answer questions about protective efficacy of a vaccine. Non-human primates like *Pan troglodytes* and several *Aotus* species can be infected with *P. falciparum* as well as *P. vivax* for challenge studies, but parasites have to be adapted to these artificial hosts for successful infection. Besides that these primate animal models are difficult to access and detailed studies on the comparability of these models with natural infection of humans are still missing. Nevertheless non-human primate models can be useful in identifying optimal vaccine formulations and for conducting preclinical safety studies<sup>19</sup>. With DNA-based heterologous prime-boost immunization strategies, varying results were obtained in different animal models and humans<sup>20</sup>.

Besides large-scale phase 3 clinical trials, experimental sporozoite challenge of human volunteers is another possibility to test vaccine efficacy. This well established method has been used extensively in the case of the sporozoite vaccine RTS,S/AS02A and the above mentioned DNA-based vaccines. Despite a high rate of sterile protection in laboratory challenge trials RTS,S/AS02A failed to confer sterile protection in field trials<sup>2,3,5,21</sup>. Even though detailed studies on the immune mechanisms have been done during artificial challenge trials no clear mechanism could be identified accounting for sterile protection by RTS,S/AS02A<sup>22</sup>. Comparable results have been reported with DNA-based prime-boost strategies developed at the University of Oxford: selected vaccine formulations have shown some protection after challenge of non-immune volunteers but failed to show efficacy in field trials<sup>23,24</sup>. Sporozoite challenge of human volunteers at an early clinical stage is thus a unique and intriguing possibility to test malaria vaccine efficacy with few volunteers in a short

time but the relevance of challenge results for predicting clinical efficacy has still to be demonstrated. Sporozoite challenge cannot be used to determine the efficacy of blood-stage vaccines, as challenged volunteers are treated as soon as they develop patent blood-stage parasitemia. Therefore phase 2b trials remain the generally accepted method of establishing proof of principle for blood-stage vaccines. Some early, promising work has been performed with blood-stage challenge by inoculation of ultra-low doses of infected erythrocytes<sup>25</sup>. If this is further developed, it may form the basis of a challenge trial framework for phase 2a trial to evaluate blood-stage candidates.

While all these experimental human and animal challenge and *in vitro* model systems provide supporting evidence that an antigen was correctly chosen and presented to the immune system to induce a potent response, the predictive value for protective efficacy of these tools cannot be determined until the results are correlated with clinical efficacy of vaccine candidates.

To improve our understanding of protection against malaria, various studies have been investigating immune responses underlying semi-immunity in endemic areas but mechanisms identified in the field have not helped in the development of a vaccine up to now. One well-studied example is Combination B, a vaccine combining MSP-1, MSP-2 and ring-infected erythrocyte surface antigen (RESA). Prospective field trials have identified a negative correlation between serum IgG levels against the vaccine components and incidence of clinical episodes in non-immunized residents of a malaria endemic region<sup>26,27</sup>. Nevertheless immunization with these components had no significant effect on the number of clinical episodes in a phase 1/2b trial in children in the same malaria endemic region<sup>28</sup>.

Overall it remains very difficult to choose the right antigens for inclusion into a vaccine and to estimate vaccine efficacy in animal models, and artificial challenge trials with malaria-naïve volunteers. Due to these missing correlates of protection, results on the efficacy of malaria vaccine candidates are obtained only very late in clinical development. And most worryingly, product development could be stopped based on failure to detect efficacy in current *in vitro* and *in vivo* models not known to be predictive of clinical efficacy. It will therefore be of crucial importance to understand the mechanism of action of partially effective vaccines.

#### **9.1.4 Definition of an effective malaria vaccine and its contribution to current methods of control**

First attempts in malaria vaccine development aimed at sterile protection from infection, which can only be achieved with a pre-erythrocytic vaccine. So far only irradiated sporozoites<sup>1</sup> and RTS,S/AS02A<sup>3</sup> succeeded in conferring sterile protection to immunized volunteers. The feasibility of a sporozoite vaccine remains doubtful and RTS,S/AS02A failed to show sterile protection in the field<sup>5</sup>. Modern malaria vaccine development therefore focuses on an anti-disease vaccine and the endpoint in efficacy trials is now rather prevention of severe disease than infection. The first vaccine showing reduction of severe malaria in the field was RTS,S/AS02A although the mechanism of this reduction is not known<sup>5</sup>.

