Vaccine development strategies applied to the *Plasmodium falciparum* malaria antigen Pf332

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Never mistake knowledge for wisdom.
One helps you make a living,
the other helps you make a life.
— Sandra Carey
The passion for science
and the passion for music
are driven by the same desire:
To realize beauty in one's vision
of the world.
— Heinz Pagels

About the cover

A classic illustration of the immune system is that of a war, with the body
defending itself against an invading threat such as bacteria or virus.
However, the way I have come to picture it is different, since the “kill or be
killed” dogma is sometimes not applicable, as in the case of malaria, and
because the concept of war disaccords with the overall beauty of the immune
system, with its imposing networks, silent chains of reactions and self-
regulating mechanisms.

Instead, I look at the immune system in the light of chaos theory, which
comprises the behaviour of chaotic dynamical systems that are sensitive
to initial conditions. To many, the word chaos suggests randomness,
unpredictability and messiness, but chaos is actually organized and follows
certain patterns. Graphic representations of such patterns, fractals, are found
virtually everywhere in nature, and are often very beautiful.

The first encounter with immunology can be overwhelming and a chaotic
experience, due to the complex nature of the immune system. But once the
underlying organization is grasped, one can discern the beauty of the system.
Since immunology shares these features with chaos theory, I decided on a
fractal for the front cover picture. It was drawn by my father, to whom
I hereby dedicate this thesis.
Summary

Malaria is one of the major infectious diseases in the world with regard to mortality and morbidity, and the development of a vaccine against the malaria parasite *Plasmodium falciparum* is considered of high priority. The aim of the work presented in this thesis was to develop and characterize recombinant vaccine constructs based on the *P. falciparum* asexual blood-stage antigen Pf332. We have studied the humoral responses in mice elicited by various types of constructs, including naked DNA plasmids, naked mRNA, alphavirus, and peptides. Immunological memory was successfully induced against the repetitive EB200 fragment of Pf332, although the antibody titers were generally low and the highest titers were unexpectedly obtained with a conventional DNA plasmid. In another study, we also demonstrated the ability to circumvent genetically restricted immune responses in mice against two malaria epitopes, one of them derived from Pf332, by inclusion of universal T-cell epitopes into multiple antigen peptide constructs. However, the overall variability of the responses stressed the importance of including several epitopes in a future malaria vaccine. Further, the recent completion of sequencing of Pf332 enabled us to identify and characterize the immunogenic properties of a non-repeat fragment of the Pf332, termed C231. Our analyses of C231 showed that antibodies raised against the recombinant protein possess an *in vitro* parasite inhibitory capacity similar to that of antibodies against recombinant EB200. Furthermore, the recognition of C231 by antibodies in sera from individuals naturally primed to *P. falciparum*, correlated well with that previously observed for the corresponding sera and EB200. When analyzing the IgG subclass distribution of anti-C231 antibodies, we noted a bias towards IgG2 and IgG3 relative to IgG1, differing from the subclass profiles of IgG binding crude *P. falciparum* antigen, which were dominated by IgG1. Taken together, the work presented herein is likely to facilitate further studies on Pf332 as a potential target for protective immune responses, and amounts to a small step towards the realization of a malaria vaccine.
This thesis is based on the following articles, which are referred to in the text by their Roman numerals:

I  

II  

III  

IV  

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Abbreviations

ADCI  Antibody-dependent cellular inhibition
AMA-1  Apical membrane antigen 1
APC  Antigen-presenting cell
Bel  B-cell leukemia/lymphoma
BCR  B-cell receptor
CD  Cluster of differentiation
CD40L  CD40 ligand
CS  Circumsporozoite
CTL  Cytotoxic T-lymphocyte
dsRNA  Double-stranded RNA
DTP  Diphteria/tetanus/pertussis
Fas  FS-7 associated surface antigen
FCA  Freund's complete adjuvant
FIA  Freund's incomplete adjuvant
GPI  Glycosyl-phosphatidylinositol
GST  Glutathione-S-transferase
HIV  Human immunodeficiency virus
HLA  Human leukocyte antigen
HRP  Histidin-rich protein
IFN; IFN-I  Interferon; Type I-interferons
Ig  Immunoglobulin
IL  Interleukin
MAP  Multiple antigen peptide
MHC  Major histocompatibility complex
MMR  Measles/mumps/rubella
MVA  Modified vaccinia virus Ankara
mRNA  messenger-RNA
MSP  Merozoite surface protein
MyD88  Myeloid differentiation primary response gene 88
NK  Natural killer
NO  Nitric oxide
ODN  Oligo-deoxyribonucleotides
PAMP  Pathogen associated molecular pattern
PMNS  Post-malaria neurological syndrome
RESA  Ring-infected erythrocyte surface antigen
SFV  Semliki Forest virus
TCR  T-cell receptor
Th  T helper cell
TLR  Toll-like receptor
TNF  Tumour necrosis factor
Introduction

Introduction to vaccines

A brief historical perspective

Few public health interventions have had such an impact on global health as vaccination. Thanks to pioneers such as Jenner and Pasteur, a handful of vaccines prevent illness or death for millions of individuals every year. The concept of immunity can be traced back as far as 430 B.C., when it was first described by the Greek general Thucydides [1]. He observed that only those who recovered from the current plague could nurse the sick, since they would not contract the disease a second time - they were immune (from Latin *immunis*, exempt). The first recorded attempts to actively induce immunity were performed in Asia during the Middle Ages, by a process known as variolation. Healthy people contracted a mild form of the disease by inhaling powdered smallpox (*Variola*) scabs, or wearing the undergarments of an infected individual [2]. Upon recovery, the individual was immune to smallpox. Variolation was associated with around two percent mortality, which was considered a far better risk as compared to the thirty percent mortality caused by natural infection. By 1700, variolation had spread to Africa, India and the Ottoman Empire, and the procedure became fashionable in Europe some decades later.

By the end of the 18th century, the English physician Edward Jenner recognized that milk-maids infected with cowpox were immune to the related smallpox-virus. He deliberately infected an eight-year-old boy with cowpox pustules and later exposed the boy to smallpox, which he failed to contract. After repeating the experiment on other children, including his own son, Jenner concluded that this process provided immunity to smallpox. In contrast to variolation, this process was safe, as cowpox did not elicit severe disease in humans. The vaccination (from Latin *vacca*, cow) provided immunity for up to ten years, and Jenner’s findings were published in 1798 [3]. In 1807, the Bavarian government became the first to require recruits to be vaccinated, and vaccination spread with the practice of war.

Some years later, the French chemist Luis Pasteur discovered that the ageing of a cholera culture had weakened (attenuated) it. He used the technique of attenuation to successfully vaccinate sheep against anthrax, and
gained tremendous distinction in 1885, when saving a boy from rabies by injecting attenuated rabies virus. Still, no one understood the molecular and cellular basis of the concept immunity. Its cornerstones were eventually laid hundred years later by Emil von Behring and Shibasaburo Kitasato, who observed the actions of antibodies after serum transfer experiments [4], and the Russian Ilya Mechnikov who discovered cell-mediated immunity, when observing phagocytosis [5].

Modern vaccines: Safety

Further vaccination history was triumphantly written in 1980, when WHO declared the global eradication of smallpox as a result of efficient vaccination. Despite the success of vaccination in eliminating disease and death, the public acceptance of even minor side-effects of vaccination is very low. This was illustrated by a gradual cease of pertussis vaccination in Britain during the 1970s. Unfortunately, the country experienced a severe pertussis epidemic as a consequence, during which over 100,000 children caught pertussis, and some died or contracted chronic neurological damages [6]. Aversions against modern vaccines may stem from coincidental associations. For instance, sudden-infant-death syndrome occurs during an age when children receive DTP vaccinations, and symptoms of autism usually manifest around the time of MMR vaccination. Scientific reports on DTP vaccination causing asthma, and MMR vaccination causing inflammatory bowel disease, Crohn’s disease or autism, have not been reproducible or have been contradicted in several follow-up studies [6]. Negative secondary effects of vaccine additives and by-products have also been subjects of public concern. In Sweden, merthiolate was withdrawn as a preservative following public concerns about the mercury content in dental fillings. Allergy towards the albumin by-product present in the MMR vaccine has also been a vaccine-related worry. However, an MMR vaccination study involving 400 children with pronounced albumin allergy confirmed the safety of this vaccine, since only one percent of the allergic children developed reactions; all of them mild [7].

During all developmental phases of a vaccine, ranging from animal models to clinical trials, recipients are closely monitored for side-effects. Common safety issues deal with balancing the adjuvant efficacy against its toxicity, and tuning of antigen dosage in order not to induce tolerance. All licensed vaccines today sort under either the first generation of vaccines, which are based on attenuated or killed pathogens, or the second generation, which are so called subunit vaccines based on defined antigens from the pathogens. Much focus is presently put on the development of third generation vaccines, which are based on nucleic acids encoding defined antigens. The emerge of this new technology has also raised a number of safety issues, that will be discussed in a later chapter.
The immune response

Adaptive immune system responses

Initiation
The purpose of vaccination is to induce long-lived immunological memory, by activating the adaptive immune system. Adaptive responses are initiated by antigen uptake, processing and subsequent presentation of the antigen-derived peptide, on cell-surface expressed class I and class II major histocompatibility molecules (MHC I and MHC II, respectively). MHC I molecules are present on nearly all nucleated cells, and when binding peptides (forming an MHC I:peptide complex), they play a vital role in activation of the CD8$^+$ subset of T cells. MHC II on the other hand, is expressed solely by antigen-presenting cells (APCs), and MHC II:peptide complexes are associated with activation of the CD4$^+$ subset of T cells. Cells with a major function as antigen presenters, so-called professional APCs, include dendritic cells (DCs), macrophages and B cells, and differ in their mechanisms of antigen uptake and expression levels of MHC and co-stimulatory molecules. DCs internalize antigen via endocytosis (ingestion of macromolecules) and phagocytosis (engulfment of pathogens); macrophages via phagocytosis only, and B cells via B-cell receptor (BCR) mediated endocytosis. DCs constitutively express both MHC II and the co-stimulatory molecules needed for sufficient activation of T cells, and are hence very efficient antigen presenters. Macrophages need to be activated by phagocytosis prior to expression of both MHC II and co-stimulatory molecules, and B cells express MHC II constitutively but need to be activated by soluble antigen and T cells, in order to upregulate their co-stimulatory molecule expression.

