Human T cell responses to a semi-conserved sequence of the malaria vaccine candidate antigen MSP-1

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Abbreviations

Ab	Antibody
ADCC	Antibody-Dependent Cell-mediated Cytotoxicity
APC	Antigen Presenting Cell
APL	Altered Peptide Ligand
CD	Cluster of Differentiantion
cDNA	complementary Deoxyribonucleic acid
Ci	Curie
CLIP	Class II-associated invariant chain peptide
ConA	Concanavalin A
cpm	counts per minute
CTL	Cytotoxic T Lymphocyte
DMSO	Dimethylsulfoxid
dNTP	Deoxyribonucleosidetriphosphate
EBV	Epstein Barr Virus
FACScan	Fluorescenc Activated Cell Scanning
FCS	Fetal Calf Serum
FITC	Fluorescein Isothiocyanate
HBSS	Hanks Balanced Salt Solution
HEPES	N-2-Hydroxyethylpiperazine-N´-2-ethansulfonic acid
HLA	Human Leukocyte Antigen
HS	Human Serum
HVS	*Herpesvirus saimiri*
Ig	Immunoglobuline
IL-2	Interleukine 2
IL-4	Interleukine 4
INFγ	Interferon γ
mAb	monoclonal Antibody
MHC	Major Histocompatibility Complex
mRNA	messenger Ribonucleic Acid
MSP-1	Merozoite Surface Protein 1
PBMC	Peripheral Blood Monomorph-nuclear Cell
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PE	Phycoerythrin
PHA	Phytohemagglutinine
RBC	red blood cell
rpm	rounds per minute
RT	Room Temperature
SI	Stimulation Index
TCR	T Cell Receptor
Th	T helper cell

Summary

Over the past decade of malaria research, there has been considerable progress in the understanding of immune mechanisms involved in conferring protection to malaria and in the identification of vaccine candidate antigens. Despite this increasing knowledge, there is still no effective malaria vaccine available. Current vaccine development concentrates on multi-component, multi-stage subunit vaccines in combination with improved delivery systems. In addition, an ideal malaria vaccine should induce both cellular and humoral immune responses and therefore requires the incorporation of T cell- as well as B cell-epitopes.

The merozoite surface protein 1 (MSP-1) of *Plasmodium falciparum* is one of the most promising vaccine candidate antigens. A semi-conserved region at its N-terminus eliciting protective immune responses in malaria models has been incorporated into the synthetic peptide vaccine SPf66. This sequence of MSP-1 was found to be a suitable human B cell epitope, eliciting parasite-binding antibodies. In this thesis the question has been addressed whether the same region of MSP-1 also represents a suitable T cell epitope and whether semi-conserved sequences are suitable elements for epitope-focussed vaccines. In addition, residues of sequence 38-58 of MSP-1 interacting with the T cell receptor (TCR) were mapped, and the potential of pseudopeptide analogues for T cell activation was explored. Furthermore, MSP-1^{38-58} and SPf66 were used to test a *Herpesvirus saimiri*-based system for T cell cloning and to assess the potency of a new adjuvant.

Human MSP-1^{38-58}-specific T cell lines and clones were generated from SPf66-vacinated volunteers. The T cell clones were CD4+, mainly of Th2 type, and exhibited a high specificity for the particular sequence variant (S^{44} Q^{47} V^{52}) present in the vaccine: None of the four other naturally occurring variants of the semi-conserved region of MSP-1 found in *P. falciparum* populations stimulated T cell proliferation or cytokine secretion, although all variants exhibited activity in HLA-DR peptide binding competition assays. Thus MSP-1^{38-58} although a potent stimulator of T cells, does not appear to be a suitable vaccine epitope.

Multiple genetic restriction elements were used by the T cell clones to recognize MSP-1^{38-58}. DR- and DP-restricted clones were found to recognize overlapping, but distinct, epitopes clustered within the core region of MSP-1^{38-58}. Substitution of individual amino acids with alanine or glycine revealed that only about nine residues of the presented peptide are "read out" by the TCR although additional epitope-flanking regions are required for T cell stimulation as well.

The contribution of the peptide backbone itself to T cell activation and HLA-DR-binding was assessed with reduced-amide pseudopeptide analogues of MSP-1. Some pseudopeptides exhibited even better stimulatory activity while others were less potent than their parent peptide. Thus the peptide backbone appears to contribute critically to MHC binding and TCR triggering. Pseudopeptides, which generally exhibit decreased protease susceptibility and a better reproduction of conformational B cell epitopes, might advantageously replace natural peptides in future vaccines.

Investigations of cellular immune responses on the clonal level during clinical vaccine trials are hampered by the limited volume of available blood samples. A method to generate antigen-specific T cell clones using *Herpesvirus Saimiri* (HVS)-transformed autologous T cells as antigen presenting cells (APCs) was established. MSP-1 $^{38-58}$ specific and SPf66-specific T cell clones were generated by using either autologous PBMCs or HVS-transformed T cells as APCs. The resulting panels of T cell clones exhibited similar characteristics and identical TCR rearrangements were found in both panels. HSV transformation is thus a useful method for detailed analysis of T cell responses in the course of clinical vaccine trials where only small amounts of blood cells are available.

The immunogenicity of a synthetic peptide vaccine depends on the delivery system or adjuvant. To investigate the immunogenicity of a new formulation of SPf66 in combination with the saponin adjuvant QS-21, SPf66-specific T cell lines were generated from SPf66/QS-21 vaccinated volunteers and compared to lines generated from SPf66/alum vaccinated persons. The T cell responses elicited by the two SPf66-formulations differed significantly: Vaccination in combination with QS-21 induced both CD4+ and CD8+ T cell responses while SPf66/alum vaccination induced predominantly CD4+ T cell responses of the Th2 subtype. QS-21 is therefore a promising candidate for the delivery of the next generation of malaria vaccines.

1 General Introduction

Malaria remains one of the major public health problems in tropical countries, causing 1.5–2.7 million deaths per year, mainly among young children in Sub-Saharan Africa (Greenwood et al. 1991; World Health Organisation 1995). Vaccination is considered as an approach that will complement other strategies for the prevention and control of this disease. Therefore the development of an effective malaria vaccine has become a major challenge for biomedical research. Individuals exposed to malarial infection over a long period of time exhibit a complex immune response against the parasite and can develop a partial immunity. Human passive immunisation studies with isolated antibodies from donors living in endemic regions have indicated that antimalarial IgG can prevent replication of the parasite (Bouharoun-Tayoun et al. 1990; Cohen et al. 1961; Endozien et al. 1962; McGregor et al. 1963). Besides the humoral immune response, cellular immunity is assumed to also contribute to protection against infection (Good and Doolan 1999). A number of cell surface proteins of sporozoites, merozoites and infected erythrocyte have been studied intensively for their ability to induce protective immune responses. Intensive efforts are made to identify B cell as well as T cell epitopes of vaccine candidate antigens.

1.1 Malaria tropica

Malaria is a severe infectious disease caused by protozoan parasites of the genus *Plasmodium*. Four species of *Plasmodium* are able to infect humans: *P. falciparum* (Malaria tropica), *P. vivax* (Malaria tertiana), *P. ovale* and *P. malariae* (Malaria quartana). Malaria tropica caused by *P. falciparum* is the most dangerous and geographically the most widespread malaria, being responsible for about 90% of the malaria morbidity and mortality world-wide (Marsh 1992).

The resistance of malaria parasites to drugs and the resistance of mosquitoes to insecticides have resulted in a resurgence of malaria in many parts of the world and a pressing need for vaccines and new drugs.

1.1.1 Life cycle of P. falciparum

The life cycle of the malaria parasite and considered stage specific vaccine-targets are shown in Figure 1. Infection in humans begins with the bite of an infected female *Anopheles* mosquito (Good et al. 1998). Sporozoites released from the salivary glands of the mosquito enter the bloodstream and quickly invade hepatocytes. There they undergo a phase of maturation and asexual reproduction (schizogony) resulting in thousands of merozoites which burst from the hepatocyte and are released into the blood. The erythrocytic life cycle is initiated by the invasion of red blood cells by the merozoites followed by schizogony. Infected erythrocytes rupture and release more merozoites into the blood, which can rapidly invade uninfected red blood cells and thus start a new cycle.

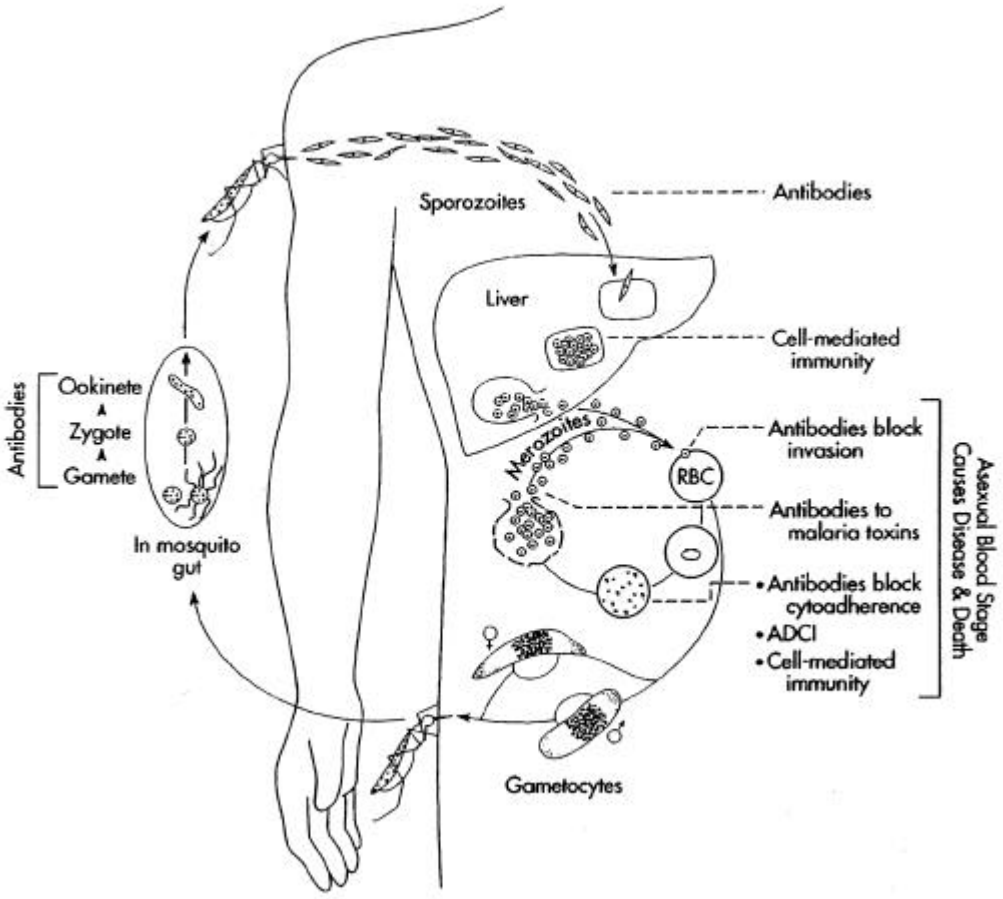

Figure 1. The life cycle of *P. falciparum*, with emphasis on targets for blood-stage vaccines (Good et al. 1998).

To enable transmission from the human host to the mosquito vector, some of the merozoites undergo differentiation into male and female gametocytes. The blood-feeding mosquito takes

up gametocytes contained within red blood cells. In the mosquito's gut the gametocytes emerge as gametes, fertilize to produce motile ookinetes and then migrate across the gut wall to form oocysts. The oocyst produces sporozoites which migrate to the mosquito's salivary gland ready for inoculation into the human host.

A high genetic variability and an extensive polymorphism together with a high degree of different proteins associated with a particular stage of the life cycle are characteristics of the malaria parasite. Different stage specific antigens are currently taken into consideration to trigger a protective immune response via vaccination.

1.1.2 Immunity to malaria

The immune response in humans against natural infections due to malaria parasites is complex and varies with the level of endemicity, genetic makeup, age of the host and parasite stage and species. The immune response involves in addition to innate non-adaptive defence mechanisms, humoral as well as cellular components of the adaptive immune system. Repeated infections and continued exposure are required to achieve clinical immunity (Marsh 1992). While children younger than 6 month are presumed to be protected by maternal antibodies, this protection is lost during early childhood, rendering ingants susceptible to severe forms of the disease with high mortality until the age of about 3 years. Once this stage of high parasitemic infection is passed, acquired immunity (premunition) develops gradually. The developed immunity is not sterile but individuals usually show low-grade parasitemia without apparent clinical symptoms (Facer and Tanner 1997; Marsh 1992) . Nevertheless, this acquired "anti-disease" immunity is not stable and requires exposure to repeated infections.

The mechanisms of acquired immunity are poorly understood but are thought to be cell-mediated as well as humoral. An early response by monocytes or macrophages to parasite toxins induce fever and other non specific immune mechanisms which lead to reduction of blood stage parasite densities. Subsequent steps leading to the protective state of premunition include the T cell mediated activation of macrophages and neutrophils as well as the induction of a broad B cell response. Extensive antigen-polymorphism, antigenic variation, immunodominance of non-protective antigenic structures in combination with low immunogenicity of epitopes critical for protection, represent obstacles for the immune system to build up a protective immunity.

Little is known about the role of T lymphocytes in mediating immunity to Plasmodium infections in humans. CD4+ αβ T cells seem to be essential in the maintenance of protective immunity by providing T cell help both for B cell maturation and induction of antibody independent defence (Good and Doolan 1999; Plebanski and Hill 2000; Troye-Blomberg 1994). Adoptive transfer experiments in murine models gave indications for the importance of CD4+ T cells directed against asexuals blood stage antigens (Good and Doolan 1999). Protection against pre-erythrocytic stage malaria is presumed to be mediated directly by CD8+ cytotoxic T cells. Cytokines like INF-γ and other factors, including nitric oxide, have also been implicated in protection (Doolan et al. 1996; Fell and Smith 1998) .

1.1.3 Protective mechanisms and immune evasion in blood-stage immunity

While in pre-erythrocytic immunity, INF- γ seems to be associated with protection, in asexual blood-stage immunity, proinflammatory lymphokines may play a more ambiguous role, with the potential to be either protective or pathogenic (Plebanski and Hill 2000). INF-γ production by CD4+ T cells in response to specific blood-stage antigens seems to be associated with protection against malaria re-infection in Africa (Luty et al. 1999). In murine malaria, INF- γ secreting T cell clones can protect by a nitrate-dependent mechanism possibly mediated by macrophages and neutrophils (Stevenson et al. 1989). T cell secretion of IFN-γ may also help to induce cytophilic blood-stage-specific IgG and assist in antibody-dependent cellular inhibitory mechanisms (Bouharoun-Tayoun et al. 1995).

Antibodies to diverse parasite antigens expressed on the surface of infected red cells, or on free merozoites can inhibit parasite growth (Good et al. 1998; Miller et al. 1998; Saul 1999). In man, T cells specific for blood stage antigens may contribute to immunity both as helpers for antibody production and as effector cells leading to parasite elimination.

Several immune evasion mechanisms might be operating in malaria infections. Plasmodium infection can lead to anergy and deletion of parasite-specific CD4+ T cells in mice (Hirunpetcharat and Good 1998). Interference with the priming of human T cell responses by naturally occurring variants could be demonstrated for a cytotoxic T cell epitope of CSP (Plebanski et al. 1999a). A similar mechanism of immune interference may also play a role in the suppression of immune responses against blood-stage antigens. Mutation of residues involved in binding of certain parasite-epitopes to certain MHC-alleles common in

the host population, could impede T cell recognition. Once epitope-specific T cells are generated, the pathogen could still modulate their effector functions by APL antagonism (Gilbert et al. 1998; Plebanski et al. 1999b). Antagonism is based on the concomitant presentation of closely related peptide variants which provide an altered activation signal to the T cell, resulting in the inactivation of some of its effector functions (Jameson and Bevan 1995; Sette et al. 1994). Antigenic polymorphism, T cell antagonism and immune interferences by altered peptide ligands (APL) have therefore been proposed as evasion mechanisms used by the parasite. It remains to be proven conclusively whether the parasite has evolved such immune evasion strategies. However, rapid advances in characterising plasmodial antigens eliciting protective responses made it possible to define some antigenic structures involved in immunity. This, together with an improved understanding of cellular mechanisms, provides some basis for the development of a malaria vaccine.

1.2 Malaria vaccines

The concept for the development of a malaria vaccine arose from experimental immunisation studies with irradiated sporozoites. Human volunteers immunised with irradiated attenuated *P. falciparum* sporozoites developed a protective immune response against subsequent malaria infections (Clyde 1975; Rieckmann et al. 1974). This observation supported the idea that vaccination against malaria infection should be possible in principle. Disadvantages associated with attenuated vaccines, like difficulties in producing large amounts of irradiated sporozoites, have led to the search for protective antigens of different life cycle stages of the malaria parasite. Despite intensive research during the last twenty years, there is still no effective vaccine available (Facer and Tanner 1997). The complexity of the parasite life cycle, imperfect tools to assess the efficacy of immune responses and limited knowledge of the factors that determine the outcome of an infection are still the main obstacles in developing such a vaccine.

1.2.1 Strategies for malaria vaccine development

There are several different strategies in the development of a malaria vaccine. The vaccine either could i) block sporozoites from invading or developing within hepatocytes (*anti-infection vaccine*), ii) block the merozoite invasion of red blood cells or inhibit development of schizonts (*anti-disease vaccine*), or iii) block the pathology inducing effects of cytokines or parasite sequestration (*disease-modifying vaccine*). Another strategy aims to block human-mosquito transmission by immunisation against the sexual stages of the parasite (*transmission-blocking vaccine*), preventing the spread of disease within a population but having no direct impact on protection of the vaccinated individual (Facer and Tanner 1997). An effective vaccine should advantageously follow different strategies (see Figure 1), by stimulating protective immune responses against several stages of the life cycle at one time (Bathurst et al. 1993).

1.2.2 Key malaria vaccine candidate antigens

Many candidate antigens for *P. falciparum* malaria subunit vaccines have been studied and identified in the last two decades of malaria research. Efficacy has been measured on the basis of protection studies in animal models (murine and ape) and on *in vitro* activities of antisera and monoclonal antibodies that block host cell invasion and/or parasite development *in vitro* (Good et al. 1998).

Circumsorozoite surface protein (CSP) of *P. falciparum* is a major surface antigen of the pre-erythrocytic sporozoites. It contains a conserved region of NANP repeats which acts as a highly immunodominant B cell epitope. Clinical trials conducted with recombinant or synthetic peptides on the basis of NANP repeats induced a dose dependent immune response, but exhibited poor protective efficacy (Anders and Saul 2000; Facer and Tanner 1997). Most success has been achieved with RTS,S which is a CSP-based recombinant fusion protein. Over 50% of naive immunised volunteers were protected against experimental sporozoite challenge, but protection was of short duration (Stoute et al. 1998). LSA-1 is expressed by the liver stage parasites and can be localised in the parasitophorous vacuole space. Together with LSA-3, CSP and SSP-2 (sporozoite surface protein 2) LSA-1 is thought to have pre-erythrocytic-stage vaccine potential due to recognition of conserved LSA-1 epitopes by cytotoxic CD8+ T lymphocytes (Hill et al. 1992). In a recent study, immunization of

chimpanzees with LSA-3 induced protection against successive heterologous challenges with large numbers of *P. falciparum* sporozoites (Daubersies et al. 2000).

Current blood stage vaccine candidates include merozoite surface antigens MSP-1 and MSP-2, Erythrocyte Binding Antigens EBA-175 and Duffy-binding protein as well as RESA (Ring-infected Erythrocyte Surface Antigen), GLURP (Glutamate-rich Repeat Protein), RAP-1 (Rhoptry-Associated Protein), RAP-2, AMA-1 (Apical Membrane Antigen), Pfs-230 and Pfs-40 (Anders and Saul 2000; Engers and Godal 1998; Good and Doolan 1999). Among these antigens, MSP-1, AMA-1 and RAP-1/RAP-2 are the most promising vaccine candidates. A vaccine composed of three recombinant proteins, MSP-1 combined with MSP-2 and RESA (Combination B), has been studied for safety and immunogenicity (Genton et al. 2000; Saul et al. 1999). Vaccination with Combination B reduced parasite densities with an efficacy of 62% and vaccinees had lower prevalence of infection with a parasite strain carrying the same MSP-2 allele used for the vaccine.

An extremely heterogeneous protein family (*var* gene family) of the erythrocyte membrane proteins 1 (PfEMP1) is involved in sequestration. Disease-modifying vaccines could make use of these antigens and prevent clinical complications associated with sequestration of parasitised red blood cells.

1.2.3 MSP-1

Among the blood-stage vaccine candidates, Merozoite Surface Protein 1 (MSP-1) of *P. falciparum* is regarded as highly promising antigen. Immunisation with purified MSP-1 could protect monkeys from malaria infection after experimental challenge with parasites (Hall et al. 1984; Patarroyo et al. 1987; Siddiqui et al. 1987).

MSP-1 is the most abundant glycoprotein found on the surface of the invasive merozoite stage. It is synthesized as an approximately 200 kDa precursor protein at the schizont stage and is further proteolytically cleaved into a number of products residing on the surface of the merozoite (Holder and Blackman 1994). A first processing step produces four major fragments of 83, 42, 38 and 28-30 kDa (Figure 2). Secondary processing of the 42 kDa fragment generates a C-terminal 19 kDa product, which is the only fragment remaining on the parasite during red cell invasion, all other fragments being shed from the merozoite surface (Blackman et al. 1991). Based on sequence analysis the glycoprotein can be divided into 17 building blocks consisting of conserved, semi-conserved and variable regions (Mackay et al.

1985; Tanabe et al. 1987). Block 1 at the N-terminus of MSP-1 contains the signal sequence (aa 1-20), a conserved region (aa 21-41) and a region of low strain-specific variability (aa 42-53/55). The latter region comprises the 83.1 epitope (aa 43-53) which was included into the synthetic peptide vaccine Spf66 (Figure 2, see also chapter 1.3).

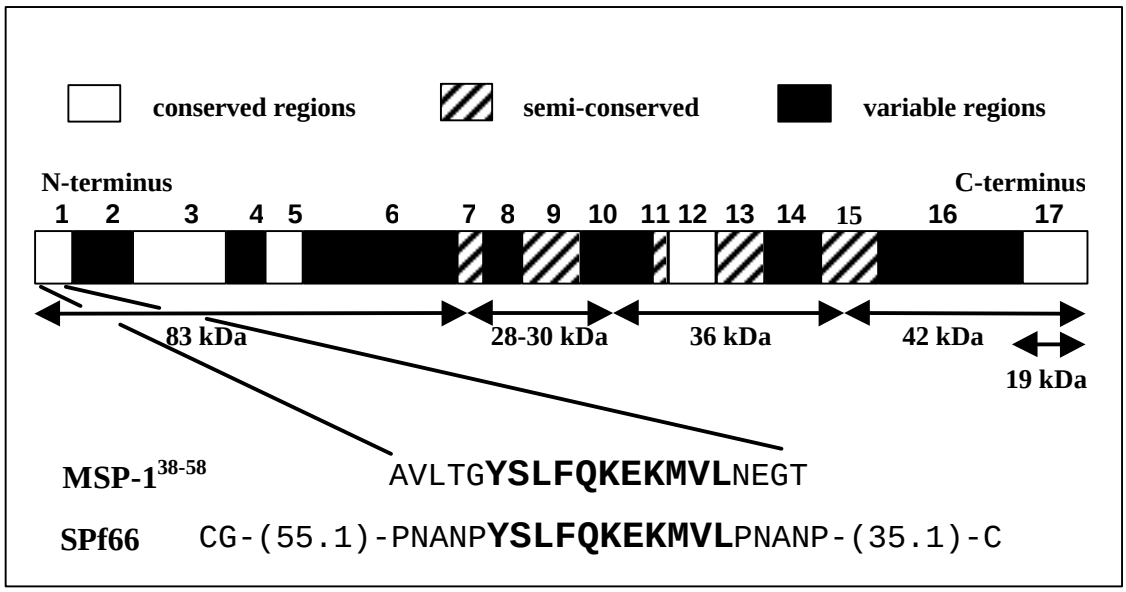

Figure 2. Schematic representation of the MSP-1 protein and its processing products (arrows). Building blocks consist of conserved, semi-conserved and variable regions. Lower part of the figure shows the MSP-1^{38-58} peptide sequence variant of the PNG-MAD20 clone and a monomeric unit of the synthetic peptide vaccine SPf66, comprising the 83.1 epitope Y^{43}SLFQKEKMVL^{53} of MSP-1.

Six different naturally occurring sequence variants of the 83.1 epitope have been described world-wide (Jiang et al. 2000; Miller et al. 1993) , with dimorphic amino acid exchanges at three defined positions: S^{44}/G^{44}, Q^{47}/H^{47} and V^{52}/I^{52} or L^{52} (Table I). Polymorphisms in other amino acid positions of block 1 have not been described indicating that variation may not be advantageous for the parasite. Lack of silent polymorphism (base exchanges but no amino acid replacement) has been attributed to evolutionary recent descent of the world populations of the parasite from a common cenancestor that lived in tropical Africa a few thousand years ago (Ayala and Rich 2000; Rich et al. 2000) .

Dimorphism of block 1 could represent an immune evasion mechanisms of the malaria parasite. Immune responses directed against one variant could be down-regulated after

infection with another variant by altered peptide ligand inhibition mechanisms (Gilbert et al. 1998; Plebanski et al. 1999b).

Table I. *Sequence variants of block 1 of MSP-1*

Strain	Amino acid sequence [a]
PNG-MAD20	Y **S** L F **Q** K E K M **V** L
Holland-NF54	Y **G** L F **Q** K E K M **V** L
Ivory-RO71	Y **G** L F **H** K E K M **I** L
Brazil-608	Y **G** L F **H** K E K M **L** L
Thai-K1	Y **S** L F **H** K E K M **I** L
IFA-9.2	Y **G** L F **H** K E K M **V** L

[a] Amino acid sequences are listed in single letter code and correspond to aa 43-53 of block 1 of MSP-1. Variable amino acids are drawn in bold letters. Variant PNG-MAD20 has been included into the synthetic peptide vaccine SPf66.