An effective malaria vaccine will need to justify its place in the overall portfolio of tools for malaria control. A vaccine could add significant benefit to current methods of control like insecticide treated bednets (ITN), indoor spraying with residual insecticides (IRS), environmental changes, and drug treatment<sup>29</sup>. Some of the advantages of a malaria vaccine over other control methods are i.) existing tools (IRS or ITNs and drug therapy) alone are unlikely to provide complete protection against malaria in highly endemic areas, ii.) drug resistance and insecticide resistance will continue to pose threats to control strategies based on treatment and vector control with insecticides iii.) the expanded program of immunization (EPI) provides an effective delivery system that is in place, and already reaches a high proportion of children in malaria endemic countries, iv.) there is greater flexibility over the time interval during which a vaccine can be given than is the case for control strategies based on treatment, which must always be available immediately upon development of disease, v.) there is increasing political support from the international community to finance the costs of malaria vaccines for poor countries<sup>29</sup>.

#### **9.1.5 Application of an effective IRIV-based vaccine in malaria endemic areas and related problems**

The success of an IRIV-based malaria vaccine may depend at least in parts on pre-existing anti-influenza immunity. Despite a lack of information anti-influenza IgG is

found in sera from residents of sub-Saharan Africa (Nicole Westerfeld personal communication) and widespread influenza outbreaks in malaria endemic regions have been reported<sup>30</sup>. IRIVs are highly immunogenic in infants, the target population for a malaria vaccine, as seen with virosomal influenza (Inflexal®)<sup>31</sup> and hepatitis A (Epaxal®)<sup>32</sup> vaccines. The IRIV-based malaria vaccine described in this thesis largely depends on CD4 T cell stimulation by influenza epitopes on the virosomes (Peduzzi, unpublished). T cell help in IRIV-immunized individuals during infection with *P. falciparum* has to come from T cells specific for parasite epitopes, which were not present in the vaccine. Several studies have shown that natural infection with *P. falciparum* indeed induces helper T cell responses<sup>27,33</sup>. Taking into account the presence of a variety of CD4 T cell epitopes on a parasite, chances are high that a helper T cell response against at least one of these antigens will develop and support a boost of the vaccine-induced immunity.

A crucial property of a successful malaria vaccine is the induction of a good immunological memory response. A successful vaccine has to induce a protective and long-lasting immune response, which depends on immunological memory. For *P. falciparum* it is known that the parasite interferes with the development of a memory response by modulating both memory T and B cell responses (reviewed in Good et al., 2004; Wykes and Good, 2006)<sup>8,34</sup>. A malaria vaccine thus has to induce an immune response, which differs from a natural response in terms of memory induction. As the mechanisms underlying interference of the parasite with immunological memory are not well understood, it will be difficult to rationally design vaccines overcoming this problem. Another concern for the development of a malaria vaccine is the observation that the presence of maternally derived antibodies at the time of immunization may interfere with the development of the infant's own active immune response (reviewed in Good et al., 2004)<sup>8</sup>. Immunization with Epaxal® induces an almost four-fold higher antibody response in infants without maternal anti-hepatitis A virus antibodies than those with maternal antibodies at baseline<sup>32</sup>. One explanation for this observation is that the passively acquired antibodies most likely inhibit the development of an antibody response by blocking the access to critical B cell epitopes<sup>35</sup>. Maternal antibodies can also affect the fine specificity of the newborn's immune response by inducing antibodies to alternate, unmasked B cell epitopes. Increasing the dose of vaccine can be sufficient to circumvent inhibition by maternal antibodies, presumably by providing additional antigenic epitopes, which

remain free and accessible to infant B cells<sup>36</sup>. Cellular responses are in general not affected by maternal antibodies and can be boosted by subsequent immunizations<sup>35,36</sup>. With an IRIV-based vaccine it would be interesting to investigate the influence of maternal anti-influenza antibodies, as these could enhance opsonization and thereby increase the immunogenicity of IRIVs in influenza-naïve infants. At the same time maternal antibodies to *P. falciparum* antigens could inhibit the development of a humoral response to *P. falciparum* peptide components. In the light of these considerations, immunization schedules will need special attention and careful evaluation to find the right balance between the presence of maternal antibodies, the infant's ability to mount an immune response and the vaccine formulation.