T-cell activation
Upon interaction with APCs in the lymphoid tissue, T cells accumulate signals from their T-cell receptor (TCR), co-stimulatory molecules and the receptors that have bound surrounding cytokines (immunoregulatory low-molecular weight proteins). The amount of signal depends on antigen dose, co-stimulation and duration, and seems to be a crucial parameter for the fate of the cell. At the two extremes are unresponsiveness (anergy) after a very weak signal, and activation-induced cell death after excessive stimulation [8]. After antigen encounter, the vast majority of CD4$^+$ T cells develop into T helper 1 effector cells (Th1) or T helper 2 effector cells (Th2) that leave the lymphoid tissue to exert their effects in the periphery. This differentiation of naïve T cells into effector cells leads to activation of the humoral and/or cellular arm of the immune system.
The humoral response

Humoral immune responses are mediated by plasma cells (effector B-cells) secreting antibodies. Antibodies constitute the main specific operator against extracellular pathogens and toxins, and are divided into five classes or isotypes: IgD, IgM, IgE, IgA and IgG. IgA and IgG are further divided into the subclasses IgA1 and IgA2, and IgG1, -2, -3 and -4, respectively. IgD is together with IgM the major membrane-bound immunoglobulin, but while no biological effector function has been identified for IgD, IgM plays an active role in the immune defense and is the first antibody class produced during the initial exposure to antigen (the primary response). IgE may bind to receptors on the membranes of basophils and mast cells. Crosslinkage of receptor-bound IgE by an antigen (allergen) induces cellular release of molecules mediating immediate hypersensitivity reactions, such as histamine. IgA is the predominant isotype found in external secretions such as saliva, tears, mucus, and breast milk. IgG constitutes about 80% of the total serum antibodies and are associated with phagocytosis (mainly IgG1 and IgG3), activation of complement system (IgG3 and IgG1, to a smaller extent IgG2), and the release of immune effectors, including highly reactive oxygen- and nitrogen compounds, and immunoregulatory polypeptides (chemokines) and proteins (cytokines). Further, IgG antibodies as well as IgM, IgE and IgA, may bind to pathogens and thereby inhibit their dispersal and invasion of host cells. IgG may also be therapeutically induced by a vaccine in order to bind autoantibodies or allergy-induced IgE and hence prevent their action.

Most antibody responses are T-cell dependent and hence require interaction between the B cells and CD4⁺ T helper cells. Naïve B cells bind and internalize antigen via BCRs (surface immunoglobulin receptors). The endocytosed antigen is then processed and presented as peptides on the surface of the B cell, in association with MHC II molecules. The immunological synapse formed between the B- and T cell involves ligation of the B-cell MHC II:peptide complex with the TCR on the T cell, and co-stimulatory molecules on B cells (CD 40 and CD 80/CD 86) with their corresponding co-stimulatory molecules on T cells (CD40L and CD28/CTLA-4, respectively). Simultaneous secretion of cytokines by activated T helper cells induces proliferation and differentiation of B cells into plasma cells, and their antibody isotype switching from IgM to IgG, IgE or IgA. However, some pathogen associated molecular patterns (PAMPS) are able to activate B cells in the absence of CD4⁺ T helper cells, leading to polyclonal B-cell activation which does not engage in isotype switching or somatic hypermutation, and hence gives rise to antibodies of a generally lower protection capacity. This phenomenon is for instance observed during an infection with the *Plasmodium falciparum* malaria parasite.
A small fraction of the plasma cells become long-lived plasma cells residing in the bone marrow [9]. Although long-lived, they have to be continuously replenished either from naïve or memory B cells. Two principal mechanisms have been suggested in the latter case; either by activation by antigen trapped by so-called follicular dendritic cells [10], or activation by polyclonal stimuli such as CpG DNA (described on page 20), lipopolysaccharide, or bystander T-cell help (i.e., CD40 ligation and cytokines secreted by T cells activated by a third-party antigen) [11].

The cellular response

Cell-mediated responses are mediated by T cells recognizing peptides in association with MHC I or MHC II, and constitute the main specific defense against intracellular pathogens, involving lysis (perforation of cell membrane leading to annihilation) of infected cells and modulation of specific immune responses via cytokine and chemokine production. Unlike B cells, T cells can only recognize antigen when associated with MHC I or MHC II.

Simplified, naïve CD4\(^+\) T cells encountering an MHC II:peptide-complex may differentiate into either a Th1 or a Th2 cell. Pro-inflammatory cytokines (IFN-\(\gamma\) and TNF-\(\alpha\)) secreted by mainly Th1 cells activate CD8\(^+\) T cells and macrophages, which further enhances the cell-mediated response. The production of these cytokines is induced by IL-12 from activated macrophages and dendritic cells, and both IFN-\(\gamma\) and TNF-\(\alpha\) are able to exert a direct effect on intracellular microbes, such as the influenza virus and *Mycobacterium tuberculosis* [12]. IFN-\(\gamma\) and IL-12 downregulate the expression of Th2 cytokines (IL-4, IL-5, IL-10 and IL-13), that stimulate B cells to undergo activation and differentiation. In turn, IL-4, IL-10 and IL-13 counteract the expression of Th1 cytokines.

Differentiation of naïve CD8\(^+\) T cells into cytotoxic T-lymphocytes (CTL) requires not only interaction with MHC I:peptide complex and costimulatory molecules, but also the pro-inflammatory cytokine environment provided by effector Th1 cells and macrophages. Several mechanisms of CTL effector functions exist. Upon ligation of the CTL T-cell receptor with the MHC I:peptide complex of a target cell, CTLs release granules containing perforin and serine proteases known as granzymes. Granzyme B proteins bind to receptors on the target cell, thereby initiating protein internalization via endosomes (vesicles formed by invagination of the cell membrane). Perforin then permeabilizes the endosome, leading to the release of granzyme B into the cell cytosol, and eventually to apoptosis (programmed cell-death) or necrosis (cell disruption) [13]. CTLs are also able to trigger apoptosis by the Fas-dependent pathway, if the Fas ligand on the CTL interacts with CD95 on the target cell. However, this pathway is believed to be more important for maintaining lymphocyte homeostasis than killing intracellularly infected cells, as CD95 is expressed only on activated T- and B cells. In addition to killing infected cells, CTL mediate effector
functions by secreting anti-microbial and inflammatory cytokines such as IFN-\(\gamma\) and TNF-\(\alpha\).

**Memory**

Only a small percentage of the lymphocytes activated during the initial exposure to antigen (the primary response), around 5-10 percent, differentiate directly into memory cells. Memory cells show distinct phenotypic differences from naïve cells, in that they require shorter time for proliferation, synthesizing cytokines, differentiating into CTL (CD8\(^+\) T-cells) or plasma cells (B cells), and migrating to non-lymphoid tissues. They also express distinct chemokine- and adhesion receptors, which are involved in directing the migration of the cells to a particular localization of the body, and maintaining them there.

Whether persistence of antigen on follicular dendritic cells is needed or not for maintenance of immunological memory, has been under debate for many years. Several studies indicate that memory B- and T cells can survive in complete absence of antigen [14-17], while work from other researchers have concluded the need for antigen persistence [10;18;19], or led to the suggestion of a vital role played by cross-reactive environmental antigens [20]. However, a current consensus of opinion is that memory T cells remain in a semi-activated state owing to stimulation by surrounding cytokines. The natural turnover (background rate of proliferation) of CD8\(^+\) memory cells, and their prolonged longevity, seems to be dependent on IL-15 and IL-15-induced Bel-2 expression, respectively (reviewed in [22]). In contrast, CD4\(^+\) memory T-cell longevity seems to depend on upregulated Bcl-X\(_L\) induced by IFN-I [23], and their natural turnover is regulated by IL-7 rather than IL-15 [24].

**Innate immunity: Toll-like receptors**

The innate immune response comprises various components and processes, including anatomic barriers (skin, mucus membranes), physiologic barriers (temperature, low pH), phagocytosis, and inflammation. While such responses are non-specific and do not elicit immunological memory per se, they may enhance the adapted immune response. Of particular interest in the context of vaccination is toll-like receptor (TLR) signalling, since many of the desired adjuvant effects stem from signalling via these receptors. The TLR family presently consist of thirteen members, denoted TLR1-13, and of those, TLR1-10 are found in humans (reviewed in [25]). The TLRs involved in the recognition of microbial products, TLR1-2 and TLR 4-6, are displayed on the cell surface. In contrast, TLR7-9 are localized intracellularly and their ligands found internally, for instance within phagolysosomes. Via association with intracellular signalling molecules, such as MyD88, ligation
of TLRs leads to upregulation of co-stimulatory molecules and the production of various cytokines, which in turn engage in the development of the adaptive immune response.

**Adjuvants**

Subunit vaccines based on synthetic peptides, proteins or plasmids are normally poor immunogens when administered alone, and require therefore the co-administration of an adjuvant in order to elicit a sufficient immune response. Adjuvants (from Latin *adjuvare*, to help) are defined as a group of structurally heterogeneous compounds, utilized to increase a specific immune response to an antigen. Relatively little is known about the mechanisms underlying their activity, as well as their structural requirements, and they are therefore surrounded by some level of obscurity. Several attempts to organize adjuvants in grouped categories have been made [26-28]. However, since the effects of many adjuvants are multiple and overlapping it is convenient to classify adjuvants into two groups only; those aimed at optimizing antigen delivery to the immune system, and those with immuno-stimulatory properties. Optimally, an adjuvant can serve both as a delivery vehicle and immuno-stimulator, leading to enhanced uptake (vehicle function) and presentation (immuno-stimulatory function) of antigen by APCs. This can be translated into higher signal strength, with a stronger response activation and memory induction as a consequence.