1.2.4 The synthetic peptide vaccine SPf66

The synthetic peptide vaccine SPf66 was developed on the basis of proteins isolated from infected human erythrocytes which exhibited protection in *Aotus* monkeys (Patarroyo et al. 1987). Several peptides derived from partial sequences of these proteins provided protection against experimental infection with *P. falciparum* in *Aotus* monkeys. Three of the most effective peptides were chemically synthesised as a continuous peptide linked by PNANP spacer-sequences derived from the repeat region of CSP (Patarroyo et al. 1987). The resulting multiple epitope peptide (MEP) designated SPf66 consists of three peptide sequences derived from proteins with molecular masses of 35.1, 55.1 and 83.1 kDa. Cysteine residues added to the ends of the MEP allowed polymerization (Figure 3).

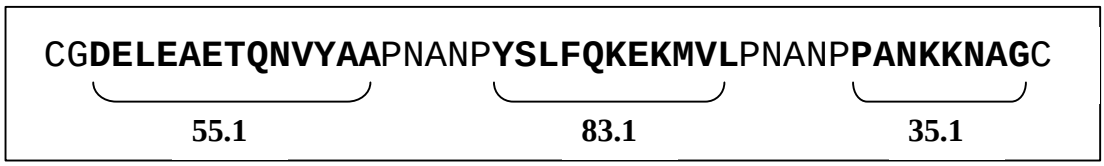

Figure 3. Amino acid sequence of a monomeric unit of SPf66. The values labelling the peptide blocks correspond to the molecular weights of the three proteins isolated from *P. falciparum* infected human erythrocytes. The PNANP spacers represent the repeat unit of CSP of *P. falciparum*.

The 83.1 epitope (YSLFQKEKMVL) included into SPf66 represents a sequence derived from block 1 of the PNG-MAD20 variant of MSP-1. The precise origin of the two other epitopes, 35.1 and 55.1, is still unknown and remains to be elucidated. Monoclonal antibodies raised against the 35.1 epitope recognise an unrelated sequence of RAP-1 (Moreno et al. 2001).

SPf66 is the first chemically synthesized vaccine that has been shown to induce a protective immune response to a parasitic disease in humans and has been extensively tested in human phase III trials (Facer and Tanner 1997; Graves and Gelband 2000; Patarroyo et al. 1988; Patarroyo and Amador 1999). While being safe and immunogenic, Spf66 showed protective immunity only in some of the phase III trials conducted so far (Alonso et al. 1994; Alonso et al. 1998; D'Alessandro et al. 1995; Nosten et al. 1996; Noya et al. 1994; Sempertegui et al. 1994; Valero et al. 1993; Valero et al. 1996). Despite the broad variation in the resulting efficacy of all trials carried out with SPf66/alum, a significant reduction in attacks of severe clinical *P. falciparum* episodes could be observed (Graves et al. 1998; Graves and Gelband 2000).

Immune responses to SPf66 have so far been evaluated in clinical trials primarily by measuring antibody titres against the entire vaccine, its peptide components and *P. falciparum* blood-stage parasites. No clear correlation between the degree of protection and the antibody titres has been observed, indicating the need for more specific criteria for humoral as well as cellular protection.

1.3 T cell recognition of antigenic determinants

Three classes of effector T cells act as defense mechanism in collaboration with other cells of the immune system. CD8+ cytotoxic T cells kill target cells that display antigenic fragments of cytosolic pathogens, most notably viruses, bound to MHC class I molecules at the cell surface. Th1 cells and Th2 cells both express CD4 co-receptor and recognize fragments of antigens degraded within intracellular vesicles, displayed at the cell sufrace by MHC class II molecules. The Th1 cells, upon activation, activate macrophages via secreted INF- γ and other effector molecules, allowing them to destroy intracellular microorganisms more efficiently; they can also activate B cells to produce strongly opsonizing antibodies belonging to certain IgG subclasses (IgG1 and IgG3 in humans). Th2 cells, on the other hand, drive B cells to

differentiate and produce immunoglobulins of all other types, and are responsible for initiating B cell responses by activating naive B cells to proliferate and secrete IgM. This B cell activation is mainly mediated by secreted growth factors like IL-4 and IL-5 (Janeway et al. 1999). Antigen recognition by both CD4+ and CD8+ T cells is mediated by the T cell receptor (TCR). Specific recognition of the peptide-MHC complex by the TCR variable domains triggers intracellular signaling cascades and subsequently activates the T cell to proliferate and differentiate into an armed effector T cell.

1.3.1 Structure of MHC class II molecules

MHC class II molecules are heterodimers, formed by two membrane-anchored glycoprotein chains (α and β chain), each containing two extracellular domains: α1 and α2, and β1 and β2, respectively. The peptide binding groove is formed as an interchain dimer by the folding of the α1 and β1 domain to create a long cleft (Figure 4). Polymorphic amino acid residues within the distal domains cluster in hypervariable regions and line the peptide binding groove (Jardetzky et al. 1996; Stern et al. 1994). The membrane proximal domains (α2 and β2) are relatively conserved and have sequence similarities to immunoglobulin constant regions.

Human MHC class II molecules are classified into three major isotypes: HLA-DR, -DQ, and DP. Multiple alleles have been described for each isotype, with DR categorized as the most polymorphic (at least two DRA and >200 DRB alleles), followed by DQ (at least 18 DQA1 and 31 DQB1 alleles), and DP with at least 10 DPA1 and 77 DPB1 alleles (Bodmer et al. 1997). APCs normally express higher levels of DR than of DQ or DP (Gorga et al. 1987). Consequently, most studies involving sequence identification of bound naturally processed peptides (Chicz et al. 1992; Chicz et al. 1993; Newcomb and Cresswell 1993; Rammensee 2001) and crystallographic structure determinations (Brown et al. 1993; Ghosh et al. 1995; Hennecke et al. 2000; Jardetzky et al. 1994; Stern et al. 1994) have focused on DR allotypes.

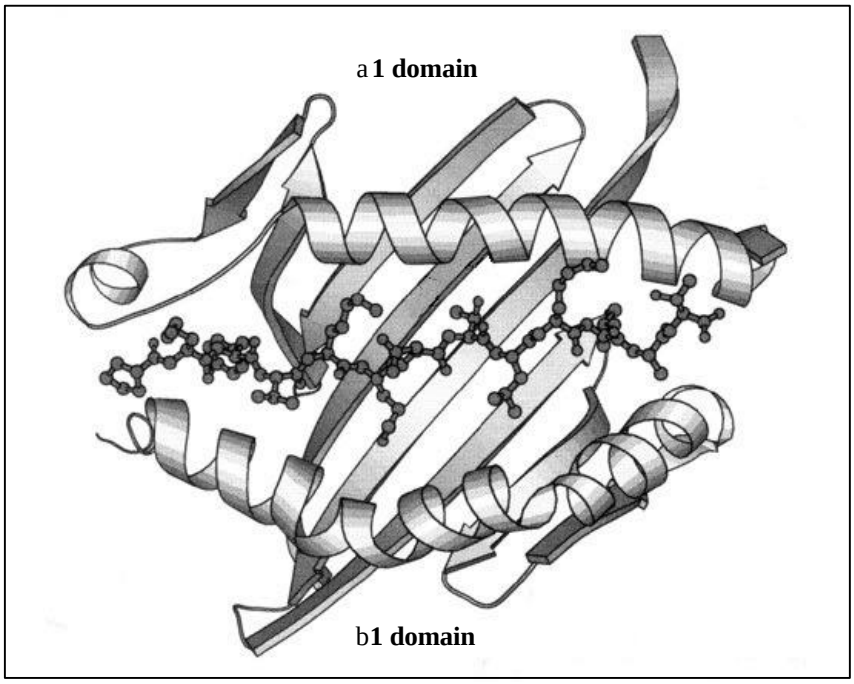

Figure 4. MHC class II molecule with bound peptide. A schematic diagram of the peptide binding groove ($\alpha 1\beta 1$ domain) of HLA-DR1 with bound $HA^{306-318}$ peptide (after Stern et al. 1994).

1.3.2 Peptide-MHC class II complex

The ability of MHC molecules to present a broad spectrum of peptide antigens for T cell recognition requires a compromise between high affintiy and broad specificity (Madden 1995). Three-dimensional atomic structures of several class II DR molecules (Ghosh et al. 1995; Hennecke et al. 2000; Smith et al. 1998; Stern et al. 1994) reveal a unique structural solution to this problem.

MHC class II molecules are able to form stable complexes with a broad range of different peptide sequences. Sequence-independent affinity for peptide ligands is achieved by the formation of hydrogen bonds between conserved MHC residues and the peptide main chain (Madden et al. 1991; Matsumura et al. 1992; Stern et al. 1994) . In addition peptide side chains contact residues within the MHC cleft and increase the overall binding affinity and specificity of the associated peptides (Falk et al. 1994; Hammer et al. 1993; Sette and Grey 1992). Polymorphic side chains of the MHC form allele specific binding pockets which exhibit strong preference for interaction with particular amino acid side chains of the peptide

(anchor residues) (Hammer et al. 1997). The binding groove of MHC II is open at both ends allowing the presentation of antigenic epitopes as large nested sets of peptides of variable length (10-35 amino acids) (Chicz et al. 1992; Chicz et al. 1993;Chicz et al. 1997; Rudensky et al. 1991). Structural studies of MHC class II-peptide complexes have shown that on either side of the minimal epitope there can be at least two peptide flanking residues that are within the groove and thus potentially accessible to the T cell receptor (Fremont et al. 1996; Stern et al. 1994). The peptide binds with hydrogen bonds throughout the entire peptide backbone to conserved residues of the MHC II molecule allowing a flatter conformation of the peptide in the class II binding cleft than in the case of MHC class I (Figure 4).

Binding efficiency of the peptide therefore depends on both, interaction of peptide residues with the MHC groove and the formation of hydrogen bonds between the peptide backbone and conserved residues from the MHC molecule.

1.3.3 The ab T cell receptor

Each T cell expresses a unique T cell receptor (TCR) which recognizes a ligand peptide bound to an autologous MHC molecule. The TCR consists of an α chain joined by a disulfide bond to a β chain. The clone-specific antigen-binding TCR αβ heterodimer is non-covalently associated with a set of invariant transmembrane polypeptide chains, called the CD3 complex. The CD3 sub-units of the complex are responsible for transducing the extracellular binding event into intracellular signalling pathways. For the activation of a T cell a variety of co-receptors also play an important role, such as CD4 or CD8 by binding to non- polymorphic sites on the MHC molecule (Chien and Davis 1993; Davis et al. 1998).

Three complementarity determining regions (CDR1, CDR and CDR3) protrude as loops at the membrane distal ends of both TCR Vα and Vβ domains, collectively forming the antigen binding site (Mazza et al. 1998). CDR1 and CDR2 are both germline encoded within V gene segments, whereas CDR3 is formed during somatic DNA recombination events by the joining of VDJ segments (β chain) or VJ segments (α chain). Due to extensive junctional diversity, the CDR3s of the α and β chain are responsible for most of the diversity observed in αβ TCRs and for antigen recognition. Structural data confirmed that the CDR3 loops are situated largely over the center of the antigenic peptide, whereas the CDR1 and CDR2 loops contact the helices of the MHC molecule (Chien et al. 1996;Hennecke et al. 2000; Mazza et

al. 1998). Crystal structures of peptide-MHC complexes have shown that only three to four side chains of the bound peptide are accessible for recognition by the TCR (Kersh and Allen 1996; Reinherz et al. 1999). The crystal structures of a TCR in complex with peptide and a mouse class II molecule (Reinherz et al. 1999), as well as the recently solved structure of a human TCR/hemagglutintin-peptide/HLA-DR1 complex (Hennecke et al. 2000) revealed an orthogonal orientation of the TCR relative to its peptide-MHC ligand and a dominance of the TCR Vα domain in atomic contacts with the peptide. Thus, a TCR recognizes a bimolecular surface, most of which is composed of the MHC molecule, with the peptide contributing key electron density in the center. There is strong evidence that flanking residues of the bound peptide protruding from the MHC groove can also stabilize the TCR-peptide-MHC-complex (Moudgil et al. 1998).

1.3.4 T cell recognition and activation

A critical event in the initiation of the adaptive immune response is the activation of T lymphocytes. Activation of T cells is mediated by interaction of the TCR with its MHC-peptide ligand. Within seconds of MHC-peptide engagement, the TCR initiates a tyrosine phosphorylation cascade that triggers multiple branching signaling pathways (Grakoui et al. 1999). However, for more complex functions of the T cell, such as proliferation, TCR engagement and signaling is required for many minutes or hours. As TCR-peptide-MHC interaction is known to have a low affinity, there must exist other mechanisms for sustained TCR engagement. Views of antigen-specific T cell junctions have revealed the formation of a specialized contact, termed the immunological synapse (Grakoui et al. 1999). This synapse is defined by a specific pattern of receptor segregation with a central cluster of TCRs surrounded by a ring of integrin family adhesion molecules (Monks et al. 1998). It has been proposed that the formation of such a synapse can provide stabilization for sustained TCR engagement and signaling.

Two complementary models try to explain T cell recognition of a foreign antigen and how T cells can productively interact with suboptimal ligands (Kersh and Allen 1996). The basis of the first model is that the TCR and its associated molecules require an accumulation of phosphorylation events after ligand binding to transmit a positive signal. In this way a T cell can survey many different ligands and specifically respond to ligands with a sufficiently

high affinity while ignoring those with only a slightly lower affinity. The other model proposes that a single peptide-MHC ligand engages multiple TCRs in a serial manner. After interaction of the peptide-MHC complex with one TCR, it detaches and can interact with another TCR. This repeated process results in the assembly of a sufficient number of TCRs in a contact cap for productive signalling to occur, even when there are only limited numbers of ligands. T cells can also be partially activated by suboptimal ligands (Kersh and Allen 1996). The TCR is not simply working as an on/off switch by recognizing a peptide. Minor sequence variations of an antigenic peptide can have antagonistic effects by specifically inhibiting the T cell response. Although the mechanism is not clearly understood, the effect of antagonism seems not to be based on differential capacity of the peptides to bind to the MHC molecule, nor due to simple competition for available MHC binding sites on the APC. Altered peptide ligands (APLs) can also induce a state of unresponsiveness or anergy of antigen specific T cells (Sloan-Lancaster et al. 1993; Sloan-Lancaster et al. 1994; Sloan-Lancaster and Allen 1996). Therefore, alteration of a single amino-acid residue can have profound consequences for the fate of the T cell response.

It has been proposed that altered peptide ligand inhibition of T cell responses may play an important role in immune evasion of malaria parasites (Plebanski et al. 1997).

1.4 Aim of this work

The induction of cellular and humoral immune responses against pathogens using peptide based vaccines requires the incorporation of T cell- as well as of B cell-epitopes. T cell epitopes should advantageously bind to a broad spectrum of different HLA molecules and by this effectively work in a high percentage of individuals within the target population. Therefore, the identification of protective B cell- as well as T cell-epitopes is a prerequisite for the development of an effective epitope-focussed subunit vaccine.

The synthetic peptide vaccine SPf66 includes a sequence which was derived from a semi-conserved region of the major malaria vaccine candidate antigen Merozoite Surface Protein 1 (amino acids 43-53). This region has been demonstrated to be a suitable human B cell epitope eliciting parasite binding antibodies. Whether this region of MSP-1 also represents a suitable human T cell epitope remained to be addressed.

The aim of this study was the functional analysis of human T cell responses specific for sequence 38-58 of MSP-1. For this purpose, human MSP-1 $^{38-58}$-specific T cell lines and T cell clones were generated from SPf66/alum-vaccinated volunteers and characterized in detail.

Different approaches were conducted to address the question of fine-specificity and flexibility of peptide recognition by MSP-1 $^{38-58}$-specific T cell clones:
Chapter 2 of this thesis deals with the high specificity of T cell clones recognizing only one out of five naturally occuring sequence variants of the semi-conserved region of MSP-1. In **chapter 3** the minimal epitope of sequence 38-57 of MSP-1 was determined, which was required to stimulate T cell clones on different restriction elements. Positions within the presented peptide important for HLA-DR binding could be deduced for the HLA-DRB1*0801 allele. The contribution of the peptide backbone itself to binding of the presented peptide into the HLA-DR-binding groove and to T cell activation was elucidated in **chapter 4**.

In parallel a method to generate antigen-specific T cell clones on HVS-transformed autologous T cells as APCs was established (**chapter 5**). This method would allow investigation of the specificity of T cell responses during the course of clinical vaccine trials where only small amounts of blood cells from each individual are available.

Finally the SPf66-specific T cell response to a new formulation of SPf66 combined with the immunological adjuvant QS-21 was investigated (**chapter 6**). The response differed significantly from the T cell response elicited by SPf66/alum-vaccination, demonstrating the strong immuno-modulatory function of the saponin adjuvant QS-21.

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2. Amino acid dimorphisms and parasite immune evasion: cellular immune responses to a semi-conserved epitope of *Plasmodium falciparum* Merozoite Surface Protein-1 are less flexible than humoral responses

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Abstract

Like most other surface exposed antigens of *Plasmodium falciparum*, the leading malaria vaccine candidate Merozoite Surface Protein-1 (MSP-1) contains a large number of dimorphic amino acid positions. This type of antigen diversity is presumed to be associated with parasite immune evasion and represents one major obstacle to malaria subunit vaccine development. In order to better understand the precise role of antigen dimorphism in immune evasion, we have analyzed the flexibility of humoral and cellular immune responses against a semi-conserved sequence stretch of the N-terminal block 1 of MSP-1. While this sequence contains overlapping promiscuous T cell epitopes and is a target for growth inhibitory antibodies, three dimorphic amino acid positions may limit its suitability as component of a multi-epitope malaria vaccine,. Parasite binding monoclonal antibodies raised against one allelic variant showed remarkable cross-reactivity with other naturally occurring sequence variants of block 1. In contrast, all human T cell lines and HLA-DR or -DP restricted T cell clones analyzed, were strictly variant specific. Competition peptide binding assays with affinity purified HLA-DR molecules indicated that dimorphism does not primarily affect HLA binding. Lack of productive triggering of the TCR by MHC/altered peptide ligand complexes thus seems to be the characteristic feature of parasite immune evasion associated with antigen dimorphism.

Introduction

Many surface exposed antigens of protozoan parasites display immunogenic epitopes which contain sequence positions that are occupied in the parasite population by two distinct amino acids. In the case of the malaria parasite *P. falciparum,* cell surface proteins like Merozoite Surface Protein-1 (MSP-1) (Miller et al. 1993), MSP 2 (Smythe et al. 1991), MSP 3 (Huber et al. 1997) and Apical Membrane Protein-1 (AMA-1) (Crewther et al. 1996; Hodder et al. 1996) commonly display this type of antigenic diversity. In contrast, allelic variants are strikingly rare in *P. falciparum* proteins that are not subject to immune selection (Ayala and Rich 2000). Based on the sequence analysis of MSP-1 genes from different *P. falciparum* isolates, this leading malaria vaccine candidate (Engers and Godal 1998; Good et al. 1998) has been divided into 17 blocks that are conserved, semi-conserved or variable (Miller et al. 1993). There is evidence that the polymorphism of MSP-1 is the result of positive natural selection (Escalante et al. 1998). The majority of the identified MSP-1 T cell epitopes are located in regions containing dimorphic amino acid positions (Crisanti et al. 1988; Egan et al. 1997; Ohta et al. 1997; Parra et al. 2000; Quakyi et al. 1994; Rzepczyk et al. 1989; Udhayakumar et al. 1995). These amino acid dimorphisms thus appear to have evolved at crucial sequence positions of immunogenic epitopes to facilitate immune evasion (Plebanski et al. 1997) and consequently this type of antigen diversity represents a major obstacle to malaria vaccine development.

Currently discussed strategies to design subunit vaccines with the potential to protect against antigenically diverse populations of parasites include the focussing of immune responses to conserved epitopes and the inclusion of several allelic variants into a vaccine formulation. However, many conserved epitopes seem to exhibit low immunogenicity and an administration of mixtures of allelic variants may not lead to broad cross-reactivity but rather to unresponsiveness through altered peptide ligand inhibition of T cell responses (Gilbert et al. 1998; Jameson 1998; Plebanski et al. 1997; Plebanski et al. 1999a; Sloan-Lancaster and Allen 1996). Therefore alternative strategies, such as the enhancement of the immunogenicity of conserved epitopes by sequence modification or the generation of cross-reactive immune responses against semi-conserved epitopes by using chemically modified pseudopeptides as vaccine components (Lozano et al. 1998) may be required. For the rational design of such epitope focussed vaccines a better understanding of the role of antigen dimorphism in immune evasion at the functional and molecular level is required. This prompted us to

analyze the flexibility of humoral and cellular immune responses against a semi-conserved sequence stretch of the N-terminal block 1 of *P. falciparum* MSP-1.

Block 1 of MSP-1 contains three dimorphic positions (amino acids 44, 47 and 52) and six combinations of these three dimorphic positions (S^{44}–Q^{47}–V^{52}, S^{44}–H^{47}–I^{52}, G^{44}–H^{47}–I^{52}, G^{44}–Q^{47}–V^{52}, G^{44}–H^{47}–L^{52} and G^{44}–H^{47}–V^{52}) have been found in *P. falciparum* populations worldwide (Jiang et al. 2000; Miller et al. 1993). These three dimorphic positions are comprised in the MSP-1^{24-67} and the MSP-1^{43-53} peptides, which have been found to elicit partial immune protection against experimental challenge with *P. falciparum* in Saimiri (Cheung et al. 1986) or *Aotus* monkeys (Patarroyo et al. 1987), respectively. Monoclonal antibodies against the MSP-1 derived 83.1 sequence (Y^{43}SLFQKEKMVL^{53}), which has been incorporated into the synthetic peptide malaria vaccine SPf66 (Patarroyo et al. 1987; Patarroyo et al. 1988) exhibit *P. falciparum in vitro* growth inhibitory activity (Lozano et al. 1998). We have been able to generate both HLA-DR and –DP restricted human T cell clones from SPf66 vaccinated volunteers by repeated stimulation with the S^{44}–Q^{47}–V^{52} sequence variant of MSP-1^{38-58} (Daubenberger et al. 2001). Furthermore, human and mouse T cell responses have also been described against the MSP-1#23 peptide (G^{44}LFHKEKMILNEEEITTKGA^{63}) (Parra et al. 2000; Quakyi et al. 1994). The sequence stretch of block 1 of MSP-1 that contains the three dimorphic positions thus seems to represent both a promiscuous T cell epitope and a target for potentially protective humoral immune responses. Here we have tested parasite-binding antibodies and HLA class II restricted T cells raised against the S^{44}–Q^{47}–V^{52} allelic variant of this sequence for cross-reactivity with other naturally occurring sequence variants. Results indicate that lack of productive triggering of the TCR by MHC/altered peptide ligand complexes is a characteristic feature of parasite immune evasion associated with this type of antigenic diversity.

Materials and Methods

Peptides

Allelic variants of MSP-1^{43-53} and MSP-1^{38-55} and the S^{44}–Q^{47}–V^{52} sequence variant of MSP-1^{38-58} were synthesized by standard solid phase synthesis using F-moc chemistry. SPf66 $(CGDELEAETQNVYAAPNANPYSLFQKEKMVLPNANPPANKKNAGC)_n$ and its polymerized 83.1 building block $(CGYSLFQKEKMVLGC)_n$ were a kind gift of M. Patarroyo.

Monoclonal antibodies

B cell hybridoma clones that secrete anti-SPf66 IgG were generated as described (Pluschke et al. 1998; Poltl-Frank et al. 1999) from spleen cells of mice immunized with SPf66. mAbs specific for the MSP-$1^{43–53}$ building block of SPf66 were identified by ELISA with plates coated with the 83.1 polymer. Eleven mAbs that stained *P. falciparum* clone MAD-20 blood stage parasites in an indirect immunofluorescence assay (IFA) and reacted with MSP-1 and its proteolytic processing products in Western blots with saponin lysates of erythrocytes infected with clone MAD-20 (Poltl-Frank et al. 1999) were included in the present analysis. Three clonally unrelated mAbs (Table 1) derived from two different mice were chosen for quantitative affinity measurements.

T cell lines

Eight volunteers were immunized with either the alum formulation (AH, CH, ToSch, TS) or a QS-21 formulation (#21, #34, #29, #89) of the SPf66 vaccine as described (Daubenberger et al. 2001; Kashala et al. 2001). All volunteers were HLA-DR typed (Table 2) either by using the HLA-DRβ BigDye Terminator Sequencing-Based Typing Kit (PE Biosystems) or by PCR amplification and DNA sequence analysis of genomic DNA as described (Daubenberger et al. 2001). Donors TS and Tosch were also HLA-DP typed by PCR amplification of the second exon of the HLA-DPA1 and DPB1 genes (Daubenberger et al. 2001). PBMC of the eight donors were isolated six weeks to four months after the last immunization by density gradient centrifugation on Ficoll-Hypaque (Pharmacia). Bulk cultures were set up using 2×10^6 PBMC together with 20 µg/ml of MSP-1^{38-58} in complete culture medium (CM) in 24-well plates. CM consisted of RPMI 1640 supplemented with 10 % heat-inactivated pooled human AB serum (Haenecke AG, Germany), 10 mM HEPES buffer, 2 mM L-glutamine, 100 U/ml

penicillin, 100 µg/ml streptomycin (Gibco BRL) and 50 µM 2-ME. After 8 days of incubation, 1 x 10^6 blasting cells were restimulated with 1 x 10^6 irradiated (30 Gy) autologous PBMC and peptide antigen at 20 µg/ml in 24 well plates. On day 3 recombinant human IL-2 (20 U/ml) was added. After two rounds of restimulation, the specificity of the resulting T cell lines was tested in a proliferation assay using irradiated autologous PBMC or autologous EBV-LCL as APC together with the relevant peptides at 20 µg/ml.