The phase 1 study with a virosomal vaccine candidate based on CSP and AMA-1 described in this thesis was a proof of concept that synthetic peptides delivered on IRIVs are safe and highly immunogenic. The final goal for a multivalent virosomal malaria vaccine is the inclusion of five or more peptide components from different life-stages of the parasite. In addition to the CSP, AMA-1 and SERA5 peptides characterized in this thesis we have other peptide components in development, including peptides-derived from blood-stage proteins MSP-1 (James et al. accepted), MSP-3, and D13<sup>37</sup>. Most of these candidates have completed preclinical optimization and characterization and are now ready for safety and immunogenicity studies in humans. The availability of genome information<sup>38</sup> together with techniques for identification of novel antigens from genome-wide analysis is currently yielding many new potential targets<sup>39</sup>.

As mentioned before, induction of both humoral and cellular immune responses to antigens from different life-stages of the parasite might be crucial for good vaccine efficacy. All above described peptide antigens are used as B cell epitopes but the IRIV technology is also suitable for the induction of cytotoxic T cell responses<sup>40,41</sup>. For a multi-component multi-stage vaccine approach it would be desirable to include T cell epitopes of liver-stage antigens for the induction of cytotoxic T cell responses in addition to B cell epitopes of blood-stage and sporozoite antigens.

### 9.1.6 Comparison with other malaria vaccine candidates

One big advantage of the IRIV system compared to other malaria vaccine approaches is the principle of single building blocks, which can be combined into a multi-component subunit vaccine. Each building block, i.e. peptide derived from a *P. falciparum* antigen presented on virosomes, can be developed and evaluated separately before combining the components in one vaccine. Combined delivery of two different virosomally-formulated antigens did not interfere with the immunogenicity of single components in the phase 1a clinical trial described in this thesis.

RTS,S/AS02A, the most advanced malaria vaccine is based on one sporozoite antigen only. It is a fusion protein containing the repeat region and flanking sequences of CSP fused to hepatitis B surface antigen (HBsAg) and is expressed together with unfused HBsAg in *Saccharomyces cerevisiae* (reviewed in Heppner et al., 2004)<sup>42</sup>. A phase 2b efficacy trial with RTS,S/AS02A in African children gave promising results: The vaccine showed 35% efficacy for time to first clinical episode and 49% efficacy for incidence of severe malaria for at least 18 month<sup>4</sup>. Although RTS,S/AS02A is the first pre-erythrocytic vaccine showing efficacy in the field it will probably need more development to be introduced as a standard vaccination. The mechanisms of protection are incompletely understood and it is generally agreed that a successful malaria vaccine should combine several antigens from different life-stages of the parasite<sup>42,43</sup>. To include more components into RTS,S one has to face all the problems associated with recombinant protein production. High A/T content of the genome, high molecular weight, protein disorder, basic isoelectric points and unusual repeat motifs make the heterologous expression of recombinant *P. falciparum* proteins very difficult<sup>44</sup>. Moreover new vaccine constructs have to go through all the steps of clinical profiling again, whereas with IRIVs it can be argued that the substitution of short defined peptides on the virosome surface are minor changes and should not affect safety of the vaccine. Another advantage of IRIVs over the other experimental adjuvants is that they are already registered and used in many countries. More than 20 million doses of the two marketed virosomal vaccines Inflexal®V (influenza) and Epaxal® (hepatitis A) have been given so far<sup>45</sup>. IRIVs are delivery system and adjuvant at the same time and have an excellent safety and immunogenicity profile. There are only two human compatible adjuvants on the

market. Alum, the most widespread adjuvant based on aluminium salts, is inferior to IRIVs in terms of immunogenicity and quality of induced antibodies<sup>10,46</sup>. RTS,S/AS02A is based on an experimental oil-in-water formulation with the additional adjuvants Monophosphoryl lipid A (MPL) and QS21 not yet registered for use in humans. MPL, the lipid moiety of bacterial LPS, and the saponin derivative QS21 (extracted from the plant *Quillaja saponaria*) are very potent immunostimulatory adjuvants<sup>47</sup>. Some of the researchers in the field have argued that some of the observed effects of the vaccine might be mediated by an unspecific stimulation of the immune system by the adjuvant and not by a sporozoite-specific immune response<sup>48</sup>.