The choice of adjuvant depends on the desired type of immune response in terms of humoral or cell-mediated immunity, as adjuvants tend to bias the response to one type or the other. It is therefore necessary to evaluate the effect of the adjuvant involved in the vaccination scheme. The evaluation may also include tuning of antigen dosage to determine the “immunological window”, since adjuvants generally permit the use of much smaller quantities of antigen, and too large or too small quantities of antigen may induce tolerance. Further, a high antigen dose within the immunological window may have a negative influence, as it allows for the expansion of T cells with low-affinity TCRs.

The discussion about specific adjuvants and their documented properties will in this thesis be limited to Freund’s adjuvant, aluminium compounds, unmethylated CpG sequences and double-stranded RNA.

**Freund’s adjuvant**

In the early 1940s, Jules Freund & Katherine McDermott developed what is known as Freund’s complete adjuvant (FCA), consisting of a mixture of a non-metabolizable oil (mineral oil), a surfactant (Arlacel A), and killed mycobacteria (*M. tuberculosis* or *M. butyricum*). It has been used for many years to enhance immunologic responses to antigens, and is even today considered to be one of the most effective adjuvants. A water-based solution
of the antigen is gradually homogenized into the oil-based FCA, which after injection constitutes a depot of micro-droplets that are gradually endocytosed by APCs. Although FCA is a very effective adjuvant for production of antibodies, there are problems associated with its use, such as chronic granulomas, sterile abscesses, and/or ulcerating tissue necrosis [29]. It is therefore not used for vaccination of humans. However, Freund’s incomplete adjuvant (FIA), has previously been widely used in human vaccination. Its composition lacks the immuno-stimulatory bacterial component, and hence it serves merely as a delivery vehicle. The use of FIA in humans was withdrawn due to the emerge of local tissue necrosis, stemming from free fatty acids release from the surfactant Arlacel A [30].

Aluminium compounds
Aluminium compounds are the only adjuvants used with routine human vaccines. They include alum, aluminium hydroxide and aluminium phosphate, and are often collectively referred to as alum, although the actual alum (potassium aluminium sulfate) is rarely used as adjuvant nowadays (reviewed in [31]). It is important to carefully select the type of aluminium adjuvant and optimize the mixing conditions (i.e., the pH) for each antigen, since the process is heavily dependent on electrostatic forces between adjuvant and antigen.

The adjuvancy provided by aluminium compounds includes the formation of a depot, enhanced antigen uptake by antigen-presenting cells as a result of particle formation, as well as activation of macrophages and the complement system. Their limitation include occasional local skin reactions, augmentation of IgE antibody responses, and inability to augment cell-mediated immune responses, especially cytotoxic T-cell responses. In addition, aluminium adjuvants cannot be frozen or easily lyophilized [31].

Unmethylated CpG sequences
The immuno-stimulatory effect of bacterial DNA was discovered rather recently [32], but an explanation eluded everyone for more than a decade. It was shown to stem from unmethylated CpG dinucleotides flanked by two 5’ purines (or a 5’ purine followed by thymidine), and two 3’ pyrimidines [33]. The CpG motifs exert their adjuvant effect by binding to TLR9 in the endosomal compartment of human plasmacytoid dendritic cells and B cells [34]. TLR9 ligation leads to upregulation of co-stimulatory molecules and the production of cytokines such as TNF-α and IFN-I in dendritic cells, and IL-6 in B cells. NK cells are activated secondarily by IFN-I to secrete IFN-γ [35;36]. Due to the expression of mainly pro-inflammatory cytokines, CpG motifs are suitable for the induction of cell-mediated immunity. CpG ODNs have been shown to override the Th2 effect of aluminium compounds in both adult and very young, already Th2-biased mice [37], but its effect is in turn over-ruled by the Th2-promoting gene gun delivery method [38].
CpG present in DNA plasmid vaccines contribute to the immunogenicity of DNA vaccines, which has been observed for instance in an *Aotus* monkey malaria vaccine trial [39]. In addition, nuclease-resistant CpG ODNs injected without any antigen in mice, one to two days prior to challenge with *Plasmodium yoelii* sporozoites, was shown to confer sterile protection against malaria infection [40].

**Double-stranded viral RNA**

Replicase-driven genome replication is a feature of many viruses and results in the formation of double-stranded RNA molecules (dsRNA), with subsequent activation of the innate immune system. At least three types of host defense enzymes are known to be activated by dsRNA: RNAse L, which inhibits protein translation and induces apoptosis, protein kinase R, which initiates IFN-I expression, and dsRNA-specific adenosine deaminase, which launches editing (modifications of the dsRNA), resulting in amino acid substitutions in proteins synthesized from edited templates [41]. DsRNA is also known to bind TLR-3, which induces production of TNF-α, IL-6 and IL-12 in murine macrophages [42]. This cytokine profile suggests a bias towards cell-mediated immunity, which was recently observed in a replicase-based vaccination study on a pseudorabies virus antigen [43]. However, enhancement of humoral immune responses following dsRNA injection has also been recorded [44]. Apoptosis triggered by replicase-based dsRNA has been shown to induce maturation in dendritic cells [45], and is hence not only a desired safety feature of replicase-based vaccine constructs.

A new application for dsRNA is recently explored within therapeutic vaccinology. Non-viral dsRNA taken up by cells is subjected to processing by the RNase III enzyme Dicer, resulting in formation of 21-22 nucleotide long dsRNA molecules termed small interfering RNAs (siRNAs). These interact with the RNA-induced silencing complex (RISC), which leads to single-strand siRNA annealing with an eventual complementary cellular mRNA sequence, specific cleavage and thereby silencing of the mRNA transcript [46]. Within the area of infectious diseases, siRNAs have been utilized for inhibiting *in vitro* HIV-1 replication and *in vitro* and *in vivo* Hepatitis B replication [47,48], although their clinical use may be limited by their IFN-I inducing effect [49].

**The future**

The future is likely to bring about new, efficient tools to achieve adjuvancy; perhaps by yet unidentified compounds or by improving the ones already existing. A mixture of two adjuvants, CpG ODNs and mineral oil, has successfully been tested [50], and the CpG adjuvant is indeed a strong candidate for future human applications, much due to its low toxicity and capacity to induce cell-mediated responses. Recent data showed that poly-L-arginine inhibits the CpG-induced systemic release of pro-inflammatory
cytokines, which further lends support to general clinical use [51]. Promising therapeutic areas for CpG include cancer, infectious diseases and allergy; the latter focus being particularly “hot” as CpG was recently shown to inhibit IgG1 and IgE class switching in B cells [52].

### Subunit vaccines

Subunit vaccines are based on one or several selected parts of a pathogen, and offer some significant advantages over attenuated or inactivated (killed) virus or bacteria. Subunit constructs exclude the risk of regained virulence, and are well-defined and in general more cost-effective as they allow production and purification in large scale. They can be designed genetically or synthetically to include the desired antigenic determinants only, and at the same time exclude epitopes inducing non-protective antibodies. Despite these advantages, there are some drawbacks associated especially with the development of recombinant protein-based vaccines. Their production may be hampered by the possible loss of correct protein folding and the lack of several post-translational protein modifications, both which may eliminate certain conformational epitopes. Further, peptide-based subunit constructs are limited to linear epitopes. The fact that both peptides and recombinant proteins need to be delivered together with an adjuvant is also a disadvantage, once called the “immunologist’s dirty little secret” by the renowned immunologist Charles Janeway [53]. Two types of subunit vaccine constructs are dealt with in further detail below; multiple antigen peptides, and nucleic acid-based vaccines which result in in vivo recombinant protein production.

### Synthetic peptides: Multiple Antigen Peptides

For efficient antigen uptake by antigen-presenting cells, the immunogen should be of some size. This requirement may constitute an obstacle for immunization with linear peptides, which often lack the complex three-dimensional structure of proteins. Multiple antigen peptide (MAP) constructs consist of linear peptide antigens synthesized on, or coupled to, a branched core of lysine residues [54]. The result is an immunogen with high epitope density, and a size sufficient to be immunogenic. Further, the design enables circumvention of immune responses limited by genetic restriction, since otherwise non-immunogenic B epitopes may be combined with T-helper cell epitopes of universal character [55;56]. MAP constructs have successfully been used for experimental vaccination against various infectious diseases such as malaria, schistosomiasis and HIV-1 [57]. A recent phase 1 trial with malaria epitopes assembled as MAPs showed that the characteristics of CD4+ T cells elicited, in terms of long-lived memory, cytokine profiles, fine
specificities and genetic restriction, was comparable to that elicited by immunization with irradiated sporozoites, the "golden standard" for malaria vaccine development [58].

**Nucleic acid-based vaccines**

Nucleic acid-based immunogens represent the third generation of vaccines and a relatively new technological approach. It combines advantages of recombinant proteins and peptides, as they are well-defined and cost-effective, with the advantage of the ability of an attenuated intracellular pathogen to induce cell-mediated immunity. Upon transfection, DNA or RNA encoding a selected antigen is expressed by the cell's translational machinery, and presented to the immune system. The nucleotide sequence can be delivered either in the form of a naked DNA plasmid, or carried by biological vehicles such as intracellular bacteria, recombinant virus and virus-like particles, or non-biological vectors such as cationic liposomes.

**DNA plasmids**

Unencapsulated (“naked”) DNA plasmids started to be explored as a vaccine technology in the early 1990’s [59], and in July 2005, a vaccine against the West Nile virus in horses was the first DNA vaccine to be licensed. The typical DNA plasmid vaccine vector is of *E. coli* origin, and encodes the antigen(s) of interest under the control of a strong viral promoter recognized by the mammalian host; usually the cytomegalovirus immediate early region promoter (reviewed in [60]). The plasmid is often delivered by needle injection into the dermis or skeletal muscle, but other routes such as gene gun or jet injector delivery, oral distribution, and intranasal inhalation are also experimented with. The delivery method strongly affects the profile of the immune response in terms of humoral or cell-mediated immune mechanisms [61;62].