T cell clones

MSP-1^{38-58} specific HLA class II restricted human T cell clones (Table 3) generated from PBMCs of SPf66/alum vaccinated volunteers TS (Daubenberger et al. 2001) and ToSch (Nickel et al., unpublished) were included in the analyses. T cell clones were established essentially as described using irradiated autologous PBMC as APCs and MSP-1^{38-58} as stimulating antigen. TCR gene expression was analyzed by RT-PCR of TCR transcripts (Moonka and Loh 1994) and sequence analysis of the amplification products. The restriction element of a particular T cell clone was determined in proliferation assays using HLA-matched homozygous EBV-LCL (BM9, Herluf, LS40 and Boleth) for presentation of the MSP-1^{38-58} peptide in the presence or absence of anti-HLA-DR (L243, IgG2a), anti-HLA-DQ (SP-LV3, IgG1), anti-HLA-DP (B7/21, IgG1) or anti-HLA-DR ,DP,DQ (HB 145, IgG1) mAbs. Isotype matched mAbs (IgG1, IgG2a) were used as negative controls. APC were incubated for 30 min with a 1:5 dilution of hybridoma cell culture supernatant prior to addition of T cells and peptide. The proliferation assays were conducted as described above. Homozygous EBV-LCL Herluf (IHW 9299), BM9 (IHW 9068), Boleth (IHW 9031) and LS40 (IHW 9300) were obtained from the European Collection of Animal Cell Cultures, Salisbury, UK and were part of the XIIth International Histocompatibility Workshop Cell Panel.

T cell stimulation assays

For T cell stimulation assays, 2 x 10^4 cloned T cells were co-cultured for 3 days with autologous irradiated PBMC (2 x 10^4 cells /well) or EBV-LCL (5 x 10^3 cells/well) together with the relevant peptide (20 µg/ml). All proliferation assays were performed in triplicate and during the last 18 h [^{3}H]-Thymidine (1 µCi/well, Amersham International) was present. The cultures were harvested by an automated harvesting device and assayed for [^{3}H]-Thymidine incorporation by liquid scintillation counting with a Betaplate counter (LKB, Sweden). Data

are expressed as mean delta cpm of triplicate cultures ± SD (mean cpm of antigen-stimulated cultures - mean cpm of control cultures), or as stimulation index (SI), which was calculated by the following equation: SI = mean experimental cpm with antigen / mean control cpm without antigen. SI > 3 were considered positive.

Affinity purification of HLA-DR molecules

Human HLA-DR8 molecules were purified from lysates of HLA-DRB1*0801 homozygous EBV-LCL BM-9 by affinity chromatography (Sinigaglia et al. 1991) using anti-HLA-DR mAb L243 cross-linked to Protein A-Sepharose CL-4B (Amersham Pharmacia Biotech AB, Uppsala, Sweden) as affinity support. Cells were lysed at a density of 10^8 cells ml^{-1} on ice for 60 min in 1% (v/v) Nonidet NP-40, 25 mM iodoacetamide, 5 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 10 μg/ml each of soybean trypsin inhibitor, antipain, pepstatin, leupeptin and chymostatin in 0.05 M sodium phosphate buffer, 0.15 M NaCl, pH 7.5. Lysates were cleared of nuclei and debris by centrifugation at 27,000 *g* for 30 min. After addition of 0.2 volumes of 5% sodium deoxycholate (DOC) to the supernatant and mixing for 10 min, the lysate was centrifuged at 100,000 *g* for 2 hr and subsequently filtered through a 0.45 μm membrane (Sartorius AG, Göttingen, Germany). For affinity purification of class II molecules the lysates were first passed over a Sepharose CL-4B pre-column, and subsequently over the protein A sepharose-mAb L243 column. The affinity column was then washed with i) 20 column volumes of 50 mM Tris-HCl, pH 8, 0.15 M NaCl, 0.5% NP-40, 0.5% DOC, ii) 5 column volumes of 50 mM Tris-HCl, pH 9, 0.5 M NaCl, 0.5% NP-40, 0.5% DOC and iii) 5 column volumes of 2 mM Tris-HCl, pH 8, 1% octyl- β-D-glucopyranoside (OG, Sigma, St. Louis, MO). HLA-DR molecules were eluted with 0.05 M diethylamine in 0.15 M NaCl, pH 11.5, containing 1% OG, 1 mM EDTA. The eluate was immediately neutralized with 1/20 volume of 1 M Tris/HCl, pH 6.8. Preparations were kept in aliquots at –80 °C until use.

Peptide binding competition assay

Peptide binding to HLA-DR8 molecules was analyzed essentially as described (Calvo-Calle et al. 1997) with an ELISA based assay using the biotinylated reporter peptides $CLIP^{82-102}$ (PKPPKPVSKMRMATPLLMQAL) or $GYR(A)_6L$, which was N-terminally labeled via two 6-aminopropionic acid spacers to biotin (Sigma- Genosis, Cambridge, UK). Purified HLA-DR molecules were diluted in freshly prepared binding buffer, containing 100 mM

citrate/phosphate buffer (pH 7), 0.15 mM NaCl, 4 mM EDTA, 4% NP-40, 4 mM PMSF and 40 μg/ml each of soybean trypsin inhibitor, antipain, leupeptin and chymostatin. Sixty microliter of an optimal dilution containing 10 – 100 ng HLA-DR molecules were added to each well of a 96-well low-binding V-bottom microtiter plate (Nalge Nunc Int., Roskilde, Denmark) together with 20 μl of biotinylated reporter peptide (final concentration 0.2 μM) in citrate/phosphate buffer (pH 7) and 20 μl of compeptitor peptide serially diluted in DMSO:PBS (1:4). After 24 hours of incubation at room temperature, the solution was transferred to wells of Immunolon-2 ELISA plates (Dynex Techn., Chantilly, VA), which had been coated with a 20 μg/ml solution of the anti- HLA-DR mAb L-243 and subsequently blocked with PBS containing 0.05% Tween-20 and 5% bovine serum albumin. After 3 hours of incubation at room temperature, plates were washed with PBS, 0.05%Tween-20. After incubation with alkaline phosphatase-labeled streptavidine (Calbiochem, La Jolla, CA) reporter peptide/HLA-DR complexes were quantified by determining conversion of the substrate 4-nitrophenylphosphate (Sigma, St. Louis, MO). Binding of peptides and pseudopeptides to the HLA-DR molecules was determined by measuring the OD in the presence versus the absence of competitor. Inhibition was calculated as percentage using the formula 100 x [1 – (ΔOD in the presence of competitor/ΔOD in the absence of competitor)]. The competitor concentrations yielding 50% inhibition of reporter peptide binding were calculated as IC50 values. Competitors yielding IC50 values <100 μM were considered positive for binding to the HLA-DR molecule. $CLIP^{82-102}$, which is known to bind to most HLA-DR alleles (Ghosh et al. 1995; Malcherek et al. 1995) was included into each assay as positive control and inhibited binding of the reporter peptide with an IC50 of 0.02 μM (data not shown). As negative controls, $(NANP)_3$ (Calvo-Calle et al. 1997) or the $MSP\text{-}1^{38-55}$ derived 21747 peptide (AVLTGYSGFQKEKMGLNE) which contains glycine substitutions at positions 45 and 52, were included in each assay.

IFA

The C-terminal sequences of MSP-1 of *P. falciparum* clones MAD 20 (MSP-1 block 1 sequence variant S^{44}–Q^{47}–V^{52}), RO71 (G^{44}–H^{47}–I^{52}), NF54 (G^{44}–Q^{47}–V^{52}), IFA9.2 (G^{44}–H^{47}–V^{52}) and K1 (S^{44}–H^{47}–I^{52}) were reconfirmed by DNA sequencing of PCR products as described (Jiang et al. 2000). Brazil-608, the only *P. falciparum* clone described so far to express the G^{44}–H^{47}–L^{52} allele (Miller et al. 1993) was not available. 12-well multitest immunofluorescence microscopy slides (Flow Laboratories, Baar, Switzerland) were pre-

treated with 0.01% poly-L-lysine (Sigma, St. Louis, MO) for 30 min at room temperature and washed five times with RPMI basal salt medium (Gibco BRL). Erythrocytes from *P. falciparum in vitro* cultures (Matile and Pink 1990) with about 10% parasitemia were washed two times in RPMI at room temperature. Cells were re-suspended in RPMI and mixed with two volumes of a solution containing 4% para-formaldehyde and 0.1% Triton X-100. Droplets of 30 µl cell suspension were added to each well, incubated at room temperature for 30 min and washed five times with PBS. Wells were incubated for 15 min at room temperature with blocking solution containing 10% fatty-acid free BSA in PBS. Immunostaining was started by incubating the wells with 20 µl of 0.1 mg/ml purified mAb in blocking solution for 1 hour at room temperature in a humid chamber. After five washes with blocking solution, 20 µl of 5 µg/ml Cy3-conjugated affinity-pure $F(ab')_2$ fragment goat anti-mouse IgG antibodies (Jackson Immuno Research Laboratories, West Grove, PA), diluted in blocking solution containing 0.01mg/ml Hoechst No. 33258 (Sigma), were added to the wells and incubated for one hour at room temperature. Afterwards slides were washed five times with PBS, mounted with 90% (v/v) glycerol containing 0.1 M TrisCl, pH 8.0 and 2 mg/ml o-phenylenediamine and covered with a cover slip. Antibody binding and DNA staining was assessed by fluorescence microscopy.

Isothermal titration calorimetry (ITC)

Calorimetric titration experiments were performed using a MCS-ITC instrument (MicroCal, Northampton, MA). The sample cell (1.34ml) was filled with a mAb solution (typically 2µM) in PBS. The injection syringe (nominal volume 250 µl) was filled with a peptide solution (typically 50 - 100µM) in PBS. The reference cell contained a solution of 0.01% sodium azide. During the experiments the sample solution was stirred by rotating at 400 rpm the injection syringe whose tip has the form of a paddle. After the baseline stability was better than 0.1 µcal/sec, 1-2 µl of solution were injected to remove possible air bubbles at the syringe openings. The preinjection was followed by a succession of injections of constant volume, typically between 5 and 15 µl at constant time intervals. The time intervals between two consecutive injections, typically 250sec, allowed the heat signal to come back to baseline. The instrument was equilibrated with an external circulating bath at least 5°C below the experimental temperature of 25°C. Prior to each experiment sample cell and syringes were rinsed with water. After each experiment sample cell and syringes were rinsed first with water, cleaned with 200 ml of 0.1% SDS-solution (Merck, Darmstadt, Germany) and finally

rinsed with at least 1 liter of double distilled water. The isothermal titration curves were registered and analyzed using ORIGIN software (MicroCal) provided with the MCS-ITC instrument.

Results

Cross-reactivity of antibody responses against the semi-conserved region of block 1 of MSP-1

Eleven mAbs elicited by the MSP-1 building block ($Y^{43}SLFQKEKMVL^{53}$) of the synthetic peptide malaria vaccine SPf66 that react with MSP-1 of *P. falciparum* clone MAD-20 (block 1 sequence variant $S^{44}–Q^{47}–V^{52}$) in IFA and Western blotting were included in the analysis. Cross-reactivity of these mAbs with *P. falciparum* blood stage parasites expressing other block 1 sequence variants was analyzed by IFA. All eleven mAbs stained all parasite clones tested, as shown for mAb 9.22 in Fig. 1. Furthermore, these mAbs also reacted in Western blots with all MSP-1 alleles in blood stage parasite saponin-lysates (data not shown).

This broad cross-reactivity of the mAbs with MSP-1 sequence variants observed in IFA and Western blotting was quantitatively analyzed by isothermal titration calorimetry (ITC). Since sequence analysis of Ig light chain transcripts of the eleven anti- $MSP\text{-}1^{43–53}$ mAb secreting hybridomas revealed a marked oligoclonality of the B cell responses (Poltl-Frank et al. 1999), three clonally unrelated mAbs designated 7.19, 7.27 and 9.22 (Table 1) derived from two different mice were selected for ITC affinity measurements. ITC measures the heat of binding generated when antigen and antibody are brought together in solution, thus avoiding labeling or solid phase adsorption of one of the reactants. Peptides representing natural sequence variants of $MSP\text{-}1^{43–53}$ were injected into a sample cell filled with a mAb solution. Titration curves were obtained only when binding affinity constants (Ka) were $>10^{6} M^{-1}$. Although reactivity with all allelic variants of block 1 had been observed in IFA, interactions of mAbs 7.19 and 7.27 with some of the variant peptides were too weak to yield a detectable signal by ITC (Table 1). In contrast, mAb 9.22 yielded ITC signals with all $MSP\text{-}1^{43–53}$ variants tested (Fig.2) demonstrating a striking flexibility of antibody binding.

Strict variant specificity of block 1 specific CD4 T cell responses

While the $MSP\text{-}1^{43\text{-}53}$ peptide turned out to be too short to serve as an efficient stimulator for HLA class II restricted T cells *in vitro* (data not shown), its C- and N-terminally extended derivative $MSP\text{-}1^{38\text{-}58}$ ($AVLTGYS^{44}LFQ^{47}KEKMV^{52}LNEGTS$) was a potent stimulator of human SPf66 primed T cells. In fact, T cell lines reactive with this peptide could be obtained from all eight SPf66 vaccinated volunteers tested (Table 4). Attempts to raise $MSP\text{-}1^{38\text{-}58}$ reactive T cell lines from PBMC of a control group of non-vaccinated volunteers (n= 4) failed (data not shown). Inhibition assays of T cell proliferation with HLA isotype specific mAbs

demonstrated that HLA-DR restricted T cells dominated the MSP-1 $^{38-58}$ specific response in most T cell lines (Table 4). However, in the case of the cell line from donor ToSch, anti-HLA-DP mAbs had the most pronounced inhibitory effect. These data obtained with an immunogenetically diverse group of volunteers (Table 2) indicate that MSP-1 $^{38-55}$ represents a promiscuous HLA class II binding sequence.

In order to analyze the fine specificity of the MSP-1 $^{38-58}$ reactive cells, T cell clones were established from two of the vaccinated volunteers. All clones obtained expressed CD4 and an αβ TCR as determined by FACS analysis (data not shown). To identify unrelated clones for detailed analysis, the functionally rearranged *TCRA* and *TCRB* genes were characterized by RT-PCR and DNA sequence analysis. Certain αβ chain combinations were found in several clones, giving evidence for oligoclonal T cell expansions (manuscript in preparation). Seven unrelated clones with different unique TCR were chosen for further detailed analysis. The inhibitory activity of HLA isotype-specific blocking antibodies demonstrated that both HLA-DR and HLA-DP restricted T cell clones were obtained (Table 5). Further analyses using EBV-LCL homozygous for class II alleles demonstrated that MSP-1 $^{38-58}$ can be presented in the context of DRB1*0801, DPA1* 0103/DPB1*02012 and DPA1*01031/DPB1*0301 molecules (Table 5).

Cross-reactivity of T cell responses elicited by the sequence variant S^{44}–Q^{47}–V^{52} of the semi-conserved region of block 1 with other naturally occurring sequence variants was tested at the clonal level in proliferation assays with variant peptides. None of the naturally occurring sequence variants tested was stimulatory (Table 6) and already the single amino acid exchange at position 44 from serine to glycine abolished the proliferative responses of all T cell lines (data not shown) and clones (Table 6) tested. Lack of proliferation was paralleled by lack of secretion of the cytokines IL-4 and INF-γ into the supernatant of activated cultures (Table 7). The absolute variant specificity of the Th responses upon vaccination with the S^{44}–Q^{47}–V^{52} variant containing SPf66 was reconfirmed by the observation that MSP-1 $^{38-58}$ reactive T cell lines could be obtained from PBMC of the vaccinated donors only with this variant peptide (data not shown).

Limited effect of antigen dimorphism on peptide binding to HLA-DR

Competition binding assays with affinity purified HLA-DRA/DRB1*0801 complexes were performed to study the impact of amino acid dimorphisms on binding to the restriction element used by clones TS-M63P and TS-M100P. Serially diluted peptides were used to

compete binding of biotinylated reporter peptides to the purified HLA class II heterodimers. IC50 values were defined as the concentration of a peptide yielding 50% inhibition of binding of the respective reporter molecule. Two negative control peptides, $(NANP)_3$ (Calvo-Calle et al. 1997) and 21747 showed no significant competitive activity. Peptide 21747 consists of a modified MSP-1^{38-55} sequence with exchanges of the two amino acids L45 and V52 by glycine (AVLTGYSG^{45}FQKEKMG^{52}LNE). These two amino acid substitutions impeded both stimulation of all DR- and DP-restricted clones tested (data not shown) and peptide binding competition activity to HLA-DR8 molecules (Table 8). In contrast, all natural sequence variants tested exhibited significant competitive activity (Table 8). With both reporter peptides used, two of the variant sequences (S^{44}–H^{47}–I^{52}, G^{44}–H^{47}–I^{52}) were slightly better and two variants (G^{44}–Q^{47}–V^{52}, G^{44}–H^{47}–L^{52}) were slightly less efficient competitors than the parent sequence S^{44}–Q^{47}–V^{52}.

Discussion

The leading *P. falciparum* malaria vaccine candidate antigen MSP-1 is synthesized by the parasite as a 190KD precursor molecule, which undergoes post-translational proteolytic processing to produce fragments which persist as a non-covalently linked complex on the surface of mature merozoites (Blackman and Holder 1992; Holder and Freeman 1984). Immunization with purified *P. falciparum* MSP-1 or portions of it has protected monkeys partially or completely from malaria after experimental challenge with homologous and/or heterologous parasites (Chang et al. 1996; Cheung et al. 1986; Etlinger et al. 1991; Kumar et al. 2000; Patarroyo et al. 1987; Siddiqui et al. 1987). Several parts of MSP-1 seem to be able to elicit immune responses that have a potential to contribute to immune protection. Immunization of *Aotus* monkeys with the C-terminal 19 kDa fragment has conferred protection in some experiments (Kumar et al. 2000; Stowers et al. 2001). Partial protection was achieved in Saimiri monkeys by immunization with a peptide comprising residues 24 to 67 of blocks 1 and 2 of MSP-1 (Cheung et al. 1986) and immunization with a peptide comprising residues 43 to 53 of block 1 delayed the onset of disease in *Aotus* monkeys (Patarroyo et al. 1987). Monoclonal antibodies for the semi-conserved part of the N-terminal block 1 of MSP-1 (Lozano et al. 1998), a variant epitope of block 2 or the epidermal growth factor-like domains of the C-terminal 19 kDa fragment of MSP-1 have been shown to inhibit parasite growth *in vitro* (Chappel and Holder 1993; Locher et al. 1996).

Our data demonstrate that the dimorphic sequence 38-55 of block 1 of MSP-1 contains both, a surface exposed B cell epitope as well as overlapping promiscuous T cell epitopes. Dimorphisms at positions 44, 47 and 52 is maintained in all parasite populations in malaria hyperendemic regions worldwide (Jiang et al. 2000), indicating that they have developed as highly efficient and specific immune evasion mechanism. Complete lack of variation at other positions of block 1 shows that the selection pressure for further diversification is low. Analyses with lymphocytes of eight immunogenetically diverse volunteers immunized with the MSP-1^{43-53} containing SPf66 peptide showed, that the S^{44}–Q^{47}–V^{52} variant of the semi-conserved portion of block 1 can be presented by multiple HLA class II alleles. Presentation was not restricted to HLA-DR, since both HLA-DR and -DP restricted MSP-1^{38-58} reactive $CD4^+$ T cell clones were obtained. Furthermore individuals vaccinated with a QS-21 adjuvanted SPf66 formulation developed in addition to Th responses also MSP-1 block 1 specific HLA class I restricted $CD8^+$ T cells responses (data not shown). T cell epitopes

binding to a broad range of restriction elements are of particular interest for the design of multi-epitope vaccines (Bona et al. 1998; Patarroyo et al. 1987) that aim to avoid the development of counterproductive or irrelevant responses against immuno-suppressive, self cross-reactive or immuno-dominant hyper-variable structures (Delves et al. 1997; Wrightsman et al. 1994). This raised our interest to investigate, which of the three key elements of antigen recognition by the adaptive immune system, antibody antigen interaction, peptide binding to MHC or TCR triggering by peptide/MHC complexes, is affected most by the dimorphism. Our analyses with a set of mAbs against the semi-conserved sequence stretch indicate that the humoral immune response to the semi-conserved epitope is strikingly flexible. In contrast, analyses with human Th cell lines and clones demonstrated a strict variant specificity of both HLA-DR and -DP restricted responses. Taken together, results of T cell stimulation and HLA peptide binding competition experiments indicate that lack of productive triggering of the TCRs by variant peptide/HLA complexes, rather than lack of variant peptide binding to the restricting class II molecules, is responsible for the absolute lack of cross-reactivity.

Selection of the semi-conserved MSP-1 block 1 (83.1) sequence for inclusion into the multi-epitope synthetic peptide vaccine SPf66 was based on its ability to protect *Aotus* monkeys against experimental *P. falciparum* malaria (Patarroyo et al. 1987). In these immune protection studies, the challenge strain FVO was used, which expresses the S^{44}–Q^{47}–V^{52} variant sequence (Moreno 2001) incorporated into SPf66. Lack of cross-reactivity of T cell responses elicited by the NPNA flanked 83.1 sequence of SPf66 may represent a factor that has limited the efficacy of SPf66 in clinical vaccine trials and contributed to heterogeneity of trial results in different epidemiological settings, such as areas in South America with unstable malaria pattern and malaria hyperendemic regions of Africa and Asia (Graves and Gelband 2000). Infections with *P. falciparum* in hyperendemic regions are characterized by the presence of a multiplicity of antigenically diverse parasite clones in the bloodstream of infected individuals. Co-infection with *P. falciparum* clones expressing sequence variants of MSP-1 block 1 thus is a common feature, as for example shown for the Kilombero District of Tanzania (Jiang et al. 2000), where two of the major SPf66 phase III clinical trials have been conducted (Acosta et al. 1999; Alonso et al. 1994). Cohabiting parasite strains may facilitate the survival of each other either by down-regulating effective adaptive immune responses or by interference with the priming of T cell responses (Gilbert et al. 1998; Hirunpetcharat and Good 1998; Plebanski et al. 1999a; Plebanski et al. 1999b). This raises the question, whether

semi-conserved sequences are suitable elements of a multi-epitope malaria vaccine. The complete lack of cross-reactivity of the MSP-1$^{38-58}$ specific T cell responses observed her may speak for an inclusion of all known allelic sequence variants into a multi-epitope malaria vaccine. However, inclusion of variant epitopes may even lead to antagonism of naturally acquired immunity, resulting in increased susceptibility. This strongly argues for the focussing on conserved sequences in the development of second generation multi-stage multi-epitope malaria vaccines such as CDC/NII MAL VAC-1 (Shi et al. 1999).

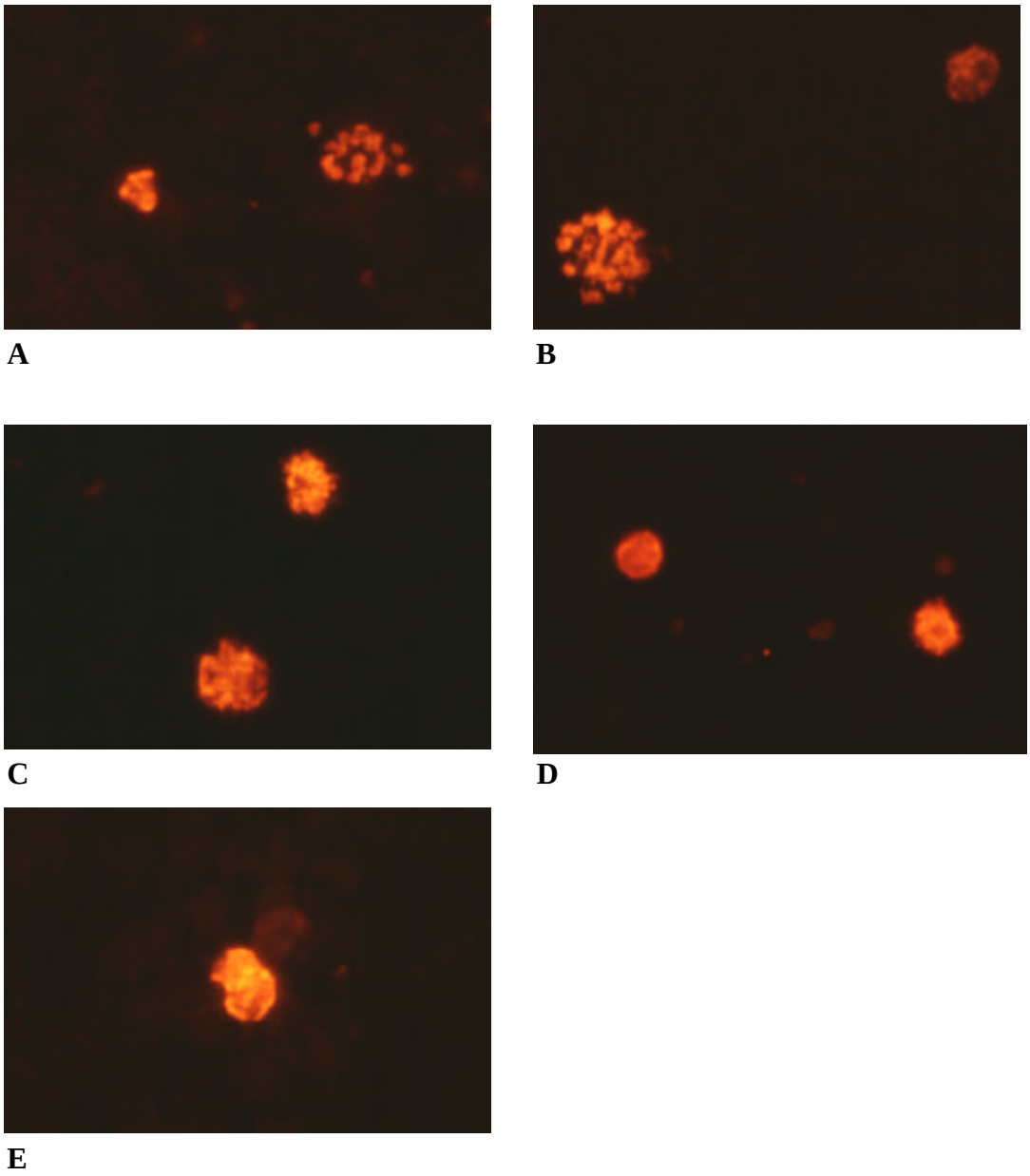

Figure 1. Indirect immunofluorescence staining of *P. falciparum* blood stage parasites by mAb 9. 22. Binding to parasite clones expressing different MSP-1 block 1 sequence variants was analyzed: **A:** MAD 20 (S^{44}–Q^{47}–V^{52}); **B:** RO71 (G^{44}–H^{47}–I^{52}); **C:** NF54 (G^{44}–Q^{47}–V^{52}); **D:** IFA9.2 (G^{44}–H^{47}–V^{52}) and **E:** K1 (S^{44}–H^{47}–I^{52}). No staining was observed with an isotype matched control mAb (not shown).