Another promising approach, which has entered clinical development is a DNA-based heterologous prime-boost regimen developed at the University of Oxford (reviewed in Moore and Hill, 2004; Hill 2006)<sup>20,49</sup>. It is also a building block system based on plasmid DNA or recombinant attenuated live viral vectors such as adenovirus, fowlpox (FP9), and modified vaccinia Ankara (MVA) for prime-boost studies<sup>50</sup>. The most advanced of these studies involve a multi-epitope (ME) string fused to TRAP. The ME portion contains two B cell, 14 CD8, and three CD4 T cell epitopes from six sporozoite and/or liver stage antigens, including CSP, liver-stage antigen 1 (LSA-1), and LSA-3. Several Phase 1 and Phase 2a sporozoite challenge studies have been conducted in healthy malaria-naive adults to identify prime-boost vaccination regimens that optimize the cell-mediated immune response and generate protective immune responses. These regimens induce very high T cell responses in *ex vivo* ELISPOT assays<sup>24</sup>. Some of the tested regimens provided a prolongation of the pre-patent period, while other regimens completely protected a portion of volunteers against heterologous strain sporozoite challenge. The same vaccine candidates were safe and highly immunogenic in a series of phase 1 trials in adults and children in The Gambia<sup>51</sup>. Despite this initial success, a subsequent phase 2b proof of concept trial of a DNA/MVA prime-boost regimen in The Gambia failed to show a reduction of the natural infection rate<sup>23</sup>. A series of new FP-9 and MVA constructs was developed and was superior in eliciting cytotoxic T cell responses compared to the above described constructs in a series of phase 1 trials in the UK<sup>52</sup>. The new constructs were safe and immunogenic in Gambian<sup>53</sup> and Kenyan adults and Kenyan children<sup>54</sup> and will now be evaluated in phase 2 efficacy trials. The heterologous prime-boost system was also used with DNA encoding full-length CSP.

Various prime-boost combinations, doses, and application routes were safe and immunogenic in the UK<sup>55</sup>, and The Gambia<sup>56</sup> but failed to show evidence of efficacy in phase 2a laboratory sporozoite challenge of malaria-naïve volunteers<sup>55</sup>. Although the building block system of this DNA approach is comparable to the IRIV system described in this thesis, DNA-immunization is focused on the induction of cellular immune responses, which is not suitable for blood-stage vaccine development. In the case of a malaria vaccine relying on cellular immunity against pre-erythrocytic stages only, one sporozoite escaping the immune response, successfully infecting a hepatocyte and developing into merozoites can be enough to cause full disease. For that reason a combination of T and B cell responses against pre-erythrocytic antigens with B cell responses against blood-stage antigens might be crucial for efficient protection against infection and/or disease. IRIVs can induce cytotoxic T cell responses against encapsulated antigens<sup>40</sup>. However the development and evaluation of a virosome-based malaria vaccine inducing both humoral and cellular immune responses will be very expensive and time-consuming. Therefore a combination of advanced approaches for the stimulation of different arms of the immune system against both parasite stages seems to be the most rational approach for the swift development of an efficient malaria vaccine. First clinical trials with combinations of two types of antigen delivery systems and different vaccine candidates have already begun. The above described ME-TRAP DNA-based prime-boost approach was combined with the IRIV-based delivery of UK-39 and AMA49-CPE in an ongoing clinical trial. Another combination including RTS,S/AS02A and MVA-CSP was safe and immunogenic in malaria-naïve subjects and showed protection against sporozoite challenge in some volunteers<sup>57</sup>. Taking into account these latest developments and findings, combination of promising and already advanced vaccine candidates seems to be the best solution for the fast development of an efficient malaria vaccine.

## 9.2 Concluding remarks

We have presented in this thesis the establishment and use of a technology platform for the development and evaluation of synthetic peptides derived from *P. falciparum* epitopes for inclusion in a virosomal malaria vaccine candidate. We have shown that it is possible to improve immunogenic properties of lead peptides in a medicinal chemistry type stepwise optimization process and highlighted the importance of a detailed pre-clinical profiling of candidate peptides. A close to natural conformation of peptides appears to be crucial for the induction of parasite-binding antibodies and was achieved in some cases by conformational restriction of the peptide. A crucial advantage of IRIVs over alum as antigen delivery system seems to be that IRIVs do not disturb the conformation of the peptide antigens hooked via a PE-anchor to the virosomal membrane<sup>10</sup>. A phase 1 proof of concept study confirmed the potential of IRIVs as a human-compatible adjuvant and delivery system for easy to synthesize peptides. Both peptides tested were safe and immunogenic at appropriate doses and no serious adverse events were reported. Immunogenicity results from this phase 1 clinical trial also confirmed the value of the animal models used during the pre-clinical profiling stage to predict the antibody responses elicited in humans. Detailed studies on the functional activity of the induced humoral responses in animal models and humans should help broadening our understanding of mechanisms underlying potential vaccine efficacy in the field.