The processes by which plasmids are internalized and located to the cell nucleus still remain to be elucidated. It has been suggested that plasmids could enter myocytes via T-tubuli, independently of disruption of the plasma membrane [63]. Since cellular uptake of the DNA plasmids is a major limiting factor for their immunogenicity [64], its optimization constitutes an important area of research. Intramuscular injection of plasmids immediately followed by electroporation increases transfection both *in vitro* and *in vivo* [64-66]. Attempts to further facilitate plasmid transport from the cytosol into the nucleus, by DNA hybridized with nuclear localization signal-conjugated peptides, have also resulted in significantly improved plasmid expression *in vitro* [67]. The majority of transfected cells expressing foreign protein after *in vivo* plasmid injection are myocytes, although APCs also take up plasmids by phagocytosis. In the latter case however, the DNA seems to be degraded within the endosomes, and therefore not lead to antigen expression,
processing and presentation by the transfected APCs [64]. DNA entry into
the cytoplasm is facilitated by adsorption of DNA onto cationic micro-
particles to form lipoplexes, which are thought to destabilize the endosomal
membrane [68]. A highly efficient way to obtain transfected myocytes as
well as APCs expressing foreign protein is to utilize the gene gun delivery
method [59;69], in which DNA plasmids are adsorbed onto gold particles
and shot into dermis or skeletal muscle. The use of this method is limited by
its relative high cost, but mainly by its powerful Th2-inducing effect on the
elicated immune response, even when immunizing with CpG-containing
plasmids normally eliciting a cell-mediated response [38].

Detailed knowledge about DNA plasmid uptake, processing and
presentation to the immune system is vital in order to rationally optimize the
induction of an immune response. Presently, three mechanisms of uptake are
under investigation; the first involving myocyte uptake, expression and
delivery of the antigen to APCs (cross-priming) [70], the second involving
transfection of APCs themselves, and the third stressing the contribution of
the myocytes themselves, upregulating MHC II molecules upon encounter
with CpG-rich DNA, with subsequent expression and presentation of the
foreign protein to T cells [71]. In general, the first two mechanisms are
regarded as more important, mainly due to the lack of co-stimulatory
molecules on myocytes. They suggest a strong role for APCs at the site of
injection, which is supported by the adjuvant effect observed with
simultaneous delivery of plasmids coding for the APC-attracting cytokine
GM-CSF [72].

Recombinant Semliki Forest virus

The Semliki Forest virus (SFV) is an alphavirus belonging to the family
Togaviridae, with avian and mammalian species (mainly rodents) as natural
hosts. Although humans may be naturally infected, the virus is not a major
pathogen of man. Human infections are usually sub-clinical, but may in
some cases cause fever and mild polyarthritis. The virus particle is spherical
with a diameter of 60-70 nm, with a surrounding lipid bilayer envelope
derived from the host cell, harboring 80 inserted viral spike trimers. The
icosahedral nucleocapsid consists of viral capsid protein, and contains the
genome which is a single-stranded, positive RNA strand. Upon infection, the
genome is released into the cytosol and transcription is immediately initiated
by the host cell’s translational machinery. The first 2/3rd of the strand
encodes a replicase, which efficiently drives replication of both the full-
length genome as well as the remaining 1/3rd of the strand. The latter
subgenomic part encodes the structural proteins, which are the nucleocapsid
and the spike proteins.

In the recombinant Semliki Forest genome, the structural protein genes
are replaced by the gene of interest, leading to its efficient replicase-driven
transcription. To enable cleavage between the replicase and the antigen
sequence in the full-length genome transcript, a proteolytic cleavage site from the foot and mouth disease virus is positioned upstream of the antigen sequence. In order to increase the biosafety of recombinant SFV particles, a split helper system was developed [73]. This strategy is based on expression of the structural genes from two separate helper RNA sequences; one expressing the capsid protein and the other the spike proteins. To produce recombinant virus particles, three separate DNA plasmids encoding the replicase plus antigen, the capsid, and the structural genes, respectively, are transcribed \textit{in vitro}. The transcripts are then simultaneously electroporated into BHK-21 cells, that enable formation of virus particles that later may be collected by repeated centrifugation. Since the helper RNA sequences deliberately lack the packaging signals necessary for encapsulation, they are not included in the harvested viral particles. Hence the particles are fully capable to infect a host cell and release the replicase-antigen RNA, but prevented from further replication ("suicidal").

Recombinant Semliki Forest virus particles induce mainly cell-mediated responses and have successfully been used for vaccination against cancer [74] and viral diseases such as influenza and louping ill virus [75;76]. Due to their RNA nature, which eliminates the risk of incorporation into the host genome, and their engineered design outlined above, SFV particles are considered relatively safe to produce and use as vaccine vehicles, and were recently approved for clinical use in HIV studies. In addition, the translational machinery of the infected host cell deals exclusively with the recombinant antigen, which inevitably leads to apoptosis and hence transient viral presence at the site of injection [77]. Further advantages include infectivity of a broad spectrum of cells including Vero cells utilized for clinical applications, and the lack of wide-spread pre-existing immunity to SFV.

\textbf{Safety of nucleic acid-based vaccines}

Within the scientific community, questions about the safety of nucleic acid-based vaccination have been raised concerning the possibility of DNA integration into the host genome, affecting oncogenes or tumor suppressor genes and thereby inducing the development of cancer. The risk of DNA integration into host genome is however considered negligible, as it has been proven very low under a variety of experimental conditions [78;79], even when foreign DNA persisted for more than 19 months in mouse muscle tissue [80]. There are studies reporting low and transient titers of DNA-specific antibodies, but no record of autoimmunity, as a consequence of nucleic acid-based immunization [81].
Figure 1. (A) Schematic view of the wild-type Semliki Forest virus genome. (B) The modified RNA encoding the replicase plus antigen of interest is simultaneously electroporated with the two helper-RNAs coding for the capsid protein C, and the precursor (p62, 6K, E1) for the spike proteins. NSP, non-structural protein.

In the light of general concerns about the introduction of nucleic acid-based immunization, and to some extent gene-modified crops, it is interesting to note that food-derived DNA was recently shown to enter the blood circulation as well as plastic-adherent blood cells, via the human alimentary tract [82]. However, in contrast to DNA of bacterial origin, vertebrate DNA is unlikely to cause immune system activation, since the immunostimulatory CpG sequences are methylated, generally flanked by bases constituting immune-neutralizing motifs, and their presence is suppressed to approximately 25 percent of the predicted random frequency [83]. No antibodies towards the protein coded by the food-derived DNA were detected in the study mentioned; probably as a result from DNA degradation rather than low immunogenicity. However, the oral route has been explored as a mean of delivering plasmid-based vaccines [84-86], and the positive results obtained are encouraging as this route possess clear advantages over injection with needle.

Lastly, in order to avoid the risk of spreading antibiotic resistance to environmental microbes, scientists are encouraged to use selection methods other than plasmid inclusion of genes conferring antibiotic resistance. One example of a successful alternative is a novel E-coli strain enabling plasmid selection and maintenance, by repressor titration [87].
augmented safety, removal of the antibiotic resistance gene may also increase the protein expression efficacy, as the expression of the resistance enzyme exerts a metabolic burden on the host cell [87].

Malaria: The disease

The parasite’s life cycle in humans
Malaria parasites belong to the kingdom *Protista*, phylum *Apicomplexa*, class *Sporozoa*, order *Eucoccidida* and genus *Plasmodium*. There are over 120 malaria species having vertebrates as hosts, but only four that cause malaria in man: *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae*. *P. falciparum* is responsible for virtually all mortality associated with malaria infections. The life cycle of *Plasmodium* parasites is complex, involving several different developmental stages in both the *Anopheles* mosquito vector and the human host. About 10-30 sporozoites are transmitted to the host by a bite of an infected female mosquito. During approximately thirty minutes, the sporozoites home to the liver, enter the hepatocytes and start multiplying by division. Depending on the species, the parasite development reaches the schizont stage after 5-15 days. In case of *P. vivax* and *P. ovale*, a small number of parasites may never reach the schizont stage but remain in hepatocytes for years in a dormant form termed hypnozoite, resulting in occasional relapses of malaria infection. Upon rupture of the infected hepatocyte, as many as ten to thirty thousands of merozoites are released into the blood circulation, where they invade erythrocytes. The entire invasion process takes about thirty seconds. The merozoite develops within the erythrocyte through the ring, trophozoite and schizont stages, before the cell ruptures and releases up to 36 merozoites that promptly re-invade new erythrocytes. The rupture of the erythrocytes is synchronized by the diurnal rhythm of the human host, and for *P. falciparum* the cycle takes around 48 hours to complete. After a few rounds of reinvasion, a small fraction of the newly invading merozoites differentiate into sexual gametocyte forms represented by macro-gametocytes (female) and micro-gametocytes (male). The gametocytes are able to escape the erythrocytes taken up by an *Anopheles* mosquito during a blood meal, and mature into gametes inside the mosquito midgut. Within a few minutes, a microgamete fertilizes a macrogamete to form a zygote, which then transforms into a mobile ookinete that crosses the midgut epithelium to the extracellular space between the epithelial cells and basal lamina. The ookinete develops into an oocyst which after 10-24 days, depending on the species and surrounding temperature, and releases thousands of sporozoites
that invade the mosquito salivary gland. These may then be inoculated next time the mosquito bites.

Figure 2. Schematic view of the part of the *Plasmodium* life-cycle that takes place in the human host. Photo detail: Lennart Nilsson/Albert Bonniers förlag AB.