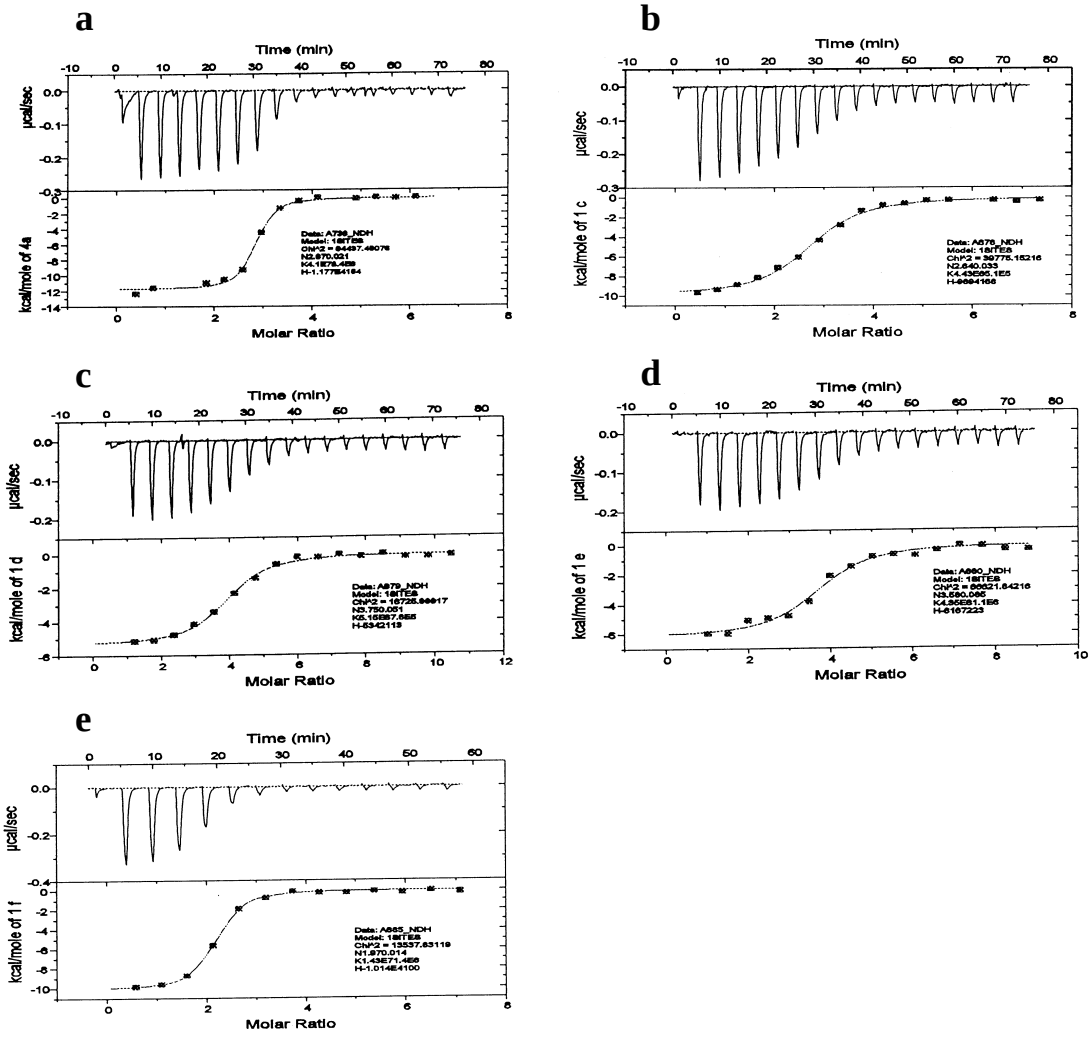

Figure 2. Isothermal titration calorimetric profiles of the binding mAb 9.22 to MSP-1$^{43-53}$ peptide sequence variants (upper curves) and integrated heats of binding in kcal mol^{-1} of injected peptide (lower curves). Sequence variants tested were S^{44}–Q^{47}–V^{52} (**a**), G^{44}–H^{47}–I^{52} (**b**), G^{44}–Q^{47}–V^{52} (**c**), S^{44}–H^{47}–I^{52} (**d**) and G^{44}–H^{47}–L^{52} (**e**).

Table 2. *HLA-DR and -DP alleles expressed by volunteers*

	HLA class II loci					
Volunteers	DRB1*	DRB3*	DRB4*	DRB5*	DPA1	DPB1
#21	13031 / 01021	0101			n.d.	n.d.
#34	1503/0701		0101 or 0103	0101	n.d.	n.d.
#29	1301/0701	0101	0101 or 0103		n.d.	n.d.
#89	11011/0701	0301	0101 or 0103		n.d.	n.d.
CH	0401/1301	0301	0101 or 0103		n.d.	n.d.
TS	0801/1201	0202			0103/02012	02011/11011
AH	03011/n.d.	03021	0101		n.d.	n.d.
ToSch	0401/0404		0101/0103		01031/01031	0401/20011

Table 3. *MSP-1* $^{38\text{-}58}$ *specific human T cell clones*

	TCR a **chain**	**TCR** b **chain**	**Restriction element**
TS-M32P	AV8-3 AJ8	BV6-5 BJ2-3	DPA1*0103 / DPB1*02012
TS-M103P	AV25 AJ52	BV19 BJ1-4	DPA1*0103 / DPB1*02012
ToSch-2	AV19 AJ11	BV27 BJ1-1	DPA1*0103 / DPB1*0401
ToSch-39	AV8-1 AJ29	BV19 BJ2-1	DPA1*0103 / DPB1*0401
TS-M40P	AV36 AJ33	BV30 BJ1-5	n.d.
TS-M63P	n.d.	BV19 BJ2-1	DRA*0102 / DRB1*0801
TS-M100P	n.d.	BV6[a]	DRA*0102 / DRB1*0801

n.d. = not determined
[a]based on staining with anti TCR-Vb13.1 mAb (Immunotech, Marseille, France)

Table 4. *HLA class II restriction of MSP 1$^{38-58}$ specific T cell lines*

	Volunteers							
	#29		**#21**		**#89**		**#34**	
Stimulation of T cell lines with	cpm ± SD	SI	cpm ± SD	SI	cpm ± SD	SI	cpm ± SD	SI
EBV-LCL	861 ± 235	1	2565 ± 559	1	1732 ± 177	1	599 ± 93	1
EBV-LCL + MSP-1$^{38-58}$	14389 ± 498	17	29892 ± 618	12	35860 ± 132	21	21142 ± 1577	35
EBV-LCL + MSP-1$^{38-58}$ + αclass II	3138 ± 468	4	7575 ± 376	3	4126 ± 184	2	6873 ± 299	11
EBV-LCL + MSP-1$^{38-58}$ + αDR	2431 ± 303	3	3375 ± 1237	1	4946 ± 533	3	964 ± 268	2
EBV-LCL + MSP-1$^{38-58}$ + αDP	12938 ± 322	15	29393 ± 2909	11	37791 ± 602	22	15129 ± 1006	25
EBV-LCL + MSP-1$^{38-58}$ + αDQ	13059 ± 174	15	25583 ± 2694	10	38913 ± 793	22	18099 ± 1877	30
EBV-LCL + MSP-1$^{38-58}$ + αclass I	14436 ± 1110	17	30190 ± 2793	12	40194 ± 1064	23	14971 ± 610	25

	Volunteers							
	TS		**ToSch**		**CH**		**AH**	
Stimulation of T cell lines with	cpm ± SD	SI	cpm ± SD	SI		SI		SI
PBMC	6514 ± 1063	1	233 ± 81	1	4535 ± 458	1	592 ± 135	1
PBMC + MSP-1$^{38-58}$	64439 ± 4444	10	46764 ± 3415	201	90019 ± 9237	20	8097 ± 1990	14
PBMC + MSP-1$^{38-58}$ + αclass II	13436 ± 757	2	376 ± 95	2	n.d.[a]		n.d.	
PBMC + MSP-1$^{38-58}$ + αDR	21727 ± 1351	4	42623 ± 5497	183	n.d.		n.d.	
PBMC + MSP-1$^{38-58}$ + αDP	50509 ± 2689	7	258 ± 60	1	n.d.		n.d.	
PBMC + MSP-1$^{38-58}$ + αDQ	68554 ± 3174	11	25465 ± 3213	109	n.d.		n.d.	
PBMC + MSP-1$^{38-58}$ + αclass I	58462 ± 2993	9	38713 ± 2561	166	n.d.		n.d.	

[a]n.d. = not determined

Table 5. *HLA restriction analysis of T cell clones derived from donors TS and ToSch*

Stimulator cells[a]	HLA class II loci[b]			mAb present[c]	T cell clones (SI)[d]				
	DRB1*	DPA1*	DPB1*		TS-M32P	TS-M100P	TS-M103P	ToSch-2	ToSch-39
TS-PBMC	0801/1201	0103/0201	02012/11011	none	7	10	17		
				anti-DR	5	3.5	10		
				anti-DP,DQ,DR	1	3.6	1		
				anti-DP	n.d.	n.d.	n.d.		
				anti-DQ	5	9	15		
Herluf	1201/1201	01/02	0401/n.d.	none	1	2	1		
				anti-DR	1	1	0.3		
				anti-DP,DQ,DR	1	0.4	0.3		
				anti-DP	n.d.	n.d.	n.d.		
				anti-DQ	1	1	1		
BM9	0801/0801	0103/0103	02012/02012	none	13	10	13		
				anti-DR	7	3	10		
				anti-DP,DQ,DR	1	2	0.3		
				anti-DP	n.d.	n.d.	n.d.		
				anti-DQ	10.2	8	12		
ToSch-PBMC	0401/0404	01031/0103	0401/20011	none				251	14
				anti-DR				201	12
				anti-DP,DQ,DR				21	2
				anti-DP				7	1
				anti-DQ				176	10
Boleth	0401/0401	0103/0103	0401/0401	none				31	7
				anti-DR				21	4
				anti-DP,DQ,DR				6	1
				anti-DP				2	1
				anti-DQ				25	4
LS40	0404/0404		0201/0301	none				84	8
				anti-DR				62	7
				anti-DP,DQ,DR				11	1
				anti-DP				1	2
				anti-DQ				74	6
SLE005			0301/0301	none				19	7
				anti-DR				14	4
				anti-DP,DQ,DR				1	1
				anti-DP				1	1
				anti-DQ				13	3

Legend to Table 5:

[a] different stimulator cells were co-cultured with T cell clones and MSP-1$^{38-58}$ for 3 days. Herluf, BM9, Boleth and LS40 are EBV-LCL homozygous at the HLA-DR locus.

[b] expression of HLA-class II alleles was determined by PCR amplification and DNA sequence analysis.

[c] mAb specific for monomorphic determinants of HLA-DR (L243), HLA-DQ (SP-LV3), HLA-DP (B7/21) and HLA-DR,DP,DQ (HB145) were included into microculture wells during stimulation of T cells.

[d] after 3 days of incubation 1 µCi of 3H-thymidine was added to each culture. Incorporated radioactivity was determined after 18 h of incubation and is expressed as Stimulation Index (SI).

Table 6. *Proliferative response of T cell clones to MSP-1 $^{38-55}$ sequence variants*

	T cell clones (cpm ± SD (SI))						
Sequence variants of MSP 1$^{38-55}$	**TS-M32P**	**TS-M40P**	**TS-M63P**	**TS-M100P**	**TS-M103P**	**ToSch 2**	**ToSch 39**
AVLTGYSLFQKEKMVLNE	8901 ± 472 (9.6)	9277 ±1248 (22.8)	11167 ± 259 (8.4)	3314 ± 242 (12.5)	28068 ± 1494 (77.8)	58265 ± 5726 (169)	54046 ± 3687 (326)
AVLTGYGLFQKEKMVLNE	731 ± 68 (0.8)	563 ± 53 (1.4)	1359 ± 341 (1.0)	279 ± 108 (1.1)	163 ± 13 (0.5)	152 ± 47 (1)	135 ± 18 (1)
AVLTGYGLFHKEKMLLNE	683 ± 65 (0.7)	429 ± 59 (1.1)	909 ± 160 (0.7)	151 ± 16 (0.6)	77 ± 12 (0.2)	100 ± 17 (1)	142 ± 1 (1)
AVLTGYGLFHKEKMILNE	701 ± 68 (0.8)	391 ± 91 (91)	894 ± 32 (0.7)	156 ± 47 (0.6)	372 ± 317 (1.0)	116 ± 32 (1)	82 ± 18 (1)
AVLTGYSLFHKEKMILNE	679 ± 73 (0.7)	373 ± 78 (0.9)	1116 ± 158 (0.8)	175 ± 18 (0.7)	292 ± 61 (0.8)	413 ± 384 (1)	409 ± 314 (2)

Table 7. *Cytokine production of T cell clones upon stimulation with MSP-1$^{38-55}$ sequence variants*

	T cell clones, IL-4 and INF-γ production (pg/ml)													
	TS-M32P		TS-M40P		TS-M63P		TS-M100P		TS-M103P		ToSch-2		ToSch-39	
MSP-1$^{38-55}$ variant	IL-4	INF-γ	IL-4	INF-γ	IL-4	INF-γ	IL-4	INF-γ	IL-4	INF-γ	IL-4	INF-γ	IL-4	INF-γ
AVLTGYSLFQKEKMVLNE	463	109	509	337	371	30	279	384	736	101	1028	107	1830	131
AVLTGYGLFQKEKMVLNE	2	0	8	11	8	9	0	0	0	0	9	1	136	46
AVLTGYGLFHKEKMLLNE	0	1	8	5	8	5	7	0	27	7	14	1	139	43
AVLTGYGLFHKEKMILNE	0	5	8	10	8	11	2	0	17	2	11	1	139	47
AVLTGYSLFHKEKMILNE	0	4	8	12	8	11	0	0	15	3	9	2	145	43

Table 8. *IC50 values of MSP-1$^{38-55}$ sequence variants in peptide binding competition assays with purified DRB1*0801 molecules*

competitor peptide	amino acid sequence	IC50 values of competitor peptide with biotinylated reporter peptide (0.2 μM)	
		GYR(A)6L reporter	CLIP$^{82-102}$ reporter
S^{44}-Q^{47}-V^{52}	AVLTGY**S**LF**Q**KEKM**V**LNE	7.9[a]	9.6
G^{44}-Q^{47}-V^{52}	AVLTGY**G**LF**Q**KEKM**V**LNE	12.5	26.4
G^{44}-H^{47}-L^{52}	AVLTGY**G**LF**H**KEKM**L**LNE	10.2	25.6
G^{44}-H^{47}-I^{52}	AVLTGY**G**LF**H**KEKM**I**LNE	6.8	7.3
S^{44}-H^{47}-I^{52}	AVLTGY**S**LF**H**KEKM**I**LNE	3.5	3
CLIP$^{82-102}$	PKPPKVSKMRMATPLLMQAL	0.02	0.2
21747	AVLTGYS**G**FQKEKM**G**LNE	>100	>100
(NANP)$_3$	NANPNANPNANP	>100	>100

[a] Results are expressed as IC50, the concentration (μM) of unlabeled competitor peptide required to inhibit 50% of the binding of a biotinylated reporter peptide. The percent inhibition was calculated based on OD at 405nm obtained in the presence of different concentrations of competitor peptide (100-0.001 μM). An IC50 >100 μM was regarded as no peptide binding. Results are mean values of duplicates of one representative experiment. All assays were performed twice.

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3. Fine-specificity of human CD4+ T cell clones raised against a promiscuous epitope of *Plasmodium falciparum* Merozoite Surface Protein-1

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Abstract

Merozoite Surface Protein-1 (MSP-1) of *Plasmodium falciparum* is a leading malaria vaccine candidate antigen. Its N-terminal block 1 contains a sequence stretch which comprises overlapping promiscuous T cell epitopes and therefore represents a candidate structure for a multi-stage multi-epitope malaria vaccine. We have generated human CD4+ T cell clones which recognize the block 1 derived MSP-1$^{38-58}$ peptide in the context with HLA-DR (DRB1*0801) or HLA-DP (DPB1*02012/DPA1*0103 or DPB1*0401/DPA1*0103). Here we describe the fine specificity of these clones and discuss implications of the findings for peptide vaccine design. Truncated variants of the MSP-1 $^{38-58}$ peptide were used to map the minimal epitopes. These comprised between 11 and 18 amino acids, reconfirming that epitope flanking regions play an important role in the stimulation of class II restricted T cells. Minimal epitopes of DR- and DP-restricted T cell clones were overlapping, but distinct. Peptides containing single alanine and glycine substitutions were used to identify amino acid residues essential for stimulation of the T cells. Between eight to ten amino acids within the minimal epitopes could not be replaced without loss of stimulatory activity. Substitutions at positions within essential epitope-flanking regions had in most cases no impact on T cell stimulatory activity. Substitutions at only three positions within the minimal epitope of the DR-restricted clones critically impeded competition of reporter peptide binding to the restricting HLA-DR8 molecule, indicating that the other non-flexible positions influence TCR triggering.

Introduction

The recognition of pathogen-derived peptides in the context of host MHC molecules is crucial for the induction and maintenance of an effective antigen-specific immune response. The association of antigenic peptides with MHC class II molecules generates the ligands required for activation of CD4+ T cells (Davis et al. 1998). Class II molecules are normally loaded with peptides derived from intracellular processing of protein antigens in endosomal compartments (Cresswell 1992; Morris et al. 1994). Short synthetic peptides can also bind directly to class II MHC molecules and the resulting peptide/MHC complex on the surface of the antigen presenting cell (APC) can successfully be recognized by T cells specific for the relevant antigen (Babbitt et al. 1986). Analysis of the allele specificity of peptide binding has shown that key anchor residues are important in determining the ability of peptides to bind to MHC molecules (Falk et al. 1994). Polymorphic side chains of the MHC form allele specific binding pockets which exhibit strong preference for interaction with particular amino acid side chains of the peptide (Hammer et al. 1997). In addition the formation of hydrogen bonds between conserved MHC residues and the peptide main chain provide sequence-independent binding affintiy (Matsumura et al. 1992; Stern et al. 1994).

Only several upward-pointing residues of the bound peptide can serve as direct TCR contacts. Crystal structures of TCR-peptide-MHC class II complexes revealed an orthogonal orientation of the TCR relative to its MHC-peptide ligand and that maximally nine residues of the presented peptide are covered by the TCR (Hennecke et al. 2000; Reinherz et al. 1999). Nevertheless, flanking residues next to the core region interacting with the TCR can dramatically influence the outcome of an individual T cell response (Moudgil et al. 1998). The triggering of an appropriate signal to stimulate T cell proliferation strongly depends on the kinetics of the TCR-ligand interaction (Lanzavecchia et al. 1999). Modified peptide variants presented on MHC class II molecules can strongly alter the quality of a resulting T cell response (Abrams and Schlom 2000; Bachmann et al. 1998). Such altered peptide ligands (APL) with single amino acid replacements or backbone modifications can exhibit changed binding affinities to the MHC molecule or change TCR-peptide-MHC interaction, leading to a modulated T cell response (Ostankovitch et al. 1998; Sloan-Lancaster and Allen 1996).

The use of peptides to induce T cell responses is of particular interest in the development of epitope-focussed subunit vaccines against infectious agents or tumors. However, polymorphism of HLA molecules represents a major obstacle for the development of such epitope-focussed vaccines. Therefore the incorporation of promiscuous T cell epitopes

which are able to bind to multiple HLA alleles (Hammer et al. 1993) is prerequisite for the effectiveness of such vaccines within immunogenetically diverse populations.

Merozoite Surface Protein 1 of *Plasmodium falciparum* is the major surface protein of the asexual blood-stage malaria parasite and represents a leading vaccine candidate antigen (Anders and Saul 2000). Block 1 of MSP-1 contains a semi-conserved sequence which has been incorporated into the synthetic peptide vaccine SPf66 (Patarroyo et al. 1988). In the present study we describe the fine-specificity of CD4 T cell clones generated by repeated restimulation of PBMC of SPf66 immunized volunteers with a peptide comprising amino acids 38-58 of *P. falciparum* MSP-1.

Materials and Methods

Synthetic Peptides

Peptide MSP-1$^{38-58}$ (AVLTGYSLFQKEKMVLNEGTS) and its N- or C-terminally truncated derivatives were synthesized by solid-phase synthesis on an automated peptide synthesizer using F-moc chemistry leading to peptides exhibiting C-terminal COOH-groups. Single alanine or glycine-substituted analogs of MSP-1 $^{38-55}$ were synthesized by the multiple solid-phase technique using *t*-butoxy-carbonyl (BOC) chemistry on a *p*-methylbenzhydrylamine resin as previously described (Lozano et al. 1998). Peptides synthesized by BOC-technique exhibit a C-terminal $CONH_2$-group. Crude products were analyzed by analytical reverse phase-HPLC and were purified to a purity of >98% by preparative reverse phase-HPLC. Composition of the purified molecules was reconfirmed by MALDI-TOF mass spectroscopy. SPf66 (CGDELEAETQNVYAAPNANPYSLFQKEKMVLPNANPPANKKNAGC)n was synthesized using the general procedure of solid phase peptide synthesis as previously described (Patarroyo et al. 1988).

Generation and maintenance of T cell clones

Six MSP-1$^{38-58}$ specific HLA class II restricted human CD4+ T cell clones (Table I) generated from PBMCs of two volunteers were included in this study. Volunteers TS and ToSch had been vaccinated with the synthetic peptide malaria vaccine SPf66 (Patarroyo et al. 1988) according to a protocol as described (Alonso et al. 1994). Ethical clearance was granted by the responsible ethical committee of the Department of Internal Medicine, Kantonsspital, Basel, Switzerland. T cell clones were established from MSP-1$^{38-58}$ specific T cell lines of both volunteers as described (Daubenberger et al. 2001) using irradiated autologous PBMC as APCs and MSP-1$^{38-58}$ as stimulating antigen. T cell lines were generated with PBMCs, isolated six weeks (TS) or four weeks (ToSch) after the last immunisation from whole EDTA-blood by density gradient centrifugation on Ficoll Hypaque (Pharmacia). PBMCs ($2x10^6$) were incubated with 40 µg/ml MSP-1 $^{38-58}$ peptide in RPMI 1640 supplemented with 10% heat-inactivated human AB serum (Haenecke AG, Germany), 100 U/ml penicillin, 100 µg/ml streptomycin (Gibco BRL), 10 mM HEPES, 2 mM L-glutamine and 50 µM 2-mercaptoethanol. This complete RPMI culture medium was used for T cell cultivation and all T cell assays. After 8 days of incubation, $1x10^6$ T cells were restimulated with $1x10^6$ irradiated (30 Gy) autologous PBMCs in the presence of 20 µg/ml of MSP-1 $^{38-58}$. Specificity of T cell lines was tested after the third restimulation by proliferation assays. T cell lines were

cloned by limiting dilution using irradiated autologous PBMC (2x10 ^{4}/well) as APC, 20 μg/ml of MSP-1 $^{38-58}$ peptide and 20 IU/ml rec human IL-2 in 96-well round bottom plates (Costar). Growing T cell clones were restimulated every second week with 20 μg/ml peptide and irradiated autologous PBMCs in complete RPMI supplemented with recombinant human IL-2.

HLA class II sequence analysis of donors was done by PCR amplification and DNA sequence analysis of HLA-DRB, DPA1 and DPB1 gene segments from genomic DNA as described (Daubenberger et al. 2001). Volunteer TS was HLA typed as DRB1*0801/1201, DRB3*0202, DPA1*0103/02011, DPB1*02012/11011 and volunteer ToSch as DRB1*0401/0404, DRB4*0101/0103, DPA1*0103/0103, DPB1*0401/20011. For the determination of the restriction elements used, peptide presentation to T cell clones in proliferation assays was selectively blocked with anti-human HLA class I (W6/32), class II (HB-145), -DR (L-243), -DP (B7/21) and -DQ (SPV-L3) mAb. The restricting class II alleles (Table I) were subsequently determined by using irradiated homozygous EBV transformed cell lines of defined haplotypes as APCs. Sequence analysis of TCR transcripts (Table I) was done as described (Moonka and Loh 1994). Specific cell-surface staining for CD4, CD8 and CD3 was performed using FITC- or PE-conjugated antibodies (Becton Dickinson) according to standard procedures. Stained cells were analyzed with a FACScan cytometer (Becton Dickinson).

T cell stimulation assays

Proliferation assays with MSP-1 $^{38-58}$ specific T cell clones were performed with 96-well round bottom-plates (Costar, Cambridge, MA) and complete RPMI medium . T cells (2 x 10 ^{4}) were incubated with 2 x 10 ^{4} irradiated (30 Gy) autologous PBMCs as APCs in the presence or absence of 20 μg/ml of peptide. After 72 h, cultures were pulsed with 1 μCi [^{3}H]-Thymidine (Movarek Biochemicals, Brea, CA) and harvested after another 16 h on glass filer mats with an automated harvesting device (Inotech, Dottikon, Switzerland). Incorporated radioactivity was measured by liquid scintillation counting using a LKB-Wallac counter (Wallac Oy, Turku, Finland). Data are expressed as mean cpm of triplicate cultures.

For the measurement of IL-4 and INF-γ, culture supernatants were collected 72 hours after primary stimulation, diluted 1:2 in PBS/0.05%Tween, and assayed by antigen capture ELISA using Maxisorp polystyrene 96-well plates (Nalge Nunc Int., Roskilde, Denmark) and

cytokine specific antibodies (Endogen, Woburn, MA), according to the manufacturer's recommendations. Recombinant human IL-4 and INF- γ (Endogen) were used as standards.