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### Education

1996 - 2000 University of Bern, Switzerland, major: medical microbiology

2000 - 2001 **Diploma** in medical microbiology: *Escherichia coli* heat labile toxin: Structure and mucosal adjuvanticity.  
R&D Immunology, Berna Biotech Ltd., Bern, Switzerland.  
Supervisor: PD Dr. Alois B. Lang

2002 - 2006 **PhD** in microbiology: Development of a synthetic malaria vaccine.  
Molecular Immunology, Swiss Tropical Institute, Basel, Switzerland.  
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### Special Duties

1998 - 2002 Substitute teacher at elementary school and high school

2003 - 2005 Member of the research committee of the Swiss Tropical Institute

2003 Organization of the Swiss meeting for PhD students in parasitology and tropical medicine

## Laboratory Skills

Recombinant protein production in bacteria  
Basic immunoassays (ELISA, Western blotting, IFA)  
FACS analysis  
Generation of monoclonal antibodies with hybridoma technology  
Cell proliferation assays from human blood samples and ELISPOT

## Presentations

- 2003 PhD student meeting of the Swiss Society of Tropical Medicine and Parasitology, Munchenwiler, Switzerland.  
Talk: Development of a virosomal formulation of a peptidomimetic of *Plasmodium falciparum* circumsporozoite protein that elicits sporozoite invasion inhibitory antibodies.
- 2003 Joint meeting of the Swiss Society of Microbiology and the Swiss Society of Tropical Medicine and Parasitology, Basel, Switzerland.  
Poster: Development of a synthetic peptidomimetic of *Plasmodium falciparum* circumsporozoite protein that elicits invasion inhibitory antibodies.
- 2004 PhD student meeting of the Swiss Society of Tropical Medicine and Parasitology, Munchenwiler, Switzerland.  
Talk: Characterization of expression and subcellular localization of a novel secretory protein of *Plasmodium falciparum*.
- 2004 Joint meeting of the 'Deutsche Gesellschaft für Tropenmedizin und Internationale Gesundheit' and the Swiss Society of Tropical Medicine and Parasitology, Wurzburg, Germany.  
Talk: Design of structurally defined peptidomimetics of a malaria protein eliciting invasion inhibitory antibodies.
- 2005 PhD student meeting of the Swiss Society of Allergology and Immunology, Wolfsberg, Switzerland.  
Talk: Development of an IRIV based malaria vaccine candidate.
- 2005 Molecular Parasitology Meeting IX, Woods Hole, USA.  
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## Publications

- 2006 Submitted to Chemistry&Biology:  
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Structure-activity based design of a synthetic malaria peptide

- 2006 eliciting sporozoite inhibitory antibodies in a virosomal formulation  
Submitted to PLoS Clinical Trials:  
**Genton B., Pluschke G., Degen L., Kammer A.R., Westerfeld N., Okitsu S.L., Schroller S., Vounatsou P., Mueller M.S., Tanner M., Zurbriggen R.**  
A randomized placebo-controlled Phase Ia malaria vaccine trial of two virosome-formulated synthetic peptides in healthy adult volunteers
- 2006 Submitted to The Journal of Infectious Diseases:  
**Okitsu S.L., Silvie O., Westerfeld N., Curcic M., Degen L., Mueller M.S., Sauerwein R.W., Zurbriggen R., Mazier D., Pluschke G.**  
A virosomally formulated malaria peptide candidate vaccine elicits a long-lasting *Plasmodium falciparum* sporozoite-inhibitory antibody response in human volunteers
- 2006 Manuscript in preparation:  
**Okitsu S.L., Mueller M.S., Vogel D., Curcic M., Zurbriggen R., Pluschke G.**  
A minimal dose of a virosome based peptide malaria vaccine candidate elicits parasite cross-reactive antibody responses in mice and rabbits
- 2007 Submitted to Vaccine:  
**Okitsu S.L., Boato F., Mueller M.S., Vogel D., Westerfeld N., Zurbriggen R., Robinson J.A., Pluschke G.**  
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