**Symptoms, diagnosis and treatment**

The initial liver-stage infection passes unnoticed in the malaria-infected individual, and hence it is not until after 1-2 weeks post-infection that symptoms appear. Initially they are easily confused with a common influenza infection, as they involve joint myalgia, headache, and fever. The clinical symptoms manifest along with the cyclic blood stage of the parasite, starting with rising fever as the erythrocytes burst. After a few hours, the temperature decreases in association with heavy sweating, only to increase again as the next fraction of erythrocytes rupture. The syndrome of severe *falciparum* malaria in African children typically involves fever, metabolic acidosis, hypoglycemia, anemia, seizures and coma. The predominant causative agent of parasite origin was identified a decade ago, as belonging to the class of glycolipids known as glycosyl-phosphatidylinositol (GPI) [88]. The malaria GPI exerts several effects on the host, and is thought to be the main mediator of the inflammatory response that causes the typical
malaria symptoms [89]. GPI induces expression of the pyrogenic cytokines TNF, IL-1 and IL-6 in human and murine macrophages [88]. TNF in turn, as well as GPI alone, upregulates vascular endothelial cell expression of adhesion molecules such as ICAM-1, VCAM-1 and E-selectin, which mediate binding of infected erythrocytes, a phenomenon called sequestration [90]. Sequestration, autoagglutination (aggregation of infected erythrocytes independent of malaria-specific antibodies) and rosetting (binding of uninfected erythrocytes to infected erythrocytes) all contribute to obstruction of the blood flow, that when occurring in the small vessels of the brain may lead to seizures and coma (cerebral malaria) and eventually death. TNF also contributes to hypoglycemia [91] and ineffective erythropoiesis [92], and its release from activated macrophages is exacerbated by GPI in synergy with IFN-γ [93]. In addition, GPI also induces TNF-independent hypoglycemia [88]. IFN-γ is thought to be produced initially by γδ T cells, NK cells, and CD1-restricted NKT cells, and as the infection proceeds, IFN-γ is also produced by conventional CD4+ and CD8+ T cells. NKT cells share some characteristics of both T cells and NK cells, as they are able to kill cells as well as express TCRs. However, the NKT cells’ TCRs recognize glycolipid antigens presented not by MHC molecules but the MHC-like molecule CD1d on dendritic cells, B- and T cells, macrophages, as well as hepatocytes.

Other malaria symptoms involve spleen and liver enlargement and pulmonary edema, as a consequence of aggregation of phagocytes and parasitized erythrocytes. Acute renal failure and jaundice may occur due to low blood volume due to fever-caused dehydration. A first description of what is called post-malaria neurological syndrome (PMNS) appeared only recently, and is described as a transient acute confusional state or psychosis after parasite clearance [94]. There is still some speculation regarding whether PMNS is a distinct clinical entity or a consequence of chemotherapy or malarial cerebral damage.

Malaria is diagnosed by examining Giemsa-stained thick and thin blood films at several occasions, to detect parasitized erythrocytes. *P. falciparum* stages later than mid-trophozoites are usually not detected due to their sequestration. Alternatively, the ParaSight™ F dipstick assay can be used for detection of *P. falciparum* infection. The test takes around twenty minutes and employs a monoclonal antibody detecting the *P. f.* histidine-rich protein 2 (HRP-2). Polymerase Chain Reaction (PCR) is usually not utilized for diagnosis as it is more time consuming and costly as compared to microscopy. Malaria is treated with chemotherapy and, in emergency situations, blood transfusion to reduce the parasitaemia and anemia.
Naturally acquired immunity to malaria

Acquired immunity to *P. falciparum* blood-stage infection is gradually built up over a period of up to twenty years by continuous exposure to the parasite, and is therefore acquired only by people living in endemic areas. The reason for this slow acquisition has been ascribed to different factors, including parasite genetic variability and parasite suppression of the host’s immune system. However, newborns do not contract disease during the first months of their life. This is generally though to depend on maternal anti-malaria antibodies crossing the placenta, rendering the newborns virtually immune, however, this explanation has been questioned [95]. Even though children in endemic areas are protected against non-cerebral severe malaria after only two infective mosquito bites [96], the mortality is still high for children under the age of five until immunity to cerebral malaria has been built up. Immunity to disease (premunition) is usually acquired in early adulthood, and is reflected by fewer clinical symptoms and eventually lower parasitaemias. Nevertheless, malaria-immune women become highly susceptible to malaria infection during their first pregnancies, due to heavy sequestration of parasites in the placenta, mediated by parasite antigen PfEMP-1 and placental chondroitin sulphate A [97] and hyaluronic acid [98]. The magnitude of sequestration decreases by each pregnancy as humoral immunity towards the placenta-binding domain of PfEMP-1 develops. Sterile immunity to disease is practically never reached in neither men nor women.

The role of antibodies in immunity to blood stages

In individuals living in malaria endemic areas, elevated levels of non-specific as well as malaria-specific immunoglobulins are observed, reflecting both polyclonal and specific B-cell activation. Passive transfer of malaria specific IgG, both in humans [99-101] and animal models [102;103] lend support to the role of antibodies in protection against *P. falciparum* malaria blood-stages. The protection seems to depend on the proportion of specific IgG1 and IgG3 antibodies, i.e. the cytophilic IgG subclasses, relative to the proportion of the non-cytophilic IgG2 and/or IgG4 antibodies (reviewed in [104]). Further, elevated IgE levels are observed in humans living in endemic areas. This is usually associated with pathogenesis, as IgE-containing immuno-complexes from malaria patients induce the release of TNF and NO from monocytes *in vitro*, by cross-linking CD23 [105]. *In vivo*, this might lead to a local overproduction of TNF in microvessels, with subsequent upregulation of adhesion molecules, and the development of cerebral malaria. However, the pathogenic role of IgE has been questioned, by some recent studies suggesting a protective role for IgE [106;107]. No specific function of IgM or IgA in malaria has been described so far.
Antibody-mediated protection against malaria blood-stages involves several different mechanisms. Antibodies may inhibit merozoite invasion of erythrocytes [108;109], cytokine induction by GPI [110], and the parasite adherence to placenta [111], and enhance the clearance of infected erythrocytes from the circulation by binding to their surface, thereby preventing sequestration and promoting elimination in the spleen by phagocytosis [112]. A potential protective role of antibodies against some blood-stage antigens, including GLURP and MSP-3, is by mediating antibody-dependent cellular inhibition (ADCI) [113;114]. The mechanism involves cytophilic antibody interaction with Fc receptors on monocytes, with subsequent release of TNF and possibly yet unidentified toxic factors [101;115;116]. Since the cooperation with monocytes depends on cytophilic antibodies, it may partly explain why cytophilic IgG subclasses predominate in protected individuals [104].

Cell-mediated responses in immunity to blood stages
Since erythrocytes do not express MHC molecules on their surface, cytotoxic CD8+ T cells are unlikely to be important for immunity against the malaria blood-stages. The important role of CD4+ T cells is however well established for experimental malaria, with evidence based on adoptive transfer of protection and increased susceptibility to infection in CD4+ T-cell depleted mice [117-119]. For *P. falciparum* malaria, both proliferation and cytokine production is observed *in vitro* upon re-stimulation of T cells from exposed individuals, but such data are in general poorly correlated with protection [120].

Studies on experimental malaria suggest that protective cell-mediated immunity is mediated by IL-12-induced production of IFN-γ, TNF and NO [121;122]. Production of these protective, yet potentially pathogenic, pro-inflammatory cytokines is counter-balanced by IL-4 and IL-10. The role of IL-10 seems to be particularly important, as a high ratio of IL-10/TNF and IL-10/IL-6 is associated with protection against lethal outcome of severe disease and anemia, respectively [123;124]. IL-10-responses to a liver-stage antigen has also been found to predict resistance against *P. falciparum* infection [125].

Macrophages play important roles in cell-mediated immunity as they phagocytose infected erythrocytes, act as effector cells in ADCI, and are early producers of the cytokine IL-12. IL-12 and IL-18 activate NK cells, which represent the earliest source of IFN-γ during a malaria infection [126]. Apart from the cytokine-mediated signal, transient direct contact between the NK cell and the parasitized erythrocyte seems to be required as well for optimal NK cell activation. The nature of the erythrocytic activation ligands in this context remains to be investigated [126]. The NK cell production of IFN-γ may also be induced by activated NKT cells, a cross-talk partly
mediated by IFN-γ released by NKT cells [127]. Activated NKT cells rapidly produce cytokines such as IFN-γ, IL-4 and TNF-α. Since their TCRs recognize glycolipid antigens they are able to bind the Plasmodium-derived GPI molecule, and NKT cells have indeed displayed inhibition of liver-stage parasite replication in murine hepatocytes, possibly mediated by cytokines or lytic pathways involving perforin and/or Fas (reviewed in [128]).

A small fraction of the T cells, less than five percent in humans, have their TCR made up of a γδ heterodimer as opposed to the common αβ heterodimer, and are referred to as γδ T cells. During malaria infection, the γδ T cell population is markedly expanded, in both mice and humans [129]. Their activation is initiated by cytokines such as IL-2, IL-4 and IL-15, and TCR recognition of peptide- and non-peptide antigens in no association with MHC molecules. Activated γδ T cells from naïve donors, but not αβ T cells, have been observed to inhibit parasite replication in vitro, by the release of granulysin [130].

Malaria vaccine development

Rationale for development of malaria vaccines

The concept of a vaccine against malaria is supported not only by naturally acquired immunity, but also experimental findings from passive transfer of IgG and injected irradiated P. falciparum or P. vivax sporozoites eliciting sterile immunity in humans. However, neither passive immunization nor injections of irradiated sporozoites are possible to implement on a larger scale, and scientists are therefore searching for other means to induce immunity. The question remains whether it is feasible for individuals in malaria endemic areas to achieve vaccine-induced sterile immunity, since no naturally acquired immunity would be standing by once the vaccine-induced immunity fades. This argument has divided the malaria vaccine strategy somewhat into two approaches, one aiming at a vaccine suitable for populations in endemic areas, which will be protective against severe disease but not infection, and one at a vaccine inducing sterile immunity, suitable for transient residents in endemic areas. The latter type has also been considered suitable for pregnant women in endemic areas. However, recent experimental data from mice suggest that vaccine-induced maternal antibodies against P. yoelii blood-stage antigens might negatively affect the neonatal response to subsequent natural challenge, by inducing anti-idiotypic antibodies and isotype skewing [131].

Since most malaria antigens are stage-specific, and the immunity to different stages of the complex parasite life-cycle requires activation of different specific immune mechanisms, most malaria vaccine research has
been focused on immunity to one single stage. A vaccine targeting the pre-erythrocytic stages would prevent sporozoite invasion of hepatocytes, or the development of the exo-erythrocytic stages within the hepatocytes. Such a vaccine would eliminate the manifestation of disease as well as further transmission, since both the malaria symptoms and gametocyte formation take place during the blood stage. A vaccine interfering with the blood stage would prevent or reduce morbidity and mortality, by inhibiting parasitaemia, sequestration, and the release of the disease-mediating molecule GPI. An altruistic vaccine acting on sexual stages would have a local impact on the transmission of the parasite. However, to ensure complete protection and prevention of vaccine-induced emergence of parasites with higher virulence, a future vaccine is likely to comprise selected antigens not only from the same developmental stage but also from several stages.