Affinity purification of HLA-DR molecules

Human HLA-DR8 molecules were purified from cell lysates of the HLA-DRB1*0801 homozygous cell line BM-9 by affinity chromatography using anti-HLA-DR mAb L243 cross-linked to Protein A-Sepharose CL-4B (Amersham Pharmacia Biotech AB, Uppsala, Sweden) as affinity support (Sinigaglia et al. 1991) . Cells were lysed at a density of 10^8 cells ml^{-1} on ice for 60 min in 1% (v/v) Nonidet NP-40, 25 mM iodoacetamide, 5 mM sodium orthovanadate and 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml each of soybean trypsin inhibitor, antipain, pepstatin, leupeptin and chymostatin in 0.05 M Na/PO_4 buffer, 0.15 M NaCl, pH 7.5. Lysates were cleared of nuclei and debris by centrifugation at 27,000 *g* for 30 min. After addition of 0.2 volumes of 5% sodium deoxycholate (DOC) to the supernatant and mixing for 10 min, the lysate was centrifuged at 100,000 *g* for 2 hr and subsequently filtered through a 0.45 μm membrane (Sartorius AG, Göttingen, Germany). For affinity purification of class II molecules the lysates were first passed over a Sepharose CL-4B containing pre-column, and subsequently over the protein A sepharose-mAb column. The affinity column was then washed with i) 20 column volumes of 50 mM Tris-HCl, pH 8, 0.15 M NaCl, 0.5% NP-40, 0.5% DOC, ii) 5 column volumes of 50 mM Tris-HCl, pH 9, 0.5 M NaCl, 0.5% NP-40, 0.5% DOC and iii) 5 column volumes of 2 mM Tris-HCl, pH 8, 1% octyl-β-D-glucopyranoside (OG, Sigma, St. Louis, MO). HLA-DR molecules were eluted with 0.05 M diethylamine in 0.15 M NaCl, pH 11.5, containing 1% OG, 1 mM EDTA and the eluate was immediately neutralized with 1/20 volume of 1 M Tris/HCl, pH 6.8. Preparations were kept in aliquots at –80 °C until use.

Peptide binding and competition assay

Peptide binding to HLA-DR molecules was analyzed essentially as described (Calvo-Calle et al. 1997) with an ELISA based assay using the reporter peptide $GYR(A)_6L$ N-terminally labeled via two 6-aminopropionic acid spacers to biotin (Sigma-Genosis, Cambridge, UK). As second reporter peptide, biotinylated $CLIP^{82-102}$ (PKPPKPVSKMRMATPLLMQAL) with a much higher binding affinity than $GYR(A)_6L$ to DR8 molecules was used for assays with Gly-substituted peptide analogs. Purified HLA-DR molecules were diluted in freshly prepared binding buffer, containing 100 mM citrate/phosphate buffer (pH 7), 0.15 mM NaCl, 4 mM

EDTA, 4% NP-40, 4 mM PMSF and 40 μg/ml each of soybean trypsin inhibitor, antipain, leupeptin and chymostatin. Sixty microliter of an optimal dilution containing 10 – 100 ng HLA-DR molecules were added to each well of a 96-well low-binding V-bottom microtiter plate (Nalge Nunc Int., Roskilde, Denmark) together with 20 μl of biotinylated reporter peptide (final concentration 0.2 μM) in citrate/phosphate buffer (pH 7) and 20 μl of competitor peptide serially diluted in DMSO:PBS (1:4). After 24 hours of incubation at room temperature, the solution was transferred to wells of Immunolon-2 ELISA plates (Dynex Techn., Chantilly, VA), which had been coated with a 20 μg/ml solution of the anti-HLA-DR mAb L-243 and subsequently blocked with PBS containing 0.05% Tween-20 and 5% bovine serum albumine. After 3 hours of incubation at room temperature, plates were washed with PBS, 0.05%Tween-20. After incubation with alkaline phosphatase-labeled streptavidine (Calbiochem, La Jolla, CA) reporter peptide/HLA-DR complexes were quantified by determining conversion of the substrate 4-nitrophenylphosphate (Sigma, St. Louis, MO). Binding of peptides and pseudopeptides to the HLA-DR molecules was determined by measuring the OD in the presence versus the absence of competitor. Inhibition was calculated as percentage using the formula 100 x [1 – (ΔOD in the presence of competitor/ΔOD in the absence of competitor)]. The competitor concentrations yielding 50% inhibition of reporter peptide binding were calculated as IC50 values. Competitors yielding IC50 values <100μM were considered positive for binding to the HLA-DR molecule. CLIP$^{82-102}$ (PKPPKPVSKMRMATPLLMQAL), which is known to bind to most HLA-DR alleles (Ghosh et al. 1995; Malcherek et al. 1995) was included into each assay as positive control. As negative control, $(NANP)_3$ peptide was included in each binding assay.

Results

Minimal epitopes of T cell clones derived from PBMC of SPf66 vaccinated volunteers by in vitro restimulation with MSP-1$^{38-58}$

T cell lines that proliferate in response to challenge with MSP-1 $^{38-58}$ peptide were established from two SPf66-vaccinated volunteers (TS and ToSch). All T cell clones derived from these lines were CD3+/CD4+/CD8- as determined by FACS analysis (data not shown). HLA-DR as well as HLA-DP restricted clones could be isolated from donor TS. In contrast, all T cell clones derived from ToSch were HLA-DP restricted. Four MSP-1$^{38-58}$ -specific T cell clones of TS (TS-M101P, TS-M33P, TS-M103P and TS-M32P) and two clones of ToSch (ToSch-2 and ToSch-39) were included in this study (Table I). They recognized the MSP-1 $^{38-58}$ peptide in the context of HLA-DRB1*0801, HLA-DPA1*0103/DPB1*02012 or HLA-DPA1*0103/DPB1*0401 (Table I). Generation of MSP-1 specific T cell lines from non-vaccinated donors failed (data not shown) indicating that MSP-1 $^{38-58}$ specific T cells were in fact elicited by vaccination with SPf66.

N- or C-terminally truncated derivatives of MSP-1 $^{38-58}$ synthesized by the F-MOC technique were used to determine the minimal epitopes required to stimulate individual T cell clones. Proliferative responses of the T cell clones to stimulation with these peptides are shown in Table II and results are summarized in Fig. 1. The minimal epitope of both DR-restricted clones investigated extended further into the C-terminal part of the MSP-1 sequence (up to residue G^{56}) than those of the DP-restricted clones (up to residues M^{51} or L^{53}). The N-terminal limit of the minimal epitope was for one of the two DR- and for three of the four DP-restricted clones V^{39}. Y^{43} and T^{41} were the N-terminal limits for the remaining two clones. The resulting minimal epitopes thus varied in length between 11 and 18 amino acids (Fig.1).

Proliferative response to single amino acid substituted analogs of MSP-1$^{38-55}$

For the analysis of the fine specificity of four selected T cell clones, single A- and G-substituted analogues of MSP-1$^{38-55}$ were tested in proliferation assays. Peptides were synthesized using the BOC-chemistry, thus having an amide group at the C-terminus. Proliferation was considered positive if values of incorporated radioactivity in the presence of peptide exceeded the background incorporation (without peptide) three times.

In the case of the DR8-restricted clone TS-M101P replacement of any of the ten C-terminal amino acids (F^{46} to E^{55}) by either an A or a G resulted in complete loss of stimulatory activity of the MSP-1$^{38-55}$ peptide (Fig. 2A). The only exception was the N^{58} to A replacement, which

did not reduce stimulatory activity. Replacements at the first eight N-terminal amino acids (A^{38} to L^{45}) caused no or only limited loss of stimulatory activity. Amino acid positions, which could not be replaced, were all located within the deduced minimal epitope (Y^{43} to G^{56}) determined by the analyses with truncated peptides (Fig. 1). Clone TS-M101P was stimulated by MSP-1^{38-55} peptide only, if the peptide contained a C-terminal amide group (BOC-synthesis). In contrast, the analog with a C-terminal carboxy-group (F-MOC-synthesis) was not stimulatory (Table II), indicating that the C-terminal amide group of residue E^{55} is essentially involved in peptide recognition. Residues Y^{43}, S^{44} and L^{45} at the N-terminus of the minimal epitope could be replaced both by A or G without loss of stimulatory activity.

For the DP-restricted clone TS-M103P, residues most critical for stimulation reached from L^{40} to V^{52} (Fig. 2B), demonstrated by complete loss of stimulatory activity when replaced by either A or G. Exceptions were the E^{49} to A and the K^{50} to G replacements, which did not abolish stimulatory activity and the replacement of G^{42} by A, which even improved the stimulatory activity. Substitutions at residues V^{39} and L^{53} of the minimal epitope (V^{39} to L^{53}) caused no loss of stimulatory activity. T cell clone ToSch-39 required the same minimal epitope as clone TS-M103P (Fig. 1) and is also HLA-DP restricted, although by a different allele (Table 1). Nevertheless significantly fewer residues (Y^{43} to K^{48}) of the minimal epitope could not be replaced without loss of stimulatory activity for clone ToSch-39 (Fig. 3A). E^{49} to G and K^{50} to G replacements also abolished activity, but in contrast to clone TS-M103P, clone ToSch-39 was still stimulated by peptides with A or G substitutions at residues L^{40}, T^{41}, M^{51} and V^{52}. ToSch-2, the third DP-restricted T cell clone analyzed, required the shortest minimal epitope (T^{41} to M^{51}) of all clones analyzed (Fig. 1). Within these eleven amino acids, only substitutions of G^{42} and M^{51} by A did not abolish stimulatory activity (Fig. 3B).

As demonstrated in Fig.5, a core of residues that could not be replaced without loss of stimulatory activity was located in the case of the HLA-DR restricted clone closer to the C-terminal end of MSP-1^{38-58} than in the case of all three HLA-DP restricted clones investigated. Results indicate that MSP-1^{38-58} contains overlapping promiscuous T cell epitopes.

To investigate whether the altered peptide ligands modulate cytokine secretion patterns, IL-4 and INF-γ production by T cell clones upon stimulation was measured in cell culture supernatants by ELISA. Cytokine production of all four clones paralleled proliferation after stimulation with A- or G-substituted analogues. The ratio of IL-4 over INF- γ production was

not altered by amino acid substitutions. IL-4 and INF- γ production by T cell clone ToSch-2 is shown in Fig 4.

Glycine scan of inhibiton of reporter peptide binding to HLA-DR8 molecules

The failure of some single amino acid substituted peptide analogues to stimulate the DR8-restricted T cell clone TS-M101P could be the result of either the inability of the peptides to bind to the restricting class II molecules, or of the inability of the MHC-peptide-complex to adequately trigger the TCR. To investigate this, we compared the relative binding affinities of unmodified parent peptide and single-G substituted analogues by competition binding assays using affinity purified DRA/DRB1*0801 molecules. Serially diluted peptides were used to compete binding of a biotinylated reporter peptide (GYR(A)6L) to the purified HLA class II heterodimers. IC50 values were defined as the concentration of a peptide yielding 50% inhibition of binding of the reporter molecule.

As shown in Fig. 6, analogues with G substitutions at positions A ^{38}, L^{40}, T^{41}, Y^{43}, S^{44}, Q^{47} or E^{55} bound to DR8 molecules with similar affinities as unmodified MSP-1 $^{38-55}$ peptide (G at position 42). G substitutions at position K ^{48}, K^{50}, M^{51} or N^{54} significantly increased the competitor activity. Glycine substitution at these positions could possibly enhance binding by allowing closer contact of the peptide backbone with the MHC-binding groove. Substitutions at residues K^{48}, K^{50} and M^{51} abolished proliferative responses although they were improving HLA-DR8 binding. These residues therefore are likely to be critically involved in TCR triggering. G substitutions at several other positions decreased the binding inhibitory activity. This effect was most strongest with amino acids L ^{45}, E^{49} and V^{52}. Similar results were obtained with biotinylated CLIP $^{82-102}$ reporter peptide which has a much higher binding affinity to DR8 than the GYR(A)6L reporter (Fig. 7). The most striking effect was observed for positions L45, E49 and V52.

To confirm the results obtained with single G substituted analogues we tested the binding affinity of two peptides containing two G substitutions (Table III). A peptide (21747) with substitutions at two positions (L ^{45} and V^{52}) that had significantly reduced the inhibitory activity, completely lost activity (IC50>100 μM). A second analogue (21748) with substitutions at two positions (Q^{47} and N^{54}) which had affected inhibitory activity less critically retained activity (Table III).

Discussion

In this report the fine-specificity of six unique CD4+ T cell clones is described, which were derived from PBMC of two SPf66 vaccinated volunteers by repeated restimulation with a peptide comprising amino acids 38 to 58 of the *P. falciparum* MSP-1. MSP-1 is a leading vaccine candidate (Anders and Saul 2000; Good et al. 1998) and a sequence stretch of the MSP-1^{38-58} sequence (amino acids 43-53) has been included in the synthetic peptide vaccine SPf66 (Patarroyo et al. 1988). Multiple genetic restriction elements, DRB1*0801, DPB1*02012/DPA1*0103 and DPB1*0401/DPA1*0103, were used by the T cell clones. Results of T cell stimulation analyses with single amino acid substituted peptides indicate that the MSP-1^{38-58} derived sequence stretches that directly interact with the binding grooves of HLA-DR and -DP, respectively, are overlapping, but not identical. In the course of a previous study we could generate MSP-1^{38-58} specific T cell lines from another six volunteers vaccinated with SPf66 (unpublished data), demonstrating that sequences present in MSP-1^{38-58} can be presented by multiple HLA-DR alleles and thus represent promiscuous T cell epitopes (Doolan et al. 2000; Hammer et al. 1993; Moreno et al. 1993; O'Sullivan et al. 1991; Sinigaglia et al. 1988) .

Positions P1, P4 and P6 are crucial anchor residues for peptide-binding to many HLA-DR-alleles (Hammer et al. 1993; Hill et al. 1994; O'Sullivan et al. 1991) . In most cases peptides bind with a hydrophobic or an aromatic residue into a large hydrophobic pocket 1 of the DR-molecule. F^{46} of MSP-1^{38-58} peptide could eventually be proposed as p1, but substitution of this position did not dramatically affect DR-binding. Although a variety of peptides with HLA-DR8 binding capacity have been reported, no allele-specific binding motive could be assigned (Chicz et al. 1993)

The length of the deduced minimal epitopes required to stimulate the individual T cell clones ranged from 11 to 18 amino acids, which is in accordance with the general length of MHC class II presented peptides (Chicz et al. 1992; Chicz et al. 1993; Madden 1995). These minimal epitopes consist of a core sequence located in the MHC peptide binding groove and of a variable number of additional essential flanking residues (Moudgil et al. 1998). The length of minimal epitopes required for stimulation of clones using the same restriction element (TS-M101P and TS-M33P, ToSch-39 and ToSch-2, respectively), but expressing different TCRs varied. This could be taken as an indication that the peptide core flanking sequences interact with the TCR. However, recently resolved crystal structures of two TCR-

peptide-MHC class II complexes revealed, that the TCR-footprint on the MHC covers maximally nine residues of the presented peptide (Hennecke et al. 2000; Reinherz et al. 1999). The DR-restricted clone TS-M101P could not proliferate in the presence of MSP-1 $^{38-55}$ if the peptide contained a C-terminal carboxy-group. In contrast, MSP-1 $^{38-55}$ with a C-terminal amide group, was stimulatory, as was MSP-1$^{38-56}$. This indicates that that TCR-triggering of TS-M101P needs at the end of C-terminal end of residue E ^{55} a hydrogen-bond donor, which is not provided by the C-terminal carboxyl group. A small structural difference at one end of the minimal epitope thus can have a dramatic effect on T cell stimulation.

In analyses with single amino acid substituted peptides, stretches of 8 to 10 amino acid side chains could not be exchanged without loss of stimulatory activity of MSP-1 $^{38-55}$. Substitutions may affect T cell triggering directly by changing binding interactions with TCR and MHC residues or by changing peptide and/or MHC/peptide complex conformation. A glycine scan of peptide binding inhibitory activity with affinity-purified DR8 molecules, the restriction element of clone TS-M101P, suggested that the impact of single amino acid substitutions on binding to DR8 is rather limited. Substitution of most positions of the minimal epitope of clone TS-M101P abolished stimulation without significantly affecting DR8 binding. Two (amino acids 44 and 47) of the three positions of the MSP-1 block 1 sequence that exhibit naturally occurring dimorphic sequence variation (Miller et al. 1993) are comprised in the minimal epitopes of all six clones tested. The third dimorphic position (amino acid 52) is additionally comprised in the minimal epitopes of four clones. Dimorphism limits the use of MSP-1$^{38-55}$ as component of a multi-epitope malaria vaccine, since T cell responses seem to be strictly allele specific (manuscript in preparation).

Table I. *MSP-1 $^{38-58}$ specific T cell clones*

	TCR gene rearrangements		Restriction element
	a **chain**	b **chain**	
TS-M101P	AV4 AJ39	BV7-2 BJ2-3	DRA*0102 / DRB1*0801
TS-M33P	AV23 AJ22	BV18 BJ2-3	DRA*0102 / DRB1*0801
TS-M103P	AV25 AJ52	BV19 BJ1-4	DPA1*0103 / DPB1*02012
TS-M32P	AV8-3 AJ8	BV6-5 BJ2-3	DPA1*0103 / DPB1*02012
ToSch-2	AV19 AJ11	BV27 BJ1-1	DPA1*0103 / DPB1*0401
ToSch-39	AV8-1 AJ29	BV19 BJ2-1	DPA1*0103 / DPB1*0401

Table III. *Inhibition of reporter peptide binding to purified DRB1*0801 molecules by MSP-1 peptide variants*

		IC50 values of competitor peptide with biotinylated reporter peptide (0.2 μM)	
competitor peptide	amino acid sequence	GYR(A)6L reporter	CLIP$^{82-102}$ reporter
MSP-1$^{38-55}$	AVLTGYSLFQKEKMVLNE	12.7[a]	39
21748	AVLTGYSLF**G**KEKMVL**G**E	5	22
21747	AVLTGYS**G**FQKEKM**G**LNE	>100	>100
$(NANP)_3$	NANPNANPNANP	>100	>100
CLIP$^{82-102}$	PKPPKVSKMRMATPLLMQAL	0.02	0.2

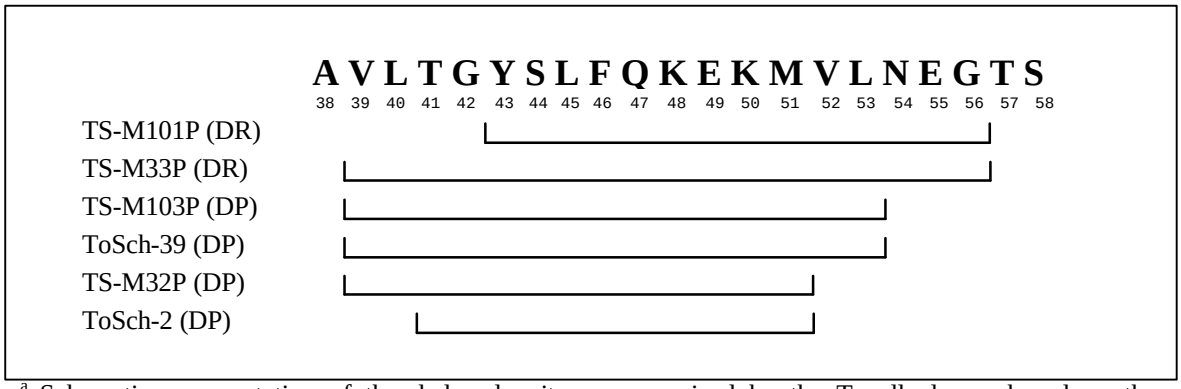
[a] Results are expressed as IC50, the concentration (μM) of unlabeled competitor peptide required to inhibit 50% of the binding of a biotinylated reporter peptide. The percent inhibition was calculated based on OD at 405nm obtained in the presence of different concentrations of competitor peptide (100-0.001 μM). An IC50 >100 μM was regarded as no peptide binding. Results are mean values of duplicates of one representative experiment. All assays were performed twice.

Table II: *Proliferation of T cell clones upon stimulation with truncated variants of MSP-1*$^{38\text{-}57}$

peptide sequence:	T cell clones (restriction element)					
	TS-M101P (DR)	**TS-M33P** (DR)	**TS-M103P** (DP)	**TS-M32P** (DP)	**ToSch-2** (DP)	**ToSch-39** (DP)
	cpm[a]) SD					
(no peptide)	99) 4	513) 21	78) 17	293) 27	499) 53	425) 48
AVLTGYSLFQKEKMVLNEGT	**305**) 18	**2922**) 860	**4845**) 233	**4126**) 320	**36917**) 1822	**9340**) 629
AVLTGYSLFQKEKMVLNEG	**629**) 106	**3970**) 1177	**17374**) 940	**9892**) 444	**67988**) 1701	**21201**) 841
AVLTGYSLFQKEKMVLNE	118) 11	735) 119	**16750**) 1266	**10216**) 715	**58265**) 5726	**11996**) 5148
AVLTGYSLFQKEKMVLN	82) 20	536) 55	**13817**) 1612	**5286**) 641	**60348**) 3704	**6146**) 318
AVLTGYSLFQKEKMVL	148) 50	544) 95	**17002**) 2382	**995**) 234	**66523**) 3906	**7384**) 383
AVLTGYSLFQKEKM	144) 42	451) 59	198) 80	**2294**) 319	**3085**) 842	443) 185
AVLTGYSLFQKEK	89) 7	503) 62	59) 15	394) 41	303) 66	197) 62
AVLTGYSLFQKE	109) 37	467) 48	74) 21	224) 30	237) 68	142) 63
AVLTGYSLFQK	112) 45	665) 18	89) 33	198) 14	65) 6	132) 66
AVLTGYSLFQ	77) 11	449) 44	73) 17	230) 56	209) 117	154) 25
VLTGYSLFQKEKMVLNEGT	**3227**) 300	**1478**) 368	**2071**) 70	**964**) 209	**45511**) 5643	**11958**) 582
LTGYSLFQKEKMVLNEGT	**3342**) 826	998) 12	46) 6	230) 52	**32003**) 2275	331) 192
TGYSLFQKEKMVLNEGT	**941**) 132	897) 251	167) 31	280) 189	**26773**) 435	155) 48
GYSLFQKEKMVLNEGT	**302**) 21	617) 226	83) 37	230) 69	1368) 835	137) 41
YSLFQKEKMVLNEGT	**841**) 107	478) 32	99) 14	322) 24	148) 29	356) 187

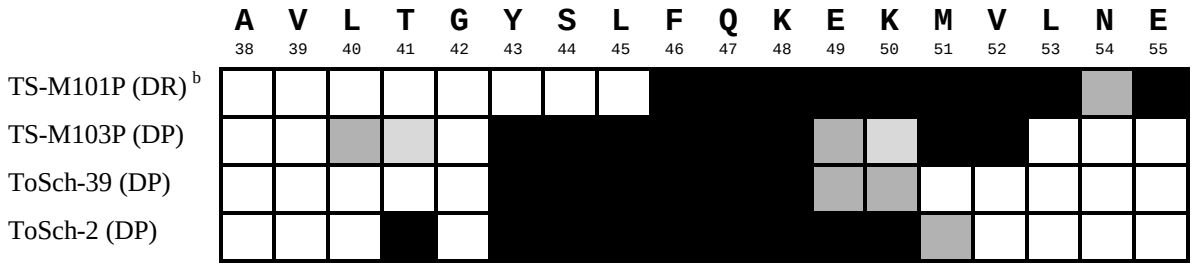
[a] Incorporation of [^{3}H]-thymidine is expressed as mean cpm) 1SD of triplicate cultures. T cell clones were incubated in the presence of APCs and 20 μg/ml of peptide. Cpm values in the presence of peptide three times higher than cpm values in the absence of peptide (no peptide) were regarded as positive proliferation (shown in bold). Proliferation assays were performed as described in Materials and Methods.

Figure 1: *Mapping of epitopes within the MSP-1$^{38-58}$ sequence*[a]



[a] Schematic representation of the deduced epitopes recognized by the T cell clones, based on the proliferative response to truncated peptides.

Figure 5. *Mapping of amino acid residues*[a] *within the MSP-1$^{38-55}$ sequence important for T cell stimulation*



[a] Open boxes represent amino acid residues which can be replaced by an Ala orGly without loss of stimulatory activity (SI>3). Black boxes represent amino acid residues which lead to complete loss of stimulatory activity after replacement by an Ala or Gly (SI<3). Grey boxes represent residues which lead to mixed stimulation pattern after substitution with Ala or Gly, dark grey: Ala-analogue stimulatory, Gly-analogue non-stimulatory, light grey: Ala-analogue non-stimulatory, Gly-analogue stimulatory).
[b] MSP-1$^{38-58}$ specific T cell clones with restriction element in parentheses.