An efficient vaccine should preferentially be based on genetically conserved antigens in order to elicit global immunity. However, there are several studies indicating that the different degrees of antigenic diversity observed for the malaria parasite does not have to hamper the development of an asexual blood-stage vaccine [132]. First, the fact that significant immunity to non-cerebral severe malaria develops after a relatively low number of infections [96], whereas immunity to mild disease takes many years, suggesting that some important targets for severe malaria have restricted diversity. Secondly, passive transfer of immunoglobulin from immune individuals living in one area dramatically reduces the parasitaemia in individuals living in very distant areas, meaning that important target antigens are relatively conserved globally [100;133]. Thirdly, women during their first pregnancy are particularly susceptible to placental malaria, but this susceptibility decreases significantly with subsequent pregnancies, i.e. the gradual increase of antibodies blocking parasite adhesion to the placenta [134]. This shows that important target antigens have low diversity and are specific to parasites adhering to the placenta. Maternal antibodies from multigravid women also recognize placental isolates from various geographic regions [135], which lends further support to antigen conservation and hence their usage as vaccine targets.

Malaria vaccines in clinical trials

Randomized, controlled clinical trials
Although several vaccines incorporating antigens from various stages of *P. falciparum* have been tested in primate models as well as human volunteers, only four types of vaccines have thus far been tested in randomized and controlled trials in malaria endemic areas. Two of them, RTS,S and CS-NANP, act on the pre-erythrocytic level, while the
MSP/RESA acts on the blood stage, and Spf66 encompasses antigens from both stages [136].

Studies in rodents and humans immunized with radiation-attenuated *P. falciparum* sporozoites indicate, that pre-erythrocytic protection may be achieved by inducing sporozoite-targeting antibodies that block hepatocyte entry, and by cell-mediated responses that eliminate parasitized hepatocytes before they release infectious merozoites. The CS protein is the pre-erythrocytic antigen against which immune responses are most clearly linked to protection. Epitopes derived from the CS protein is included in the RTS,S, CS-NANP and the Spf66 vaccines.

Protection at the blood-stage level is antibody-mediated, and some of the most well-studied target antigens include MSP-1, MSP-2, MSP-3, RESA, and AMA-1. Antibodies to these proteins block the merozoite invasion of erythrocytes, except for antibodies to MSP-3 which are involved in monocyte-mediated mechanisms. Epitopes from the MSP-1, MSP-2 and RESA antigens are included in the MSP/RESA vaccine [137].

Most trials to date have emanated from the Spf66 vaccine, which was under much focus during the 1990s. It is composed by three to five individual peptides that are joined by disulfide bridges, in order to form a circular and hence more bulky immunogen. The peptide sequences originate from different 83, 55 and 35 kDa merozoite stage-specific protein fragments, and are linked by the NANP repeat derived from the CS protein [138]. The peptide is solubilized in sterile saline solution and adsorbed onto aluminium hydroxide prior to needle injection. Results with Spf66 in reducing new malaria infections have been heterogeneous, as it was not effective in four African trials, but reduced the clinical malaria episodes during five trials outside Africa [136]. The large variation in protection has been ascribed to HLA polymorphism in humans, and it is now generally accepted that Spf66 is not effective enough for routine use.

RTS,S vaccines comprise a fusion between half of the CS protein and the hepatitis B surface antigen, which is expressed in yeast and used with the oil-in-water adjuvant ASO2 (previously called SBAB2) [139]. The hepatitis B surface antigen forms a virus-like particle that efficiently delivers the CSP protein to the cell cytosol, resulting in a cell-mediated immune response. When the vaccine efficacy was tested in semi-immune adults residing in the Gambia, the first analysis showed a significant delay in time to first infection, but this effect waned to zero already after 15 weeks [140]. A later analysis showed that after adjustments for confounders, the vaccine efficacy against infections was estimated to be 34 percent in the first year, and 47 percent in the second year [136]. The RTS,S vaccine was later evaluated in children aged 1-4 in Mozambique, and during the first six months it was associated with a 30 percent reduction in the risk of clinical malaria, delayed time to first infection by 45 percent, and reduced incidence of severe malaria by 58 percent. Importantly, the follow-up showed that the disease reduction
did not wane over at least 18 months [141]. A clinical trial on Gambian children is presently under way [142].

CS-NANP-based vaccines contain a minimum of three repeats of the NANP peptide sequence from the CS protein, conjugated with a protein carrier from the tetanus toxoid or toxin A from *Pseudomonas aeruginosa*. So far there is no evidence for a reduction in clinical malaria episodes by this type of vaccine [136].

The MSP/RESA vaccine is a mixture of three recombinant asexual blood stage antigens: block 3 and 4 of MSP-1, the 3D7 form of the polymorphic MSP-2, and the last 70 percent of RESA [137]. In a clinical trial at Papua New Guinea, there was no evidence for effect of the vaccine against episodes of clinical malaria. However, vaccinees had a significantly lower prevalence of the 3D7 type of parasite, as well as reduced number of infections with the 3D7 type. Neither prevalence nor incidence of FC27 type of infections was significantly affected by vaccination [137], stressing the importance of including several allelic types of the same antigen.

**Future prospects**

Increased funding and attention over the last decade has undoubtedly contributed to intensified malaria vaccine research. The CS protein as well as MSP-1, particularly the 19 kDa and 42 kDa fragments of the antigen, continue to be in focus, although strategies other than peptide constructs are currently being explored. Early clinical trials with DNA plasmids [143;144], and prime-boost strategies with priming DNA and boosting with protein [145;146] or recombinant viruses [147;148] have been performed and are generally regarded as safe. Since vaccines based on nucleic acids are well-defined and can be designed to harbor several epitopes as well as adjuvant features, they are likely to continue to receive attention. Nonetheless, such vaccines are still facing difficulties in translating their efficacy in preclinical studies into the clinical field.

**The Pf332 vaccine candidate antigen**

One of the largest proteins of *P. falciparum* is the asexual blood-stage antigen Pf332, identified in 1988 by Berzins *et al.* at Stockholm University together with Mattei *et al.* at the Pasteur Institute in Paris [149]. The recent completion of the sequencing of the parasite genome revealed an impressive total of 5508 amino acids, and a heavy overrepresentation of glutamic acid (30 percent) and valine (13 percent) [150]. The protein is expressed during the trophozoite stage, and transported from the parasitophorous membrane to the outer erythrocyte membrane during schizogony [151]. It was recently discovered that the antigen is co-localized with other parasite antigens such as RIFIN and PfEMP-1 during transport, and the same study also reported on an intriguingly distinct, central position of the Pf332 protein within the
transport vesicles [152]. The role of Pf332 in the parasite life cycle still remains to be elucidated.

The interest in Pf332 as a malaria vaccine candidate derives partly from the observation that the Pf332-reactive human monoclonal antibody 33G2 inhibits parasite growth and cytoadherence in vitro [153]. However, 33G2 is highly cross-reactive with other repetitive malaria antigens, and thus the involvement of Pf332 in cytoadherence is uncertain. Nevertheless, direct parasite-inhibitory action has been confirmed with various rabbit polyclonal antibodies specific for Pf332 [154;155], and human polyclonal IgG antibodies affinity-purified on Pf332 repeats also display similar parasite growth inhibitory capacity [156]. In addition, increased titers of Pf332-reactive IgG antibodies in humans are associated with decreased number of malaria incidents [157].

The current knowledge about the variation of the Pf332 gene is rather limited. The protein is expressed from a single gene located subtelomERICally on chromosome 11 [158]. Since genes positioned at subtelomeric regions are known to be influenced by frequent breakage and healing [159], partial or complete gene exclusion is a conceivable event, and previously documented for antigens such as HRP [160], Pf155/RESA [161] and Pf11.1 [162]. However, although the Pf332 gene appears to be subject to some degree of breakage-induced variation [163], irreversible loss has never been detected in any of the parasite strains analyzed so far [164;165] In addition and contrast to what has been observed for the RESA antigen, the expression of Pf332 is stable during parasite passage in monkeys [166].

As for variations within the expressed protein, we compared a central region of the 3D7 strain with the longest corresponding sequence reported in another strain (unpublished data), Palo Alto [167]. The sequence constitutes 13 percent of the antigen, and the alignment revealed a high degree of identity between the two fragments, with occasional point mutations only and few variations in copy number of repeats (two copies missing in 3D7, three in Palo Alto). This, along with our other comparisons involving shorter fragments which resulted in the same low polymorphism frequency (unpublished data), is however not sufficient data for any conclusions regarding general polymorphism of the Pf332 antigen.