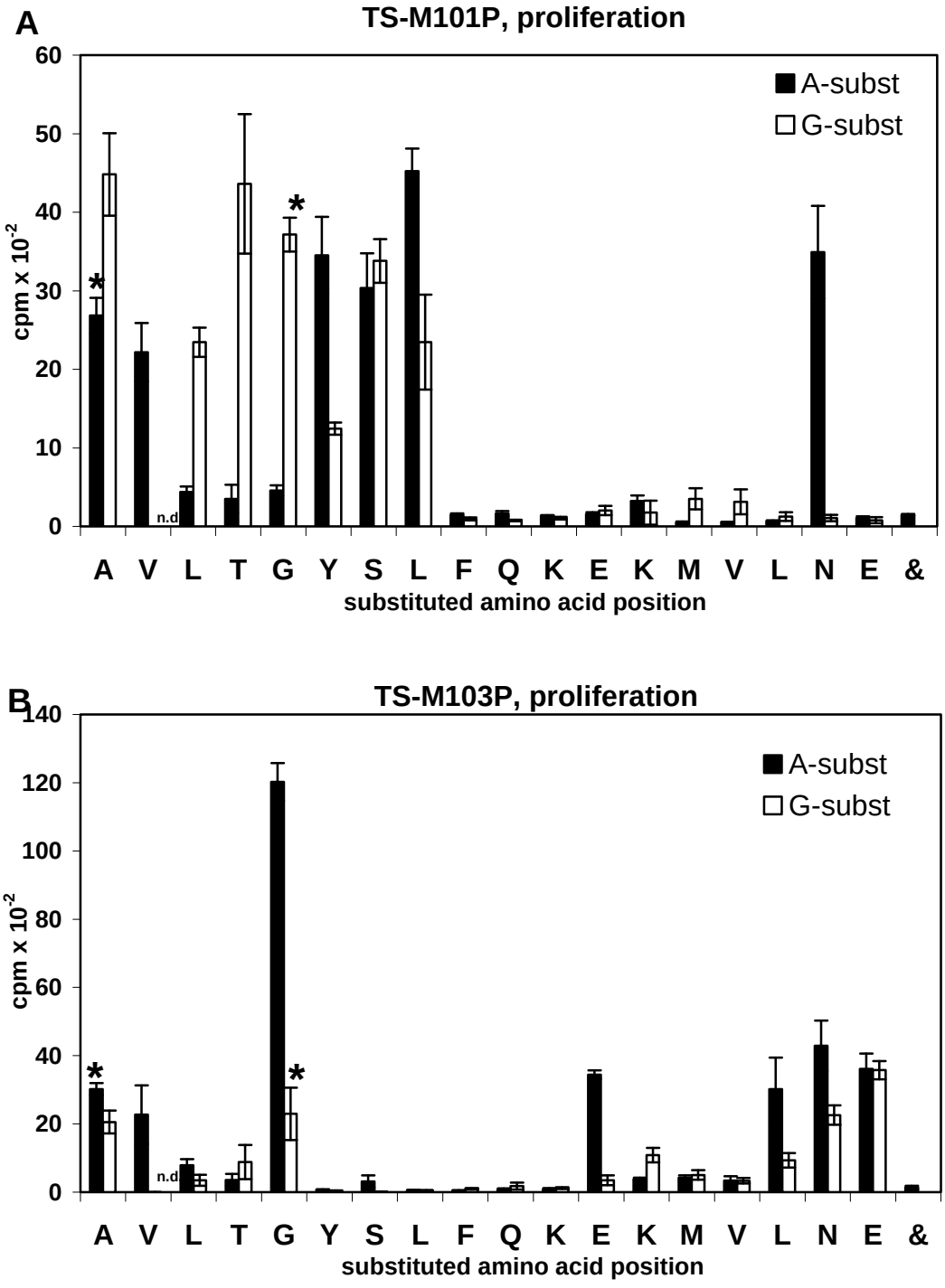


Figure 2: Proliferative response of the DR restricted T cell clone TS-M101P (A) and the DP restricted clone TS-M103P (B) upon stimulation with single position substituted analogue peptides of MSP-1 sequence 38-55 and with medium alone (&). Each substituted amino acid position of the MSP-1 peptide by an alanine (filled bars) or by a glycine (open bars) is listed at the x-axis (single letter code). Stimulation with corresponding unmodified parent peptide is marked by a star. T cells were incubated in the presence of APCs and 20 µg/ml of peptide. Incorporation of radiolabeled thymidine is expressed as mean cpm and 1SD of triplicate cultures. n.d. = not determined.

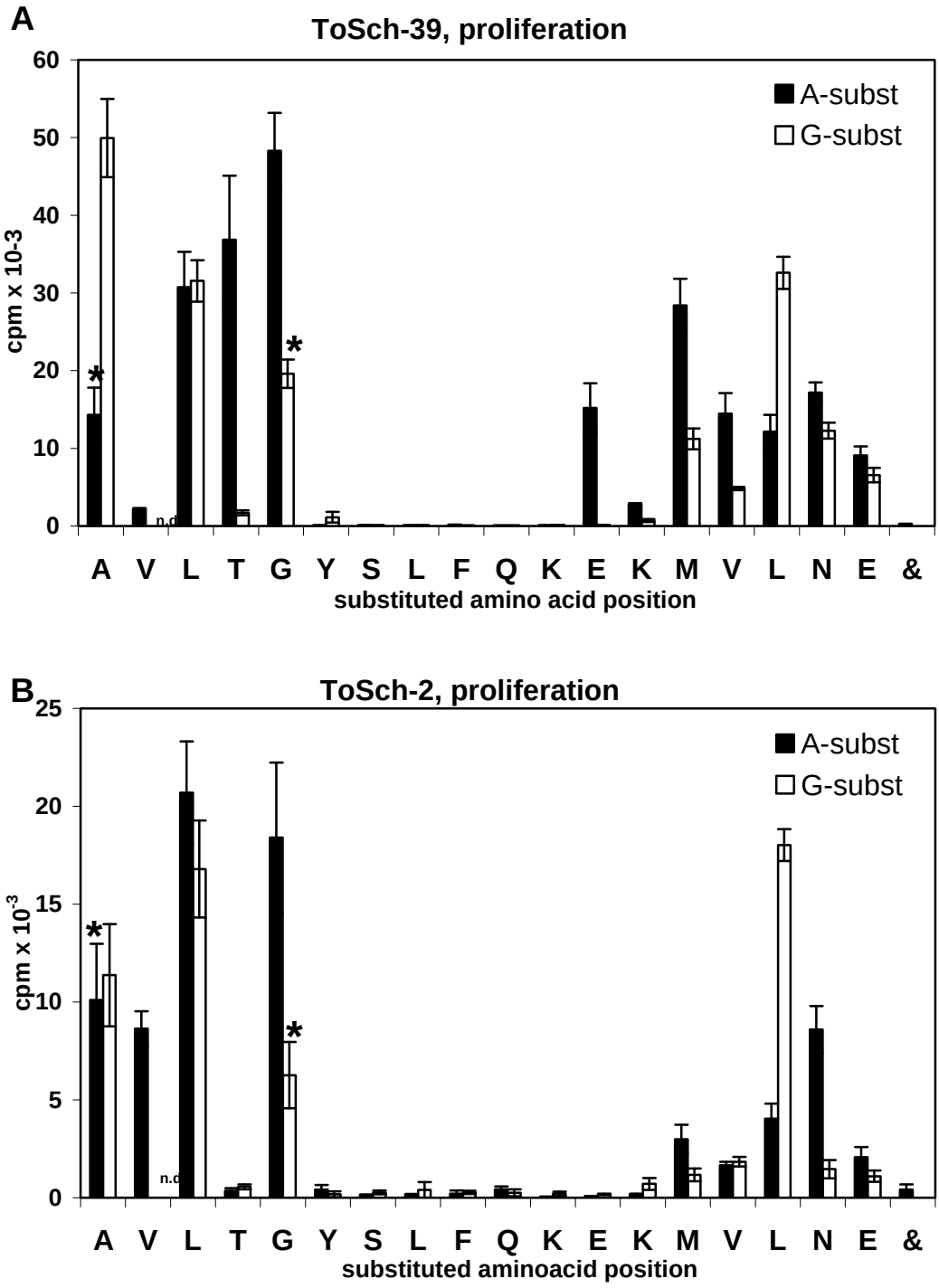


Figure 3: Proliferative response of the DP restricted T cell clones ToSch-39 (A) and ToSch-2 (B) upon stimulation with single position substituted analogue peptides of MSP-1 sequence 38-55 and with medium alone (&). Each substituted amino acid position of the MSP-1 peptide by an alanine (filled bars) or by a glycine (open bars) is listed at the x-axis (single letter code). Stimulation with corresponding unmodified parent peptide is marked by a star. T cells were incubated in the presence of APCs and 20 μg/ml of peptide. Incorporation of radiolabeled thymidine is expressed as mean cpm and 1SD of triplicate cultures. n.d. =not determined.

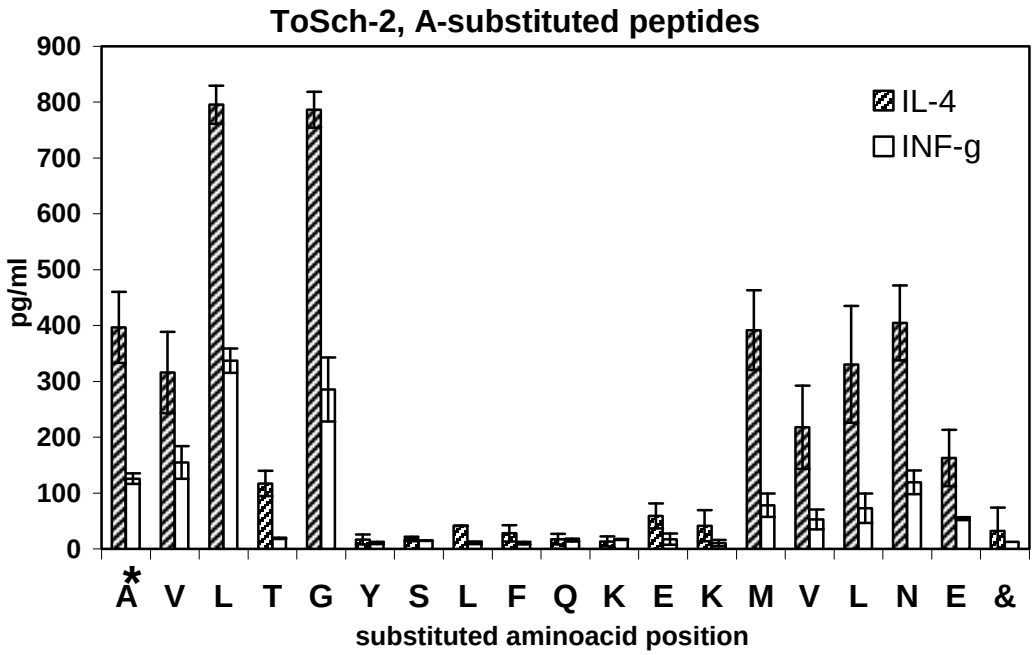


Figure 4: Cytokine production of T cell clone ToSch-2 upon stimulation with position substituted analogue peptides of MSP-1 sequence 38-57 and with medium alone (&). Each single substituted amino acid position of the MSP-1 peptide by an alanine (A) is indicated at the x-axis (single letter code). Unmodified parent peptide is marked by a star. See corresponding proliferation data in Fig. 3 B.

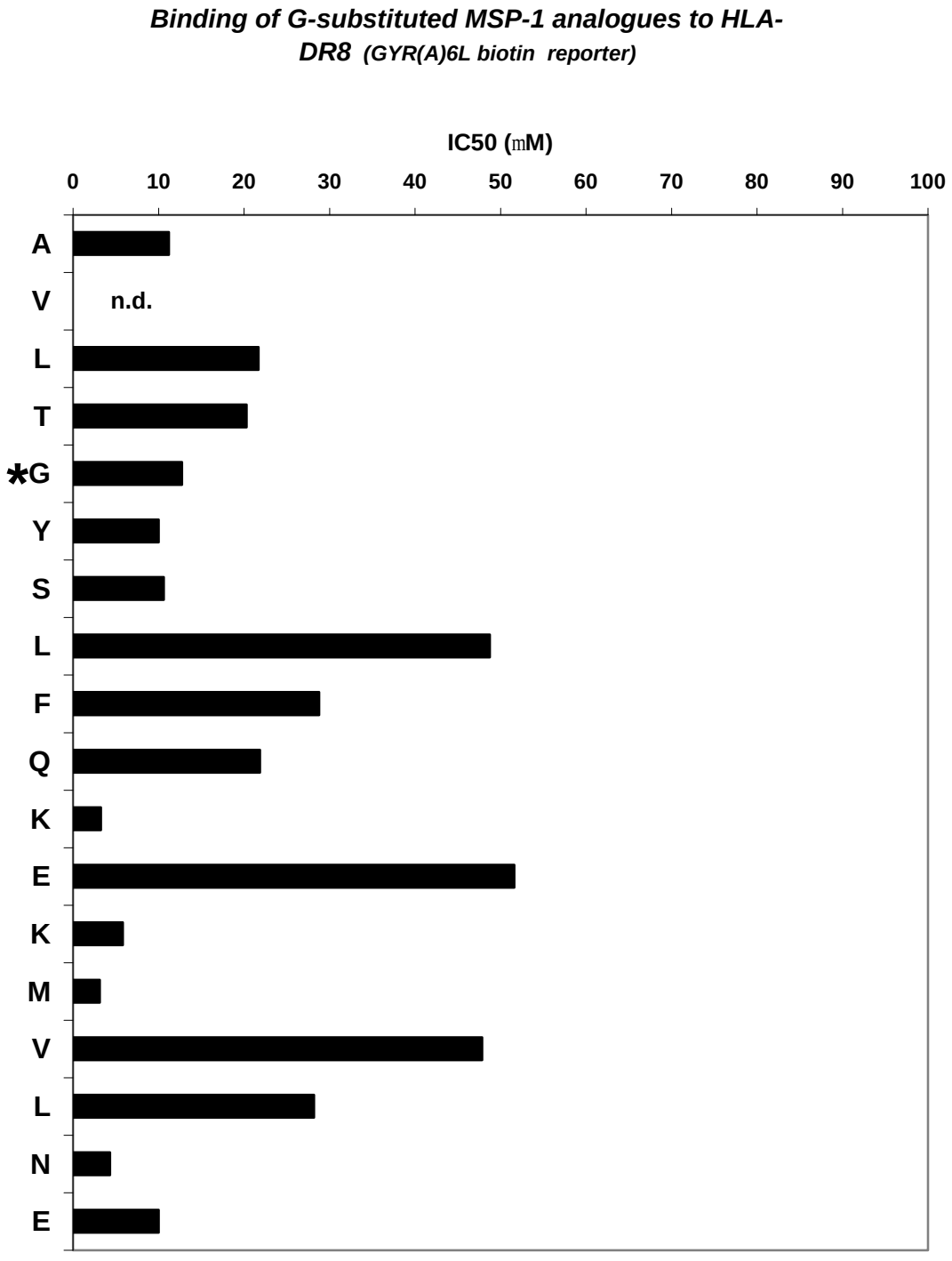


Figure 6: Effects of single glycine-substituted amino acid positions in the MSP-1 sequence 38-55 (AVLTGYSLFQKEKMVLNE) on the binding to affinity-purified HLA-DR8 molecules. Each Gly-substituted amino acid position of the MSP-1 peptide is listed at the y-axis and represents the corresponding peptide analogue. Serial dilutions (0.1 to 200 μM) of unmodified parent peptide (marked by a star) and substituted analogues were incubated with affinity-purified HLA-DR8 molecules in the presence of 0.2 mM biotinylated GYR(A)6L reporter peptide. IC50 values are given as mean of duplicates of one representative experiment out of three experiments. n.d. = not determined.

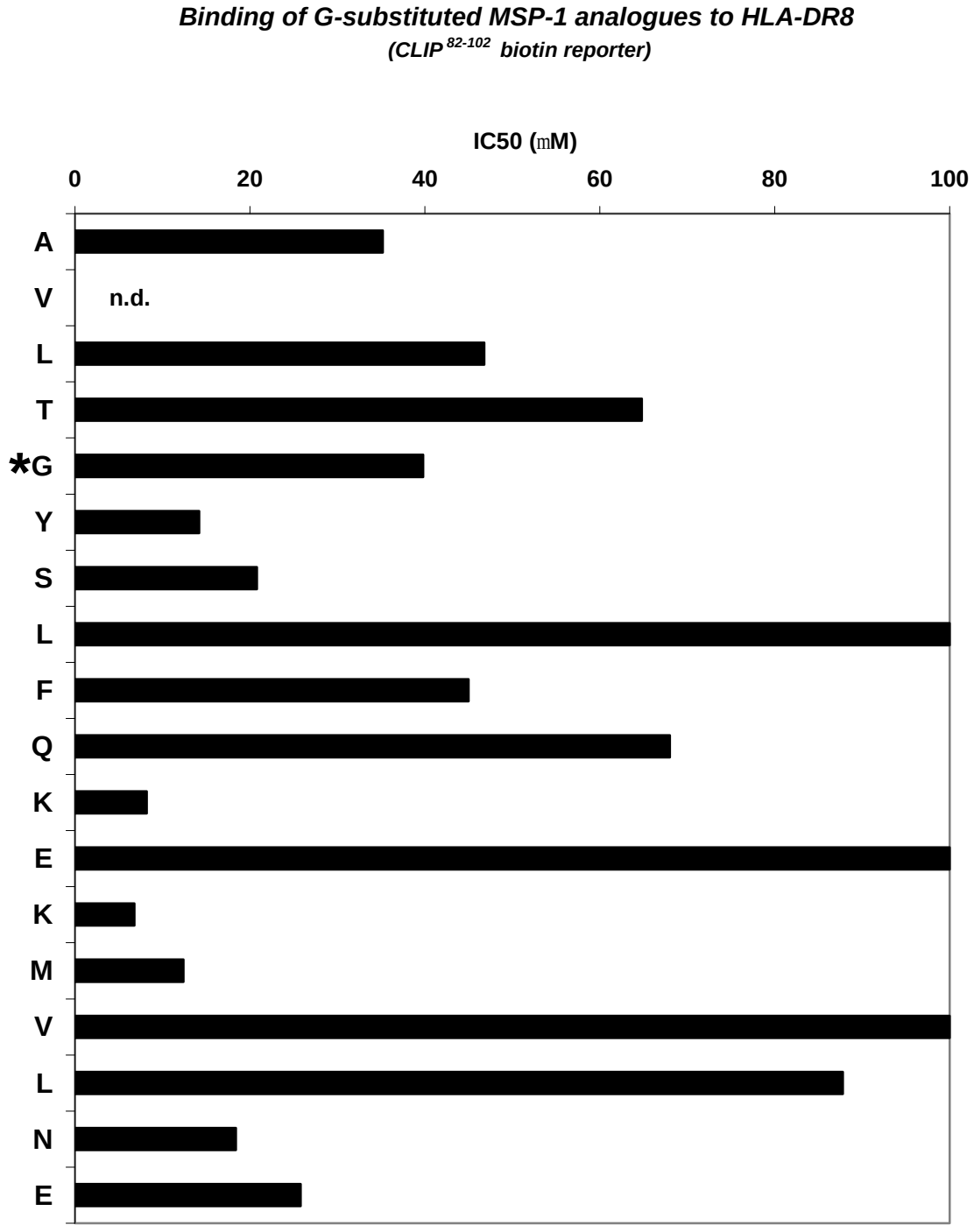


Figure 7: Effects of single glycine-substituted amino acid positions in the MSP-1 sequence 38-55 (AVLTGYSLFQKEKMVLNE) on the binding to affinity-purified HLA-DR8 molecules. Each Gly-substituted amino acid position of the MSP-1 peptide is listed at the y-axis and represents the corresponding peptide analogue. Serial dilutions (0.1 to 200 μM) of unmodified parent peptide (marked by a star) and substituted analogues were incubated with affinity-purified HLA-DR8 molecules in the presence of 0.2 mM biotinylated CLIP $^{82-102}$ reporter peptide. IC50 values are given as mean of duplicates. n.d. = not determined.

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4. Efficient antigen-specific stimulation of human HLA class II-restricted T cell clones by reduced amide pseudopeptide analogues of a sequence derived from the *Plasmodium falciparum* malaria vaccine candidate antigen Merozoite Surface Protein-1

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Abstract

Pseudopeptide analogues of antigenic peptides derived from block 1 of *Plasmodium falciparum* Merozoite Surface Protein-1 were obtained by replacing single amide bonds by a reduced Ψ [CH_2–NH] peptide bond. The altered peptide ligands were compared with their parent peptides for their capacity to stimulate HLA-DP or -DR restricted human T cell clones. Measurements of T cell proliferation and cytokine (IL-4 and IFN-γ) yielded comparable results. All three HLA-DP restricted T cell clones studied exhibited a remarkably similar response pattern to the set of altered ligands, although they were derived from two different individuals, expressed different TCR and were restricted by two different HLA-DP alleles. A different methylene-amino scan reaction pattern was observed with a HLA-DR restricted clone. The effects of individual peptide backbone modifications thus appear to be more specific for a particular type of restriction elements than for an individual T cell clone. Backbone modifications at some positions strongly reduced binding to the restricting HLA class II molecule and impeded T cell stimulat ion. However, good binders did not necessarily stimulate T cells, demonstrating that main chain modifications can also dramatically influence TCR triggering. Interestingly, some pseudopeptides were better T cell stimulators than their parent peptides. These findings together with decreased protease susceptibility and a better reproduction of conformational B cell epitopes indicate that pseudopeptides with reduced peptide bonds may advantageously replace natural peptides in vaccine design.

Introduction

There is increasing interest in synthetic peptides both as components of epitope-focussed subunit vaccines against infectious or neoplastic diseases and as immunoregulatory agents to selectively down-regulate undesirable immune responses in immune-associated pathologies, such as allergy and autoimmunity. However, a major disadvantage of peptides is their sensitivity to proteases, which reduces their lifespan in biological fluids. Non-natural peptides, including pseudopeptides with backbone modifications exhibit increased resistance to proteolytic degradation (Bianco et al. 1998; Calbo et al. 2000; Lozano et al. 1998; Stemmer et al. 1999) and have therefore been proposed to advantageously replace natural peptides in immunoprophylactic and therapeutic strategies (Kieber-Emmons et al. 1997).

Due to conformational restriction, pseudopeptides with reduced $\Psi[CH_2\text{-}NH]$ bonds in the backbone may reproduce B cell epitopes of native target proteins more efficiently than the corresponding linear peptides (Lozano et al. 1998). Since Th cell activation is essential for the induction of protective immune responses, this has raised the question, whether pseudopeptides can serve both as B and as T cell epitopes in human vaccines. Backbone modifications do not necessarily lead to loss of binding to MHC class I and class II molecules (Calbo et al. 1999; Cotton et al. 1998; Guichard et al. 1995) and stimulation of human HLA class I restricted cytotoxic T cells by pseudopeptides with reduced bond has in fact been described (Ostankovitch et al. 1998). The stimulatory potential of pseudopeptides for MHC class II restricted Th cells has, however, been investigated so far only in murine model systems (Cotton et al. 1998).

MHC class II molecules form stable complexes with a broad range of different peptide sequences. The formation of hydrogen bonds between the peptide main chain and conserved MHC residues along the length of the open ended peptide binding groove provides sequence-independent baseline affintiy for peptide ligands (Madden 1995). In addition, polymorphic side chains of the MHC molecules form allele-specific binding pockets which exhibit strong preferences for interaction with particular amino acid side chains of the peptide (Hammer et al. 1997). Matrix-based predictions that rely on mathematical processing of individual peptide side chain effects have turned out to be powerful tools to predict MHC class II ligands (Sturniolo et al. 1999).

Several upward-pointing residues of the deeply buried peptide can serve as direct TCR contacts. Two crystal structures of TCRs in complex with peptide and class II molecules (Hennecke et al. 2000; Reinherz et al. 1999) revealed an orthogonal orientation of the TCR relative to its peptide-MHC ligand and a dominance of the TCR variable region CDR3 domains in atomic contacts with the peptide. In both a human and a murine TCR/pMHCII complex, the TCR presented a relatively flat surface. Its contacts spanned only nine residues of the peptide. TCR recognition of the peptides was found to be largely hydrophobic in one case (Reinherz et al. 1999) or dominated by salt bridges in the other (Hennecke et al. 2000) and involved a number of associations with backbone and side chain structures, with the solvent-exposed P5 residue of the peptide being particularly important (Reinherz et al. 1999). The triggering of an appropriate signal to stimulate T cell proliferation strongly depends on the kinetics of the TCR-ligand interaction (Lanzavecchia et al. 1999). The quality of a T cell response can be altered by stimulation with modified peptide variants (Abrams and Schlom 2000; Bachmann et al. 1998). Such altered peptide ligands (APL) with structural alterations such as single amino acid replacements or backbone modifications can exhibit changed binding affinities to the MHC molecule or change TCR-peptide-MHC interaction, leading to a modulated T cell response (Ostankovitch et al. 1998; Sloan-Lancaster and Allen 1996).

In this report we investigated, whether human HLA class II restricted T cells can be stimulated by pseudopeptides containing single reduced $\Psi[CH_2\text{-}NH]$ bonds. Pseudopeptides and their corresponding parent peptides were derived from an immunogenic sequence of the N-terminal block 1 of *Plasmodium falciparum* Merozoite Surface Protein-1 (MSP-1), which is regarded as a major malaria vaccine candidate antigen (Good et al. 1998). Immunisation with the block 1-derived sequences MSP-$1^{24\text{-}67}$ and MSP-$1^{43\text{-}53}$ has been shown to confer partial sterilising protection against experimental challenge with *P. falciparum* in *Saimiri* monkeys (Cheung et al. 1986) or to delay the onset of parasitemia in *Aotus* monkeys (Patarroyo et al. 1987), respectively. Furthermore, the $K^{48}EKMV^{52}$ sequence of block 1 seems to be relevant for the binding of malaria parasites to red blood cells. Pseudopeptide analogues of MSP-$1^{42\text{-}61}$ have been shown to mimic bioactive conformations of MSP-1 (Lozano et al. 1998) and to elicit antibodies, which inhibit *in vitro* invasion of erythrocytes by *P. falciparum*.

These findings raised our interest to investigate, whether introduction of reduced $\Psi[CH_2\text{-}NH]$ bonds into the backbone of peptides comprising the critical $K^{48}EKMV^{52}$ sequence is compatible with the stimulation of human MSP-1 specific T helper cells.

Therefore a methylene-amino scan was performed to study the contribution of the peptide backbone in TCR mediated stimulation of HLA-DR and -DP restricted human T cell clones raised against MSP-1$^{38-58}$. Results demonstrate a contribution of particular peptide backbone positions to MHC binding or TCR triggering. Interestingly certain pseudopeptide derivatives turned out to be better T cell stimulators th an their parent peptides.

Materials and Methods

Synthetic Peptides

Parent peptides MSP-1^{38-58} and MSP-1^{42-61} and the corresponding pseudopeptide analogues (Table I) were synthesized by the multiple solid-phase technique using *t*-butoxy-carbonyl chemistry on a *p*-methylbenzhydrylamine resin as previously described (Lozano et al. 1998). Crude products were analyzed by analytical reverse phase-HPLC and were purified to a purity of >98% by preparative reverse phase-HPLC. Composition of the purified molecules was reconfirmed by MALDI-TOF mass spectroscopy. In the case of the MSP-1^{42-61} derivatives, both monomeric and polymerized versions with cysteine residues at both termini were synthesized.

T cell clones

Four MSP-1^{38-58} specific HLA class II restricted human T cell clones (Table II) generated from PBMCs of two volunteers (manuscript in preparation) were included in the analyses. Volunteers TS and ToSch had been vaccinated with the synthetic peptide malaria vaccine SPf66 (Patarroyo et al. 1988) containing the MSP-1^{43-53} sequence YSLFQKEKMVL. Ethical clearance was granted by the responsible ethical committee of the Department of Internal Medicine, Kantonsspital, Basel, Switzerland. T cell clones were established essentially as described (Daubenberger et al. 2001) using irradiated autologous PBMC as APCs and MSP-1^{38-58} as stimulating antigen.

HLA class II DRB, DPA1 and DPB1 sequences were obtained by PCR amplification and DNA sequence analysis of genomic DNA as described (Daubenberger et al. 2001). Volunteer TS was HLA typed as DRB1*0801/1201, DRB3*0202, DPA1*0103/02011, DPB1*02012/11011 and volunteer ToSch as DRB1*0401/0404, DRB4*0101/0103, DPA1*0103/0103, DPB1*0401/20011. For the determination of the restriction elements used, peptide presentation to T cell clones in proliferation assays was selectively blocked with anti-human HLA class I (W6/32), class II (HB-145), -DR (L-243), -DP (B7/21) and -DQ (SPV-L3) mAb. The restricting class II alleles (Table II) were subsequently determined by using irradiated homozygous EBV transformed cell lines of defined haplotypes as APCs. Sequence analysis of TCR transcripts (Table II) was done as described (Moonka and Loh 1994).

T cell stimulation assays

Proliferation assays with MSP-1^{38-58} specific T cell clones were performed with 96-well round bottom-plates (Costar, Cambridge, MA) and RPMI 1640 medium supplemented with 10% heat-inactivated human AB serum (Haenecke AG, Germany), 100 U/ml penicillin, 100 µg/ml streptomycin (Gibco BRL, Life Technol., Paisley, Scotland), 10 mM HEPES buffer, 2 mM L-glutamine and 50 µM 2-mercaptoethanol. T cells (2×10^4) were incubated with 2×10^4 irradiated (30 Gy) autologous PBMC as APC in the presence or absence of peptides or pseudopeptides. After 72 h, cultures were pulsed with 1 µCi [^{3}H]-Thymidine (Movarek Biochemicals, Brea, CA) for 16 hours. Subsequently cultures were harvested on glass filers mats with an automated harvesting device (Inotech, Dottikon, Switzerland) and radioactivity incorporated into the DNA was measured by liquid scintillation counting using a LKB- Wallac counter (Wallac Oy, Turku, Finland). Data are expressed as mean cpm of triplicate cultures.
Levels of IL-4 and INF-γ were determined by antigen capture ELISA using Maxisorp polystyrene 96-well plates (Nalge Nunc Int., Roskilde, Denmark) and cytokine specific antibodies (Endogen, Woburn, MA), according to the manufacturer's recommendations. Cell culture supernatants collected 72 hours after primary stimulation and diluted 1:2 in PBS/0.05%Tween were tested. Recombinant human IL-4 and INF-γ (Endogen) were used as standards.

Affinity purification of HLA-DR molecules

Human HLA-DR8 molecules were purified from cell lysates of the HLA-DRB1*0801 homozygous cell line BM-9 by affinity chromatography using anti-HLA-DR mAb L243 cross-linked to Protein A-Sepharose CL-4B (Amersham Pharmacia Biotech AB, Uppsala, Sweden) as affinity support (Sinigaglia et al. 1991) . Cells were lysed at a density of 10^8 cells ml^{-1} on ice for 60 min in 1% (v/v) Nonidet NP-40, 25 mM iodoacetamide, 5 mM sodium orthovanadate and 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 µg/ml each of soybean trypsin inhibitor, antipain, pepstatin, leupeptin and chymostatin in 0.05 M Na/PO_4 buffer, 0.15 M NaCl, pH 7.5. Lysates were cleared of nuclei and debris by centrifugation at 27,000 *g* for 30 min. After addition of 0.2 volumes of 5% sodium deoxycholate (DOC) to the supernatant and mixing for 10 min, the lysate was centrifuged at 100,000 *g* for 2 hr and subsequently filtered through a 0.45 µm membrane (Sartorius AG, Göttingen, Germany). For affinity purification of class II molecules the lysates were first passed over a Sepharose CL-4B containing pre-column, and subsequently over the protein A sepharose-mAb column. The

affinity column was then washed with i) 20 column volumes of 50 mM Tris-HCl, pH 8, 0.15 M NaCl, 0.5% NP-40, 0.5% DOC, ii) 5 column volumes of 50 mM Tris-HCl, pH 9, 0.5 M NaCl, 0.5% NP-40, 0.5% DOC and iii) 5 column volumes of 2 mM Tris-HCl, pH 8, 1% octyl-β-D-glucopyranoside (OG, Sigma, St. Louis, MO). HLA-DR molecules were eluted with 0.05 M diethylamine in 0.15 M NaCl, pH 11.5, containing 1% OG, 1 mM EDTA and the eluate was immediately neutralized with 1/20 volume of 1 M Tris/HCl, pH 6.8. Preparations were kept in aliquots at –80 °C until use.

Peptide binding and competition assay

Peptide binding to HLA-DR molecules was analyzed essentially as described (Calvo-Calle et al. 1997) with an ELISA based assay using the reporter peptide GYR$(A)_6$L N-terminally labeled via two 6-aminopropionic acid spacers to biotin (Sigma-Genosis, Cambridge, UK). Purified HLA-DR molecules were diluted in freshly prepared binding buffer, containing 100 mM citrate/phosphate buffer (pH 7), 0.15 mM NaCl, 4 mM EDTA, 4% NP-40, 4 mM PMSF and 40 µg/ml each of soybean trypsin inhibitor, antipain, leupeptin and chymostatin. Sixty microliter of an optimal dilution containing 10 – 100 ng HLA-DR molecules were added to each well of a 96-well low-binding V-bottom microtiter plate (Nalge Nunc Int., Roskilde, Denmark) together with 20 µl of biotinylated reporter peptide (final concentration 0.2 µM) in citrate/phosphate buffer (pH 7) and 20 µl of competitor peptide serially diluted in DMSO:PBS (1:4). After 24 hours of incubation at room temperature, the solution was transferred to wells of Immunolon-2 ELISA plates (Dynex Techn., Chantilly, VA), which had been coated with a 20 µg/ml solution of the anti-HLA-DR mAb L-243 and subsequently blocked with PBS containing 0.05% Tween-20 and 5% bovine serum albumine. After 3 hours of incubation at room temperature, plates were washed with PBS, 0.05%Tween-20. After incubation with alkaline phosphatase-labeled streptavidine (Calbiochem, La Jolla, CA) reporter peptid/HLA-DR complexes were quantified by determining conversion of the substrate 4-nitrophenylphosphate (Sigma, St. Louis, MO). Binding of peptides and pseudopeptides to the HLA-DR molecules was determined by measuring the OD in the presence versus the absence of competitor. Inhibition was calculated as percentage using the formula 100 x [1 – (ΔOD in the presence of competitor/ΔOD in the absence of competitior)]. The competitor concentrations yielding 50% inhibition of reporter peptide binding were calculated as IC50 values. Competitors yielding IC50 values <100µM were considered positive for binding to the HLA-DR molecule. $CLIP^{82-102}$

(PKPPKPVSKMRMATPLLMQAL), which is known to bind to most HLA-DR alleles (Ghosh et al. 1995)(Malcherek et al. 1995) was included into each assay as positive control and inhibited binding of the reporter peptide with an IC50 of 0.02 μM (data not shown).). As negative controls, $(NANP)_3$ (Calvo-Calle et al. 1997) or the MSP-1^{38-55} derived 21747 peptide (AVLTGYSGFQKEKMGLNE) which contains glycine substitutions at positions 45 and 52 , were included in each assay.

Results

Synthetic pseudopeptides

In order to investigate, how single amide bond modifications influence the capacity of a peptide to stimulate HLA class II restricted human T helper cells, we have synthesized pseudopeptide derivatives of antigenic peptides derived from block 1 of the *P. falciparum* malaria vaccine candidate antigen MSP-1. Table I shows the sequences of parent peptides and their pseudopeptide derivatives with reduced peptide bonds ($\Psi[CH_2\text{-}NH]$), which were introduced by reductive alkylation of *N*-Boc-α-amino aldehydes as described (Lozano et al. 1998). Of the MSP-$1^{42\text{-}61}$ sequence (GYSLFQKEKMVLNEGTSGTA) five pseudopeptide analogues with $\Psi[CH_2\text{-}NH]$ modifications between amino acids K48-E49, E49-K50, K50-M51, M51-V52 and V52-L53 were produced. Activities of monomeric (m) and of polymeric (p) variants with additional terminal cysteine residues were compared in the analyses. A second panel of polymeric pseudopeptides with $\Psi[CH_2\text{-}NH]$ modifications between amino acids Y43-S44, S44-L45, F46-Q47 and V52-L53 was derived from the MSP-$1^{38\text{-}58}$ sequence (AVLTGYSLFQKEKMVLNEGTS).

Stimulation of an HLA-DR restricted T cell clone by pseudopeptides

Pseudopeptide analogues of MSP-$1^{42\text{-}61}$ and MSP-$1^{38\text{-}58}$ were compared with their parent peptides for their capacity to stimulate the HLA DRB1*0801 restricted human T cell clone TS-M101P (Table II), which has been derived from a T cell line propagated with MSP-$1^{38\text{-}58}$ (Daubenberger et al. 2001). The minimal sequence required for stimulation of TS-M101P cells ($Y^{43}SLFQKEKMVLNEG^{56}$, Nickel et al., manuscript in preparation , see chapter 3) is comprised in both groups of pseudopeptides. Fig. 1 shows the proliferative and cytokine expression responses of TS-M101P cells to stimulation with monomeric (Fig. 1A, B, C) and polymeric (Fig. 1D, E, F) pseudopeptide derivatives of MSP-$1^{42\text{-}61}$ at peptide concentrations between 0.1 and 1000 µg/ml. The unmodified monomeric parent peptide mMSP-$1^{42\text{-}61}$ stimulated incorporation of [^{3}H]-thymidine at concentrations above 20 µg/ml. One pseudopeptide, m(E49-K50), was more effective than the parent peptide, stimulating proliferation at lower concentrations and leading to significantly higher incorporation of radiolabel (Fig 1A). In contrast, reduction of peptide bonds K48-E49, K50-M51, M51-V52 and V52-L53 completely abrogated stimulation of proliferation. To investigate whether the altered ligands modulate cytokine secretion patterns, IL- 4 and INF-γ production by TS-M101P cells upon stimulation with parent peptide or pseudopeptides was compared by

analyzing cell culture supernatants by ELISA (Fig. 1B and C). After stimulation with the parent peptide mMSP-1$^{42-61}$, both IFN-γ and IL-4 levels were low (30 pg/ml of IL-4 and <10pg/ml of IFN-γ at a peptide concentration of 160 μg/ml). Compared to the parent peptide, the stimulatory pseudopeptide m(E49-K50) caused a dramatically higher production of both cytokines (420 pg/ml of IL-4 and 200 pg/ml of IFN-γ at 160 μg/ml). All four non-stimulatory pseudopeptides m(K48-E49), m(K50-M51), m(M51-V52) and m(V52-L53) failed to induce production of detectable levels of both cytokines.

The polymerized versions of both the MSP-1$^{42-61}$ parent peptide and its pseudopeptide derivatives induced considerable higher proliferative responses than their monomeric counterparts (Fig. 1D). Similar to the results observed with the monomers, both the parent peptide pMSP-1$^{42-61}$ and the pseudopeptide with the Ψ[CH_2-NH] bond between amino acids E49-K50 had significant stimulatory activity. The higher relative efficacy of the pseudopeptide was less pronounced than in the case of the monomers. In the case of the stimulatory pseudopeptide, cytokine levels induced by m(E49-K50) and p(E49-K50) were roughly in the same range. In contrast, the polymeric parent peptide pMSP-1 $^{42-61}$ induced higher cytokine levels than its monomeric variant, but still lower levels than the stimulatory pseudopeptide. Stimulation with the four other polymeric pseudopeptides p(K48-E49), p(K50-M51), p(M51-V52) and p(V52-L53) was associated with incorporation of some [^{3}H]-thymidine at high ligand concentrations (Fig. 1D) but not with secretion of significant levels of IL-4 or INF-γ (Fig. 1E and F).

Also in the case of the pMSP-1$^{38-58}$ derived pseudopeptides, one altered ligand, p(Y43-S44), induced a stronger proliferative and cytokine expression response than the parent peptide at peptide concentrations >10 μg/ml (Fig. 2). The stimulatory activity of a second pseudopeptide, p(S44-L45) was comparable to that of the parent peptide, whereas reduction of peptide bonds F46-Q47 or V52-L53 led to complete loss of stimulatory activity (Fig. 2). Lack of activity of p(V52-L53)* reconfirmed the results obtained with the pseudopeptide derivatives p(V52-L53) and m(V52-L53) of MSP-1$^{38-58}$.

Binding of pseudopeptides to HLA-DR molecules

Taken together, proliferation and cytokine expression analyses showed, that the introduction of a Ψ[CH_2-NH] bond between amino acids Y43-S44 or E49-K50 augmented stimulation of

T cell clone TS-M101P, modification of the backbone between S44-L45 had no significant effect and modifications between F46-Q47, K48-E49, K50-M51, M51-V52 or V52-L53 largely impeded stimulation (Table III). Loss of stimulatory activity could be either due to the inability of a modified peptide ligand to bind to the restricting DRB1*0801 molecule or to the failure of the HLA-peptide complex to adequately trigger the TCR. To investigate this, we compared the relative binding affinities of parent peptides and their pseudopeptide derivatives by competition binding assays using affinity purified DRA/DRB1*0801 complexes. Serially diluted parent peptides and pseudopeptides were used to compete binding of a biotinylated reporter peptide ($GYR(A)_6L$) to the purified HLA class II heterodimers. IC50 values were defined as the concentration of a peptide yielding 50% inhibition of binding of the reporter molecule.

As shown in Fig. 3, the parent peptide pMSP-1 $^{42-61}$ inhibited reporter binding in a dose-dependent manner, while two negative controls, peptides $(NANP)_3$ (Calvo-Calle et al. 1997) and 21747 showed no significant competitive activity. Peptide 21747 consists of a modified MSP-1^{38-58} sequence with exchanges of the two amino acids L45 and V52 by glycine (AVLTGYS**G**^{45}FQKEKM**G**^{52}LNE). These two amino acid substitutions impede stimulation of clone TS-M101P (data not shown) and binding competition activity. The pseudopeptide p(K48-E49) and the parent peptide pMSP-1 $^{42-61}$ had comparable activity (Fig. 3), with IC50s of 9 μM and 5 μM, respectively. The pseudopeptide p(M51-V52), which had no stimulatory effect on TS-M101P T-cells (Fig. 1D), exhibited no significant binding (Fig. 3).

Results of competition assays with all parent peptides and pseudopeptides tested in the T cell stimulation analyses are summarized in Fig. 4. Polymeric peptide concentrations are expressed as molarity of the monomeric units. Compared to their respective monomeric or polymeric MSP-1^{42-61} parent peptides, pseudopeptides with a $\Psi[CH_2\text{-}NH]$ bond between amino acids K48-E49 or E49-K50 exhibited binding affinities that were only slightly reduced (Fig. 4A and B). In contrast, modification of the peptide bond K50-M51 weakened the affinity strongly and modification of the bonds M51-V52 or V52-L53 resulted in a virtual loss of binding. Analyses with the pseudopeptide derivatives of the parent peptide pMSP-1 $^{38-58}$ revealed, that reduction of the peptide bonds Y43-S44, S44-L45 or F46-Q47 had little effect on binding affinity, while modification of the bond V52-L53 reduced affinity significantly (Fig. 4C). The finding, that pMSP-1^{42-61} and its pseudopeptide derivative p(V52-L53) were weaker binders than their respective counterparts pMSP-1 $^{38-58}$ and p(V52-L53)* indicates,

that the additional N-terminal flanking sequence (AVLT) of the latter ones stabilizes the HLA-ligand complexes.

In summary, pseudopeptides with reduced bonds between amino acids Y43-S44, S44-L45 or E49-K50 were both stimulatory for TS-M101P T cells and good DRA/DRB1*0801 binders. Pseudopeptides with reduced bonds between amino acids F46-Q47 or K48-E49 were good binders, but not stimulatory. Modification of peptide bonds K50-M51, M51-V52 or V52-L53 both impeded the T cell stimulatory activity and reduced the HLA class II binding affinity.

Stimulation of HLA-DP restricted T cell clones by pseudopeptides

In order to assess, whether the effects of an introduction of $\Psi[CH_2\text{-}NH]$ bonds into peptide ligands are specific for individual T cell clones or restriction elements, the polymeric pseudopeptides were tested for their capacity to stimulate three different HLA-DP restricted human T cell clones (Table II). While the HLA-DPA1*0103/DPB1*02012 restricted human T cell clone TS-M103P was derived from the same T cell line as the HLA-DR restricted clone TS-M101P (Daubenberger et al. 2001), two DPA1*0103/DPB1*0401 restricted clones (ToSch-2 and ToSch-39) were derived from a MSP-1$^{38\text{-}58}$ specific T cell line propagated from PBMC of another individual.

All three clones showed proliferative responses to pMSP-1$^{42\text{-}61}$ and its pseudopeptide derivatives p(K50-M51), p(M51-V52) and p(V52-L53). Reduction of peptide bonds K50-M51, M51-V52 or V52-L53 decreased stimulation of clone TS-M103P, whereas the proliferative responses of ToSch-2 and ToSch-39 were not significantly affected by modifications at these positions (Fig. 5). In contrast, modification of peptide bond K48-E49 completely abolished recognition by all three clones. The three HLA-DP restricted T cell clones only differed in their response to p(E49-K50). While TS-M103P showed no proliferative response and ToSch-2 was stimulated only by relatively high concentrations (100 μg/ml and more) of this pseudopeptide, stimulation of ToSch-39 was almost as good as that observed with the parent peptide pMSP-1$^{42\text{-}61}$.

p(V52-L53)* was the only pseudopeptide derivative of the parent peptide pMSP-1$^{38\text{-}58}$, that was stimulatory for the HLA-DP restricted T cell clones (Fig. 6). In contrast, reduction of peptide bonds Y43-S44, S44-L45 or F46-Q47 completely abolished stimulation. Cytokine production of all three clones paralleled proliferation (data not shown).

Discussion

It has been demonstrated that pseudopeptide analogues with single $\Psi[CH_2\text{-}NH]$ modifications in the peptide backbone can reproduce B cell epitopes of the *P. falciparum* malaria vaccine candidate antigen MSP-1 more efficiently than the corresponding linear peptide (Lozano et al. 1998). Using the same sequences from the N-terminal block 1 of MSP-1 we analysed in the present study, whether such pseudopeptides can also serve as ligands for HLA class II restricted human T cells. In the case of an HLA-DR restricted T cell clone two out of eight backbone modifications studied resulted in improved stimulation of both proliferation and cytokine expression, demonstrating that pseudopeptides can be even better T cell activators than their unmodified peptide homologues. The reason for this increased stimulatory property could be due to a more favourable interaction of the TCR with important side chains of the peptide or with the backbone itself as a result of the introduction of an additional degree of freedom into the peptide. Modification at one of the other six positions studied had little effect on either T cell stimulatory properties or HLA-DR binding, while three other modifications impeded both HLA binding and T cell stimulation. Loss of HLA binding is consistent with results of X-ray crystallographic studies, which demonstrated, that interactions of certain carbonyl groups with conserved residues along the length of the peptide binding groove of MHC class II molecules contribute significantly to peptide binding . Interestingly two pseudopeptides were good HLA-DR binders, but failed to stimulate the HLA-DR restricted T cells. This indicates that TCR triggering was in these cases strongly modified by the introduction of the single $\Psi[CH_2\text{-}NH]$ modifications.

Pseudopeptide stimulation of three human HLA-DP restricted T cell clones was studied to assess, whether the effects of peptide backbone modifications are highly specific for individual T cell clones or rather characteristic for a particular type of restriction element. Although they were derived from two different individuals, expressed different TCR and were restricted by two different HLA-DP alleles, the three HLA-DP restricted T cell clones studied exhibited a remarkably similar response pattern to the altered ligands (Table III). Wide variation in response to the pseudopeptide p(E49-K50) was the only marked exception. The stimulation pattern observed with the HLA-DR restricted clone TS-M101P differed considerably from that of the three HLA-DP restricted clones (Table III), which indicates that the response pattern to backbone modifications is more strongly influenced by the type of restriction element used than by the structure of the individual TCR. Differences in the

peptide binding characteristics for HLA-DP and HLA-DR molecules has been described by analyzing the binding motifs for eluted peptides. Analysis of DPw4 (HLA-DPA1*0201/HLA-DPB1*0401) bound ligands reveals binding properties with aromatic/aliphatic anchors at positions 4 and 10, and an additional, mostly aliphatic, anchor at positions 12 or 13. The relative spacing of anchors is 1, 7, 10 and this is different from that observed for DR and DQ molecules (Falk et al. 1994).

Peptide carbonyls are in a position allowing direct contact with the TCR or MHC ligands (Madden 1995)(Reinherz et al. 1999). Our data demonstrate that particular peptide backbone carbonyls contribute critically to MHC binding or TCR triggering of Th cells. To influence TCR binding, carbonyls of the peptide backbone do not necessarily have to contact the TCR directly. Changes in the backbone structure may rather lead to changes in the conformational structure of the MHC bound peptide or the MHC molecule itself. This can affect kinetic and thermodynamic features of TCR interaction with the MHC peptide complex (Calbo et al. 1999). It is well established that TCRs interact more efficiently with multimerized than with single MHC/peptide complexes. Tetrameric MHC/antigen complexes generated by the cross-linking of biotinylated MHC molecules with avidin, bind to the TCR so efficient that this approach is now employed broadly for the identification of antigen-specific T cells (Altman et al. 1996). In this study we also compared the stimulation of one HLA-DR restricted T cell clone by a monomeric vs. polymeric form of its epitope. Our results clearly show that the polymerization of this MSP-1 derived epitope increases its stimulatory capacity as has been described (Rotzschke et al. 1997).

Taking together, the demonstration that pseudopeptides can be more efficient stimulators of human malaria antigen-specific Th cells encourage attempts to profile such altered ligands as elements of synthetic epitope-focussed vaccines.

Table I. *Sequences of MSP-1 pseudopeptides used in this study*

Peptide code	*Sequence*
mMSP-1 $^{42-61}$	**GYSLFQKEKMVLNEGTSGTA**
m(K48-E49)	GYSLFQK-Y **[CH_2NH]**-EKMVLNEGTSGTA
m(E49-K50)	GYSLFQKE-Y **[CH_2NH]**-KMVLNEGTSGTA
m(K50-M51)	GYSLFQKEK-Y **[CH_2NH]**-MVLNEGTSGTA
m(M51-V52)	GYSLFQKEKM-Y **[CH_2NH]**-VLNEGTSGTA
m(V52-L53)	GYSLFQKEKMV-Y **[CH_2NH]**-LNEGTSGTA
pMSP-1 $^{42-61}$	**CGYSLFQKEKMVLNEGTSGTAGC**
p(K48-E49)	CGYSLFQK-Y **[CH_2NH]**-EKMVLNEGTSGTAGC
p(E49-K50)	CGYSLFQKE-Y **[CH_2NH]**-KMVLNEGTSGTAGC
p(K50-M51)	CGYSLFQKEK-Y **[CH_2NH]**-MVLNEGTSGTAGC
p(M51-V52)	CGYSLFQKEKM-Y **[CH_2NH]**-VLNEGTSGTAGC
p(V52-L53)	CGYSLFQKEKMV-Y **[CH_2NH]**-LNEGTSGTAGC
pMSP-1 $^{38-58}$	**CGAVLTGYSLFQKEKMVLNEGTSGC**
p(Y43-S44)	CGAVLTGY-Y **[CH_2NH]**-SLFQKEKMVLNEGTSGC
p(S44-L45)	CGAVLTGYS-Y **[CH_2NH]**-LFQKEKMVLNEGTSGC
p(F46-Q47)	CGAVLTGYSLF-Y **[CH_2NH]**-QKEKMVLNEGTSGC
p(V52-L53)✶	CGAVLTGYSLFQKEKMV-Y **[CH_2NH]**-LNEGTSGC

Table II. *MSP-1 $^{38-58}$ specific T cell clones*

	TS-M101P	**TS-M103P**	**ToSch-2**	**ToSch-39**
TCR b **chain**	BV7-2 BJ2-3	BV19 BJ1-4	BV27 BJ1-1	BV19 BJ2-1
TCR a chain	AV4 AJ39	AV25 AJ52	AV19 AJ11	AV8-1 AJ29
Restriction element	DRA*0102 DRB1*0801	DPA1*0103 DPB1*02012	DPA1*0103 DPB1*0401	DPA1*0103 DPB1*0401

Table III. *Stimulatory activities*[a] *of T cell clones stimulated with pseudopeptides*

	T cell clones (restriction element)[b]			
Position of Y [CH_2-NH] modification	**TS-M101P (DR)**	**TS-M103 (DP)**	**ToSch-2 (DP)**	**ToSch-39 (DP)**
Y43-S44	++	-	-	-
S44-L45	+	-	-	-
F46-Q47	-	-	-	-
K48-E49	-	-	(+)	-
E49-K50	++	-	(+)	+
K50-M51	-	+	+	+
M51-V52	-	+	+	+
V52-L53	-	+	+	+

[a] Data are presented as stimulatory activity of pseudopeptides in relation to unmodified parent peptide, as a result of proliferation assays. ++: better stimulatory activity than parent peptide; +: comparable stimulatory activity; (+): decreased stimulatory activity; -: no stimulation.
[b] Restriction element was determined as described in materials and methods.