Experimental animal vaccinations with Pf332 have mainly been conducted with a 157 amino acid long central fragment termed EB200, identified in 1992 by Mattei and Scherf [168] and defined as a target of opsonizing antibodies in hyperimmune sera from P. falciparum-exposed Saimiri monkeys [169]. An injection with recombinant EB200 given to Saimiri monkeys previously exposed to P. falciparum boosted the levels of opsonizing anti-EB200 antibodies, and the presence of these correlated with protection against disease [170]. A study on sera from Senegalese adults displayed EB200-reactive antibodies of mainly the IgG3 subclass [171], the
subclass most efficient in mediating antibody-dependent cellular inhibition in vitro [172].
The present study

Aims

A vaccine for routine use in humans needs to meet many requirements. It should be based on at least one adequate antigen, which in turn should be efficiently delivered to the immune system, achieve satisfactory levels of activation in a broad population, as well as inducing the proper type of immune response. The overall aim of the study presented in this thesis was to develop and characterize recombinant vaccine constructs based on the *Plasmodium falciparum* asexual blood-stage antigen Pf332, attending to each of the features specified above. The study had as specific objectives:

- To compare antibody responses in mice by immunization with DNA- and RNA based vectors coding for the repetitive EB200 fragment of Pf332 (I)
- To compare the immunogenicity in mice of two genetically restricted malaria B-cell epitopes when assembled with universal T-cell epitopes in multiple peptide antigen constructs (II)
- To characterize the antigenic properties in mice of the non-repetitive C231 fragment of Pf332 (III)
- To analyze the antibody responses to C231 in individuals naturally primed to the parasite (IV)

Results and complementary discussion

**Antigen delivery**

For nucleic acid-based vaccines, the cellular transfection rate is a crucial parameter, since a poor transfection results in a low antigen dose and a weak immune response. In our first study we evaluated how DNA and RNA constructs in association with the Semliki Forest virus replicase compared with a conventional DNA plasmid for the induction of antibody responses in mice against the EB200 subfragment of Pf332 (I). Viral transfection is superior to naked plasmid transfection, since the former is not dependent on cellular uptake across two membranes but readily fuses with the cell and delivers its contents. As a consequence, the actual priming dose of nucleic
acids may be significantly lowered, which is reflected by the 20 µg plasmid DNA versus 12 pg RNA (the content of 10⁶ virus particles) used in our study. However, the use of recombinant virus as an efficient malaria vaccine vehicle is not optimal from a logistic point of view, since the socio-economic setting of many malaria endemic areas may for instance not meet the requirements of an unbroken cold chain. An even bigger obstacle is the fact that pre-existing immunity to the employed virus may hamper the immune response to the target antigen, something that previously has been observed for adeno-virus and poxvirus vectors. However, the vaccine efficacy of poliovirus and alphavirus vectors seems not to be impaired in immune hosts (reviewed in [173]). In addition, phase I trials of the hepatitis B virus-based RTS,S malaria vaccine showed no inhibition of the development of immune responses to the malaria antigen in cases of pre-existing immunity to hepatitis B [140;174]. Speaking in favor of the utilization of viral vectors is also the accumulating evidence for heterologous prime-boost regimens, i.e. priming with DNA and boosting with recombinant virus or vice versa, being superior to homologous repeated immunizations. So far, the mechanisms directing the outcome of different prime-boost protocols are unknown and subject to investigation. Their understanding will surely enable a more rational vaccine design.

In order to obtain the high transfection rates that are prerequisite of strong immune responses, it is important to limit the amount of nucleic acids included in the plasmid. We observed significantly lower antibody titers for DNA plasmids having the SFV replicase added to enhance the antigen expression, most likely as a result from a size more than the double in comparison to the conventional plasmid (6.2 kbp and 13.1 kbp, respectively). In other words, the poorer transfection rate was not compensated by the replicase-enhanced antigen expression and the intrinsic adjuvancy of the replicase. This conclusion is in contrast to other reports using other antigens or other similar virus expression system [175-177]. However, our results do not exclude the possibility that the replicase-containing plasmid induced stronger cell-mediated responses than did the conventional. Indeed, a recent study on SFV replicase-containing plasmids reported similar absence of enhanced expression efficacy over the conventional plasmid, but superior induction of cell-mediated immune responses, attributed to the presence of dsRNA [43].

Once the host cell is transfected, the expression of the antigen should be efficient in order to obtain large amounts of antigen. An inevitable obstacle is the fact that the mammalian protein translational machinery is not optimized for protozoan proteins. Since many malarial proteins contain tandemly repeated sequences, reflecting a low-complexity nucleic acid sequence heavily biased towards adenosine [178], expression in mammals may be significantly hampered by the exhaustion of tRNA molecules associated with the frequently repeated amino acids. One third of the EB200
antigen is made up of glutamic acid residues, possibly leading to the depletion of tRNA molecules corresponding to the GAA and GAG codons, and a subsequent retardation of the translational process. In addition, it has been shown in *E. coli* that when ribosomes pause during translation, the mRNA becomes prone to endonuclease degradation [179], or the ribosomes may shift frame [180], leading to not only a more slow translation but its actual failure. Scientists have taken two main approaches to improve protein expression efficacy. One is codon optimization, where the antigen sequence is optimized to employ the most frequently used codons in mammals. This method was recently shown to greatly enhance the expression and immunogenicity in mice of the two malaria antigens EBA-175 and MSP-1 [181]. The other way to improve protein expression is to include the relevant tRNA molecules in the construct (plasmid) itself, which has successfully been done for proteins derived from the malaria parasite [182], as well as from other pathogens. In this case however, the expression host targeted has been *E. coli* rather than mammals. The titers of antisera to EB200 obtained by plasmid immunization in our study were generally low (I) and could possibly be elevated by either method. As for codon optimization, it is known that both mice and humans utilize the GAG codon more frequently than GAA, while the opposite is true for the malaria parasite [183]. Another possible approach would be to circumvent the translation-related obstacles by targeting the less repetitive region of Pf332, a strategy we set out to explore in another study (III).

The low titers of anti-EB200 antibodies we obtained by immunization with naked mRNA (I) may reflect the sensitivity of ribonucleases to degradation, rather than codon bias. Vaccines based on naked mRNA are theoretically safer than DNA plasmids, as there is no risk of gene integration or autoimmunity, but their practical use is seriously hampered by their proneness to degradation. Researchers have tried to prevent this by various approaches, including delivering the mRNA embedded in liposomes [184] or delivering it intra-cytosolically using a gene gun [185], by an *ex vivo* method using transfected dendritic cells [186], by immunizing mRNA flanked by stabilizing untranslated regions at both ends [187], and by condensing the mRNA with protamine or modifying its phosphodiester backbone [188]. However, although stark naked and delivered by traditional needle-immunization, this method of mRNA immunization has been proved sufficient for vaccination against cancer. Ying *et al.* used an experimental setup similar to ours, in which mice were immunized intramuscularly with naked mRNA coding for the SFV replicase and the LacZ antigen [189]. Unlike us, they were aiming for cell-mediated responses, and showed that even a small amount of mRNA (10 µg) conferred protection in mice against tumor establishment, as a result of strong cell-mediated responses. Their results indicate that degradation of mRNA was hardly a limiting parameter, possibly because of the very rapid antigen expression associated with the
SFV replicase, in combination with its inherent adjuvant effect ascribed to dsRNA formation. Assuming that the mRNA transcripts in our study were not significantly degraded, we are left with the possibility that a strong cell-mediated rather than humoral response was elicited, or that other immunogens in the transcription mix, transcription reaction components or impurities, were able to dominate the response.

**Genetic restriction**

Genetic variability of humans is a limiting factor for one of the malaria vaccines that have undergone extensive field trials, the synthetic peptide-based Spf66 vaccine. Distributed with aluminium compounds, it elicited high levels of protection in areas of South America, but its efficacy was seriously impeded in other populations of the world [190]. In the context of genetic restriction, absence of a humoral response often reflects HLA polymorphism and consequently the lack of relevant T-cell epitopes. We therefore set out to investigate how different T-cell epitopes compare in terms of their capacity to induce immune responses, by analyzing diepitope MAP constructs containing either of four different universal T epitopes in combination with an otherwise non-immunogenic B epitope (II). As we expected, the genetic restriction in mice was successfully circumvented by the inclusion of either of the T-cell epitopes, and we went on with scrutinizing the different natures of the elicited responses (as commented further below). In fact, the strategy of universal T-cell epitope inclusion was used when designing the CS-NANP vaccine, which like the Spf66 vaccine has undergone several clinical trials.

Of equal importance is the fact that a future vaccine against malaria should target genetically different populations of the malaria parasite. This was stressed after conducting a clinical trial with the MSP/RESA vaccine construct, in which the 3D7 allelic type of the MSP-2 antigen was included, but not the FC27 allelic type [137]. Vaccinees had a significantly lower prevalence of the 3D7 type of parasite, as well as reduced number of infections with the 3D7 type, while neither the prevalence nor incidence of FC27 type of infections was significantly affected [137]. It is worth to note though that 3D7 did induce T-cell responses against FC27, although these were generally weaker in comparison to 3D7. In addition, the vaccine-induced reduction of 3D7 parasites could possibly have given way for FC27 parasites to increase in amount, as a consequence of some kind of reduced cross-protection [137]. Parasite antigen polymorphism was a focus of our third and fourth study (III, IV).

**Inducing the right type of response**

Not only does one desire sufficient levels of activation in a broad population of vaccinees, the immune response induced should also be of the proper type in terms of cell- versus antibody-mediation. Hence, rational vaccine design
involves the understanding of not only mechanisms of protective immunity, but also mechanisms biasing the response in either direction. Generally, protection against malaria depends mainly on cell-mediated immunity during the liver-stage, and antibody-mediated immunity during the blood stage. Among the parameters determining if a response will be skewed towards a cell-mediated (involving Th1 cells) or antibody-mediated (involving Th2 cells) response, are the antigen dose and type of co-stimuli [191]. The accumulating signal from antigen and co-stimulation is integrated by DCs that in turn drive the differentiation of naïve T cells to IFN-γ secreting Th1 cells or IL-4 secreting Th2 cells. The two subsets of DCs, myeloid and plasmacytoid DCs, differ in their expression of MHC II and co-stimulatory molecules, and hence their “proneness” to induce either Th1 or Th2 biased responses. They also differ in their location in the body, and from this follows that a third parameter, the immunization route, is also involved in determining the Th1/Th2 bias. In addition, our second study sheds light upon yet another important parameter, the genetic background of the vaccinees (II). As previously mentioned, we investigated the type of immune response induced by various T-cell epitopes. We were able to circumvent the genetic restriction, and found also that the nature of the immune responses in terms of total IgG antibodies and their subclass distribution, T-cell proliferation and IFN-γ production, varied with the mouse strain, in this case C57BL/6 and BALB/c. The response profiles also varied with the type of T-cell epitope employed, which may be explained by differential DC processing of antigen, resulting in different amounts of MHC II:peptide-complex on the DC surface and hence different effective antigen doses. In general, a high dose of antigen induces the development of Th1 responses, whereas a low dose favors Th2 cell development, regardless of the DC subset [192;193].

As correlates of Th1 and Th2 responses, we analyzed the IgG2a and IgG1 antibodies in mice immunized with the EB200 fragment of Pf332 (I). In the case of DNA plasmids, we investigated the effects on Th1/Th2 bias when including the SFV replicase, versus the replicase with an additional protein secretion signal for the antigen. The inclusion of a secretion signal has previously been shown to modulate the immune response towards Th2 [194]. Unfortunately our results were not conclusive, since the obtained antibody titers were generally low, and the Th1/Th2 profiles from our first immunization series differed from those of the two subsequent series. One could speculate that an eventual effect of the secretion signal would be overshadowed by that of the SFV replicase. In turn, the strong immunostimulatory effect of the replicase was probably not able to compensate for the poorer transfection rate (as reflected by lower antibody titers) obtained by its inclusion. However, as previously mentioned, the induction of cell-mediated rather than antibody-mediated responses could possibly also explain that lower antibody titers were observed for the replicase-containing plasmids.
We also observed that even in the case of relatively low preceding titer peaks, SFV particles as well as DNA plasmids induced specific humoral responses that were swiftly recalled by a booster injection, a finding consistent with the functional definition of immunological memory. The IgG subclass profiles of antibodies induced by all immunogens remained relatively unchanged after the protein booster injection. This was expected, since the cytokine polarization of mouse CD4\(^+\) T cells leads to irreversible silencing of either the IL-4 gene (in Th1 cells) or IFN-\(\gamma\) gene (in Th2 cells) [195], in contrast to human CD4\(^+\) T cells, which maintain some flexibility of cytokine gene expression even after pronounced polarization [196].

The IgG subclass distribution is indeed an important focus for malaria vaccine optimization, as non-cytophilic antibodies might compete with cytophilic and thereby hamper the efficacy of ADCI and phagocytosis (reviewed in [104]). Of the four T-cell epitopes we investigated in the second study, three were derived from \textit{P. falciparum} and the fourth from \textit{Clostridium tetani} (II). When analyzing the subclasses of the induced antibodies, we found that only the latter T-cell epitope elicited cytophilic antibodies to the associated B-cell epitope (in C57BL/6 mice). This T-cell epitope was also the only to induce higher titers of IgG2a, IgG2b and IgG3 in comparison with IgG1 (in BALB/c mice), a phenomenon previously observed by others [197], which may indicate a skewing towards the Th1 type of response. Having said this, it is however important to note that our main conclusion from this study was that the resulting responses varied both qualitatively and quantitatively, depending on the T-cell epitope used as well as the genetic background of the vaccinated mice. Keeping in mind that data obtained in mice have to be extrapolated to humans with caution, this is still likely to hold true of a human setting as well. Thus, in order to obtain an immune response of the required quality and quantity for protection in a genetically heterogeneous human population, one probably has to include a combination of different universal T-cell epitopes in a future malaria vaccine.

The mechanism whereby the cytophilic antibodies IgG1 and IgG3 mediate protection against malaria involves their ligation with Fc\(\gamma\) receptors on monocytes. However, there are studies suggesting a protective role of the non-cytophilic IgG2 \textit{in vivo} [198;199], which possibly may be explained by the presence of a polymorphism in the Fc\(\gamma\) receptor IIA (Fc\(\gamma\)RIIa). As a result, also IgG2 are cytophilic in individuals carrying the H131 allele coding for Fc\(\gamma\)RIIa [200]. When analyzing the subclasses of C231 specific IgG in sera from individuals naturally primed to \textit{P. falciparum} (IV), we observed a bias towards IgG2 and IgG3 relative to IgG1. However, a possible correlation to a preferential expression of the H131 allele in the study population remains to be studied.
Choosing the right immunogen

Throughout the years, many pathogens have evolved ingenious ways of escaping their host’s immune system, and the malaria parasite is far from an exception. Its many stratagems include rosetting (binding of uninfected erythrocytes to infected erythrocytes) and cytoadherence (binding of infected erythrocytes to endothelial cells) mechanisms, variation and diversity of antigens, inhibition of macrophage phagocytosis and dendritic cell maturation, induction of immunosuppressive cytokines and prostaglandins, development of hypnozoites and resistance to antimalarial drugs, and last but not least, the mere fact that it harbors inside erythrocytes which lack MHC molecules. Yet another phenomenon believed to be a strategy of host immune evasion is the fact that many malaria parasite antigens contain extensive arrays of tandemly repeated short amino acid sequences. Such repeats may induce T-cell independent responses, which are less protective [201], as well as function as an “immunological smoke-screen” by diverting responses away from more critical epitopes [202].

A majority of the immunogenicity studies on the Pf332 antigen have so far been performed with the EB200 fragment. It comprises a vast number of glutamic acid-rich repeats, resulting in prominent cross-reactivity with other blood-stage antigens, as well as a proneness to polymorphism. Antigenic variation and diversity is the most troublesome hurdle to overcome in the development of an effective malaria vaccine. For instance, substitution of only one amino acid in a protective T-cell epitope can result in failure to activate the proper T cells [203]. In our third and fourth study, we characterized the immunogenic properties of a non-repetitive part of the Pf332, termed Pf332-C231 (C231) (III, IV). Its primary sequence is more complex as the contribution of each type of amino acid is more equal, and the C231 sequence also includes two cystein residues. Theoretically, both of these features may contribute to sequence conservation. As a result of its higher complexity, C231 has additional advantages over EB200, such as higher probability of providing Pf332-specific monoclonal antibodies, and significantly improved possibility of sequencing, designing stringent primers, as well as staining the expressed protein after gel electrophoresis. So far our characterization of the immunogenic properties of C231 shows that the recombinant protein is reactive with sera from malaria immune humans, and that antisera raised in rabbits inhibit parasite growth in vitro with a capacity similar to that of antibodies to recombinant EB200 (III). Immunoblotting analyses performed with SDS-PAGE separated polypeptides from late stage P. falciparum parasites showed, that antibodies to C231 and EB200 reacted with the same high-molecular weight polypeptides assumed to represent Pf332 (IV). They also gave rise to similar patterns of vesicle-like dots when performing immunofluorescence staining of infected erythrocytes (IV). When analyzing individual patterns of IgG antibody
recognition in 100 Senegalese donors from 4-87 years of age, the reactivity with C231 correlated well with that previously seen in the corresponding sera for antibodies to EB200 (IV). However, while high levels of antibodies reactive with EB200 or C231 tended to be predictive of fewer future clinical malaria attacks, this tendency was statistically significant only for antibodies reactive with EB200 [157], which possibly may be explained by the higher cross-reactivity of such antibodies.

**With a little help from our friends**

Detailed studies on the mechanisms of protective immunity to malaria, as well as on the effects of a vaccine, are most readily approached by the use of mouse models. However, since the human malaria parasite strains are not pathogenic in mice, and since the immune system of mice differs from that of humans, one could argue that mouse models may not be relevant to the human infection. One illustrative example is the technology of DNA vaccines, which first seemed very promising in mice, but later it became evident that DNA immunization alone is insufficient for inducing protection in humans [204]. It is important to determine to what extent the mouse models resemble the features typical of human malaria, before one can extrapolate the data to humans. For instance, we now know that cerebral malaria in mice is caused by sequestered monocytes, rather than infected erythrocytes [205]. Apart from the various immunological differences between the two species (reviewed in [206]), the lack of translation from mouse to man may also be a result of the fact that humans, unlike laboratory animals, often have concomitant infections.

In some cases, mouse models are simply insufficient. Since no analogue to the asexual blood-stage antigen Pf332 has been identified in any of the mouse malaria species, we have not been able to analyze our vaccine constructs in terms of protection. However, we have utilized three other parameters that may serve as indicators of vaccine efficacy. Firstly, antibody titers and their inhibition of parasite growth/reinvasion *in vitro* [207]. Secondly, IgG subclass profiles, knowing that protection against malaria in human is dependent on antibodies of the cytophilic subclasses [104]. Thirdly, T-cell responses, as strong T-cell responses are likely to push T cells into the memory pool [208].

We can also conclude, that even though immune responses to EB200 are genetically restricted in mice (II), there is no indication suggesting that genetic restriction limits the responsiveness to EB200 in humans. No correlation between high or low responsiveness with particular HLA molecules was found when analyzing sera from malaria-exposed Senegalese adults [209], and analyses of sera from Senegalese and Liberian individuals showed that low reactivity with EB200 concurred with low antibody levels to crude malaria antigens as well [155].
Nevertheless, while caution in interpreting data in mice is clearly warranted, mice have been and will continue to be the foremost experimental *in vivo* model for malaria immunology and vaccine research.
During the 1970’s, three discoveries with great impact on the development of a malaria vaccine were made; genetic engineering, monoclonal antibody production, and the possibility to maintain in vitro cultures of *Plasmodium falciparum*. It is probably not presumptuous to add the recent completion of the *P. falciparum* genome sequencing, which unveiled 5,268 parasite genes [210]. Not only does this massive effort facilitate vaccine target identification, but also drug development, functional gene studies and the understanding of parasite evasion mechanisms.

Sequencing of the Pf332 gene revealed a giant protein rich in glutamic acid residues. During the last decade and before sequence completion, the Pf332-derived fragment EB200 has undergone several analyses of its immunogenicity, in which the vaccine candidate potential of the Pf332 antigen were repeatedly confirmed. Our studies herein on Pf332 have focused on characterization and optimization of the humoral responses elicited by various types of vaccines constructs, including DNA, naked mRNA, alphavirus, and peptides. The results can be summarized as follows:

- Comparative immunization with naked DNA, naked RNA and recombinant SFV particles showed that immunological memory was successfully induced against the repetitive EB200 fragment, although the antibody titers were generally low and the highest titers were unexpectedly obtained with a conventional DNA plasmid (I).
- We demonstrated the ability to circumvent genetically restricted immune responses in mice against two malaria epitopes, one of them derived from Pf332, by inclusion of universal T-cell epitopes into multiple antigen peptide constructs. However, the variability of the responses stressed the importance of including several epitopes in a future malaria vaccine (II).
- We identified and characterized the immunogenic properties of a non-repeat Pf332 fragment termed C231. Anti-C231 antibodies possessed an in vitro parasite inhibitory capacity similar to that of antibodies against EB200 (III), and the reactivity of C231 with sera from naturally primed individuals correlated well that previously observed for the corresponding sera and EB200 (IV). When analyzing the IgG subclass distribution of anti-C231 antibodies, we noted a bias towards IgG2 and IgG3 relative to IgG1, differing from the subclass profiles of IgG binding crude *P. falciparum* antigen, which were dominated by IgG1 (IV).
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GODDAG, YXSKAFT

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