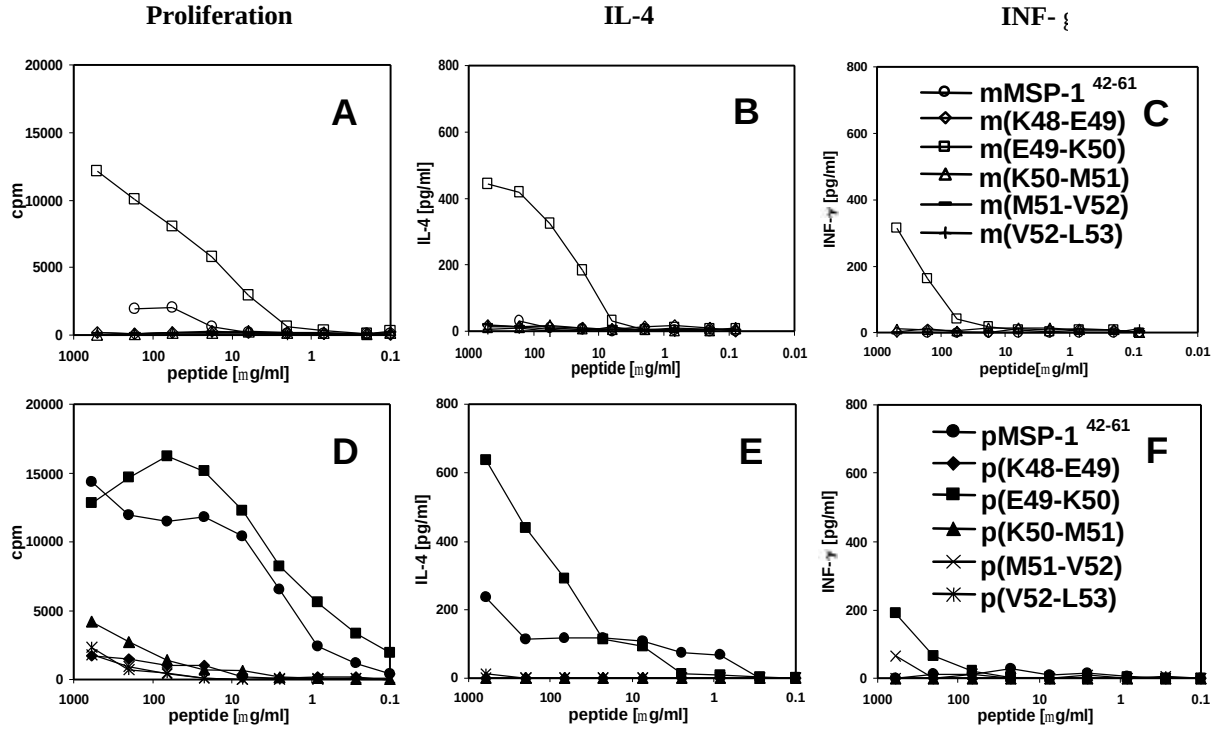

Figure 1. Responses of the HLA-DR restricted T cell clone TS-M101P to stimulation with pseudopeptides. T cells were stimulated with different concentrations of either the monomeric (A-C) or the polymeric versions (D-F) of MSP-1$^{42-61}$ and its pseudopeptide derivatives. Proliferation (A, D), IL-4 (B, E) and INF- γ (C, F) production is shown. The legend key for A-C is shown in C and the key for D-F is shown in F. No significant proliferation or cytokine production occurred in the absence of peptide (not shown). All assays were carried out in triplicates and were repeated twice. Mean values of one representative experiment are shown.

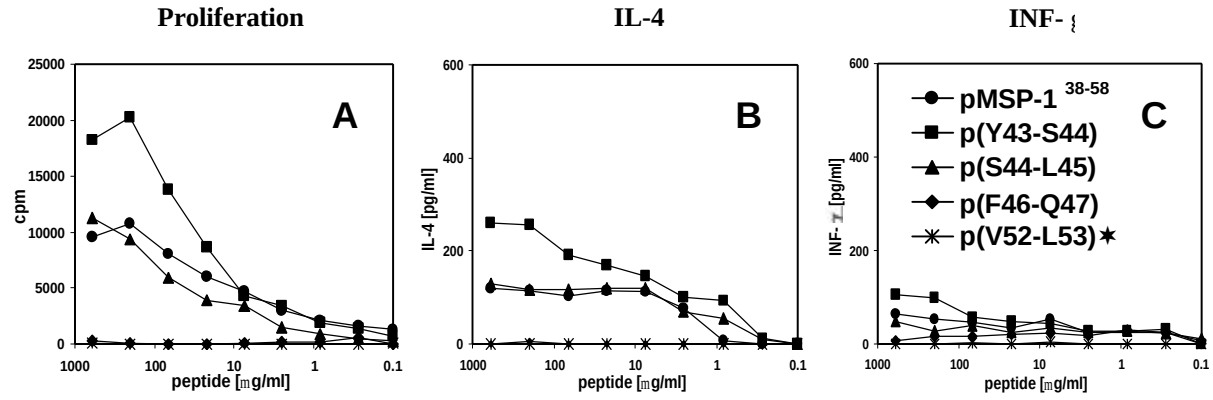

Figure 2. Proliferative responses (A), IL-4 (B) and INF-γ (C) production of the HLA-DR restricted T cell clone TS-M101P induced by stimulation with different concentrations of peptide ligands. T cells were stimulated with pMSP-1$^{38-58}$ and its pseudopeptide derivatives. All assays were carried out in triplicates and were repeated twice. Mean values of one representative experiment are shown.

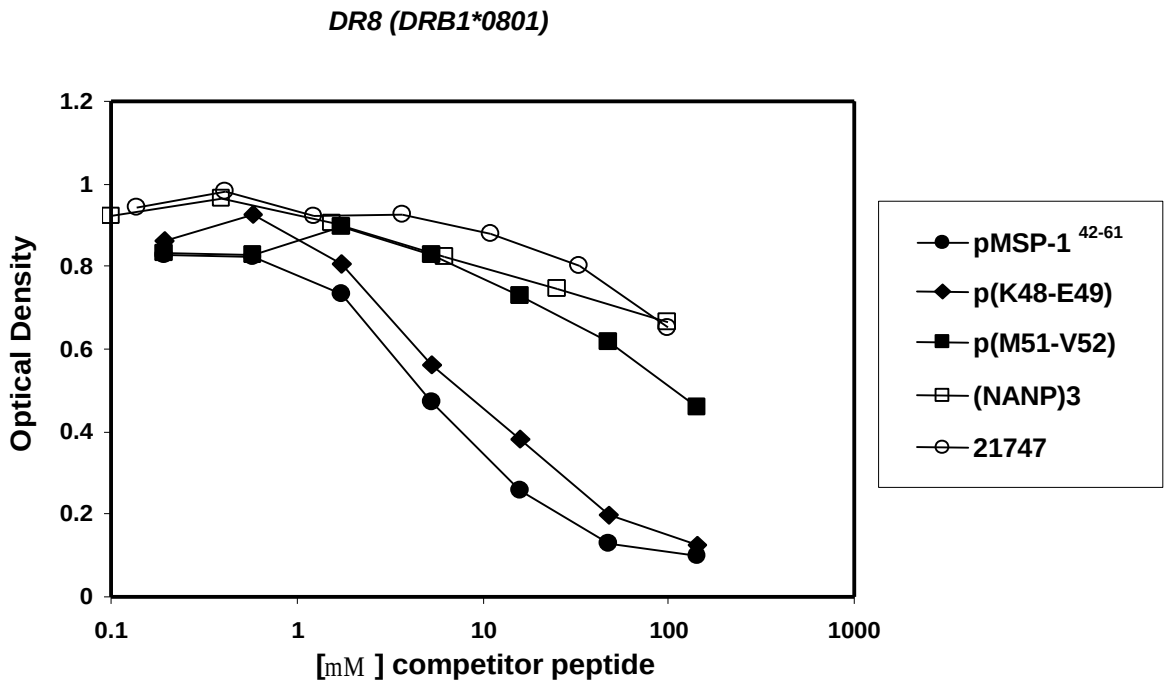

Figure 3. Inhibition of binding of the biotinylated reporter peptide $GYR(A)_6L$ to affinity-purified HLA-DR8 (DRB1*0801) molecules. Serial dilutions of the unlabeled parent peptide pMSP-1 $^{42-61}$, its pseudopeptide derivatives p(K48-E49) and p(M51-V52) and the negative control peptides $(NANP)_3$ and 21747 were used as competitors. Results are shown as ΔOD following subtraction of background OD measured in the absence of HLA-DR molecules. Mean values of one representative assay carried out in duplicate are shown.

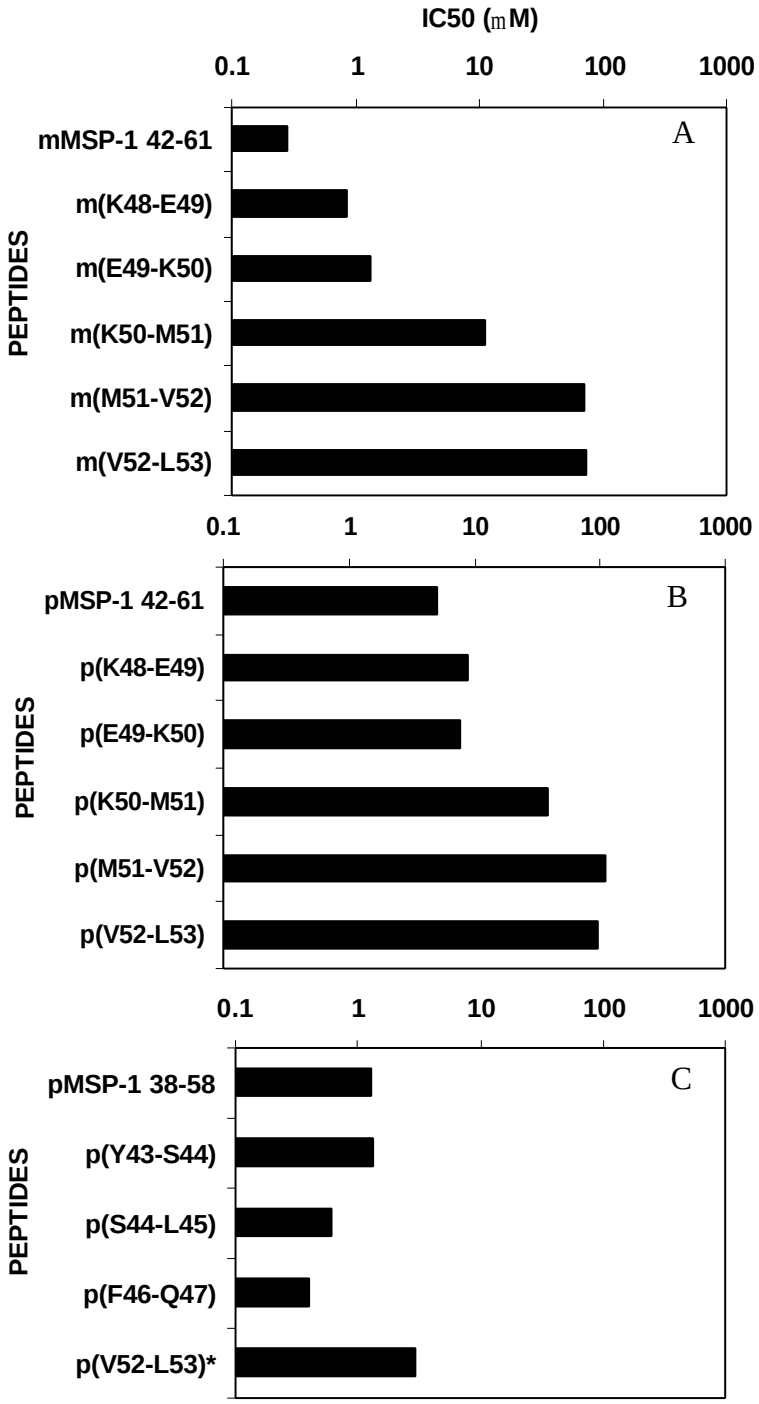

Figure 4. Effects of the introduction of reduced peptide bonds into MSP-1 derived peptides on the IC50 values in peptide binding inhibition assays with affinity-purified HLA-DR8 molecules. Serial dilutions (0.1 to 200 μM) of unmodified parent peptides and their pseudopeptide derivatives were incubated with affinity-purified HLA-DR8 molecules in the presence of 0.2 μM biotinylated GYR$(A)_6$L reporter peptide. IC50 values are given as mean of duplicates of one representative experiment.

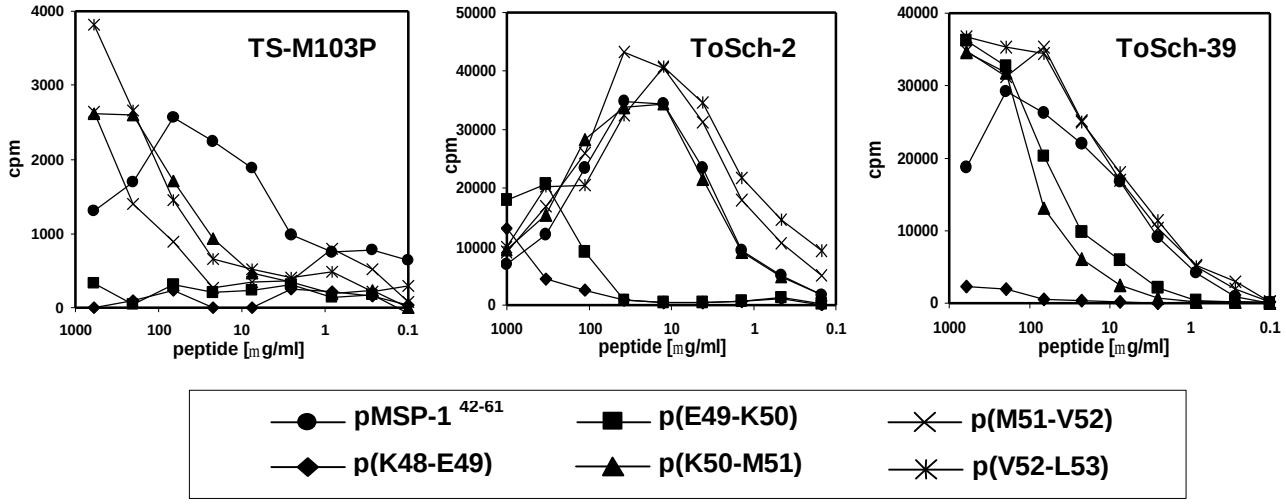

Figure 5. Proliferative responses of three DP-restricted MSP-1 specific T cell clones to stimulation with the parent peptide pMSP-1$^{42-61}$ and its pseudopeptide derivatives. All assays were carried out in triplicates and were repeated twice. Mean values of one representative experiment are shown.

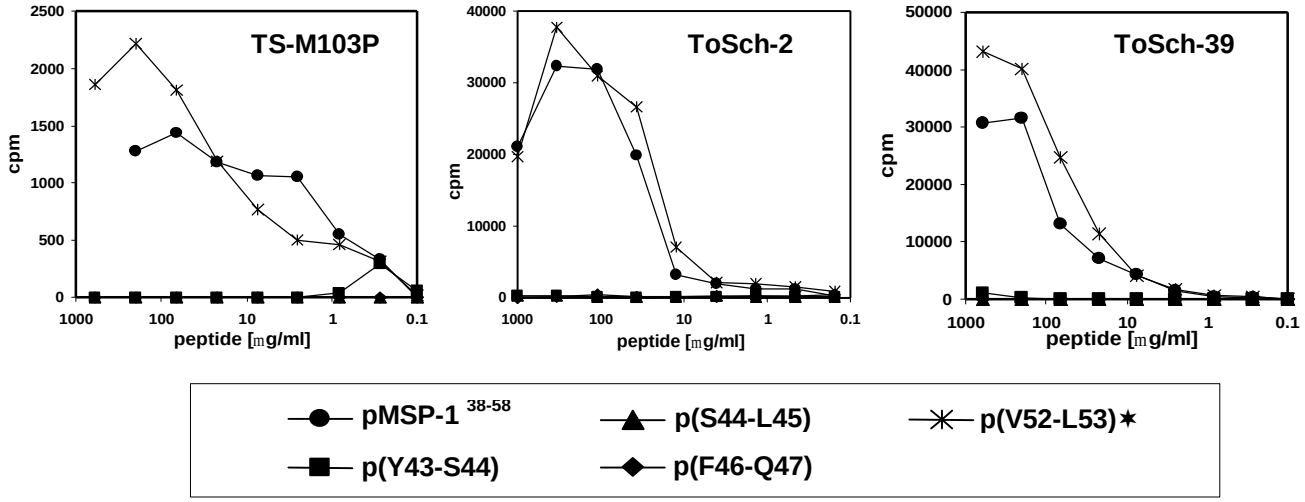

Figure 6. Proliferative responses of three DP-restricted MSP-1 specific T cell clones to stimulation with the parent peptide pMSP-1$^{38-58}$ and its pseudopeptide derivatives. All assays were carried out in triplicates and were repeated twice. Mean values of one representative experiment are shown.

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5. *Herpesvirus saimiri* transformed T cells and peripheral blood mononuclear cells restimulate identical antigen-specific human T cell clones

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Abstract

Panels of human antigen-specific T cell clones (TCC) have been established by limiting dilution using *Herpesvirus saimiri* (HVS) subtype C transformed T cells as antigen presenting cells (APC). They showed antigen-specific proliferation when peripheral blood mononuclear cells (PBMC), HVS-transformed T cells and Epstein Barr Virus transformed lymphoblastoid B cell lines (EBV-LCL) were used as APC. All T cell clones were CD4+ and HLA class II restricted. For a detailed analysis, two panels of T cell clones specific for an epitope located in the N-terminus of the Merozoite Surface Protein 1 (MSP-1) of *Plasmodium falciparum* (*P. falciparum*) were established from the same founder T cell line using either PBMC or HVS-transformed T cells as APC. TCR analysis of the two panels of TCC demonstrated that the same founder cells could be propagated in both culture systems. Furthermore, no difference in the cytokine expression pattern or antigen processing and co-stimulatory requirements was observed between TCC established on PBMC or HVS-transformed T cells. Based on the finding that HVS-transformed T cells can replace PBMC as APC for isolation and propagation of antigen-specific TCC a protocol was developed and successfully executed which allows to establish and maintain vaccine-specific T cell clones from 20 ml of blood. This method might be particularly significant in clinical trials of immune intervention strategies.

Introduction

Antigen-specific T cell lines and clones represent indispensable tools to study the quality and fine specificity of cellular responses associated with immune protection against infectious diseases and cancer, immuno-pathology and auto-immunity. Human antigen-specific T cell lines and clones can be propagated *in vitro* provided they are restimulated regularly with the respective antigen and antigen presenting cells carrying appropriate restriction elements (Märker-Hermann and Duchmann, 1998). Ideally, autologous PBMC or dendritic cells are used as APC. However, the supply with these cell types is limited in humans. Alternatively, EBV-LCL expressing relevant MHC molecules can replace PBMC or dendritic cells for *in vitro* cultures. However, one of the drawbacks associated with this approach is that the presence of EBV-specific T cells in most people leads to the *in vitro* restimulation and potential outgrowth of T cells recognizing EBV-derived epitopes (Rickinson and Moss, 1997). The establishment of TCC using a strong polyclonal stimulator like phytohemagglutinin often necessitates the laborious screening of a large panel of cultures before the desired antigen-specific TCC can be identified.

Human activated T cells display characteristics of professional APCs in that they are able to capture soluble antigen, present it in the context of newly synthesized MHC class II molecules and deliver co-stimulatory signals to other T cells (Barnaba et al., 1994). *H. saimiri* strain C488 transforms human T lymphocytes and the resulting T cell lines express differentiation and co-stimulatory molecules like CD2, CD3, CD58, CD80, CD154 and MHC class I and class II molecules (Meinl et al., 1995). Therefore, we investigated whether HVS-transformed T cell lines can be used as unlimited supply of APCs for the propagation of antigen-specific TCC. We reasoned that humans are not exposed to *H. saimiri* and therefore virus-specific memory T cells that could be restimulated *in vitro* are not likely to exist (Fickenscher and Fleckenstein, 1998). We report here that panels of TCC restimulated with PBMC or HVS-transformed T cells are comparable. This is demonstrated by TCR rearrangement analysis, proliferation and cytokine production assays. Based on our results we propose a protocol for the routine establishment and maintenance of human antigen-specific TCC in immune intervention studies using a single blood donation of roughly 2×10^7 PBMC.

Materials and Methods

Antigens

MSP-1^{38-58} (AVLTGYSLFQKEKMVLNEGTS) is a component of the semi-conserved block 1 of MSP-1, a major malaria vaccine candidate (Engers and Godal, 1998). Part of the peptide MSP-1^{38-58} (YSLFQKEKMVL) is an element of SPf66. SPf66 $(CGDELEAETQNVYAAPNANPYSLFQKEKMVLPNANPPANKKNAGC)_n$ (Patarroyo et al., 1988) consists of a 45 amino acid synthetic peptide which is polymerized through N- and C-terminal cysteine side chains to yield a product ranging from 10 to 25 kDa (Lopez et al., 1994).

Establishment of HVS-transformed T cell line and EBV-LCL

PBMC of donor TS were isolated from freshly drawn blood by Ficoll-Hypaque gradient centrifugation (Pharmacia) and transformed with *H. saimiri* strain C488 as described (Biesinger et al., 1992). The resulting cell line TS-HVS was cultivated essentially as described (Biesinger et al., 1992). EBV-LCL were established and cultivated following standard protocols (Hudson and Hay, 1989).

Establishment of T cell lines and TCC

Donor TS has been exposed extensively to *P. falciparum* and additionally was immunized with the synthetic peptide vaccine SPf66 (Patarroyo et al., 1988) as described (Alonso et al., 1994). He was a member of a group of non-immune Caucasians (group 1a) who had received three 0.5 ml subcutaneous immunizations with SPf66 within the framework of the KIVAC malaria vaccine trial (Teuscher et al., 1994). A fourth immunization was done four years later (ethical clearance was granted by the responsible ethical committee of the department of Internal Medicine, Kantonsspital, Basel). TS was typed for HLA class II expression as DRB1*0801/DRB1*1201/DRB3*0202; DPA1*0103/DPA1*02011; DPB1*02012/-DPB1*11011. For molecular typing, the relevant exons of HLA-DRB, -DPA1 and -DPB1 were amplified by PCR from genomic DNA, cloned and sequenced as described (Rozemuller et al., 1995; Bugawan et al., 1990; Tiercy et al., 1990; Nino-Vasquez et al., 1999). For the generation of T cell line TS-A, PBMC of donor TS were isolated six weeks after the fourth immunization by density gradient centrifugation on Ficoll-Hypaque (Pharmacia). Bulk cultures were set up in 24 well plates using 2×10^6 PBMC together with 20 µg/ml of SPf66 or MSP 1^{38-58}, respectively. Culture medium (CM) consisted of RPMI 1640 supplemented with

10 % heat-inactivated pooled human AB serum (Haenecke AG, Germany), 10 mM HEPES buffer, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin (Gibco-BRL) and 50 µM 2-ME. After 8 days of incubation, 1 x 10^6 blasting cells were restimulated with 1 x 10^6 irradiated (30 Gy) autologous PBMC and the peptide antigen at 20 µg/ml in 24 well plates. After two rounds of restimulations, the specificity of the resulting T cell lines was tested in proliferation assays. T cell line TS-B was established similarly as TS-A except that blasting cells were restimulated with 1 x 10^5 irradiated HVS-transformed T cells instead of PBMC.

TCC were generated from the antigen-specific T cell lines by limiting dilution using either TS-HVS or autologous PBMC as APC. Briefly, TS-HVS (5 x 10^3 cells/well) and irradiated allogeneic PBMC as feeder cells (1 x 10^4 cells/well) or autologous PBMC (2 x 10^4 autologous PBMC/well) were co-cultured with T cells seeded at 50, 5 and 0.5 cells/well in 96-well round-bottom plates plus 20 µg/ml of the relevant antigen and 20 U/ml rhIL-2. Growing cells were expanded in CM supplemented with rhIL-2, transferred to 24-well plates and restimulated with autologous EBV-LCL. Culture wells containing irradiated allogeneic feeder cells without TS-HVS did not support the outgrowth of TCC. Clones were maintained in culture by weekly restimulation with autologous EBV-LCL and the antigen. Antigen-specificity of TCC was determined using irradiated PBMC, EBV-LCL and TS-HVS, respectively, as APC and the relevant antigens. The probability of clonality of the established TCC was calculated according to the Poisson distribution. Clones were designated as follows: TS nominates the donor, S and M stands for the antigens SPf66 or MSP-1 $^{38-58}$, respectively, and P and H stands for PBMC or TS-HVS used as APC for TCC establishment, respectively.

T cell stimulation assays and MHC restriction analysis

For T cell stimulation assays, 2 x 10^4 cloned T cells were co-cultured with autologous irradiated PBMC (2 x 10^4 cells /well), TS-HVS (5 x 10^3 cells/well) or EBV-LCL (5 x 10^3 cells/well) together with the antigen (20 µg/ml) for 3 days. In the case of clone TS-M8P whose proliferation and incorporation of radioactivity was strongly dependent on the presence of exogenous rhIL-2 (20 U/ml) was added during the last 24 h to cultures. All proliferation assays were performed in triplicate and during the last 18 h ^{3}H-thymidine (1 µCi/well, Amersham International) was present. The cultures were harvested by an automated harvesting device (Inotech AG, Switzerland) and assayed for ^{3}H-thymidine incorporation by liquid scintillation counting with an LKB- Wallac counter (LKB, Sweden). Data are expressed as mean cpm of triplicate cultures or as stimulation index (SI), which was calculated by the

following equation: SI = mean experimental cpm with Ag / mean control cpm without Ag. The restriction element of a particular TCC was determined in proliferation assays using HLA-DR matched homozygous EBV-LCL as APC in combination with mAb specific for HLA-DR (L243), HLA-DQ (SPV-L3), HLA-DR/DQ/DP heterodimers (HB145) or MHC class I (W6/32). Homozygous EBV-LCL Herluf (IHW 9299) and BM9 (IHW 9068) which are part of the XIIth International Histocompatibility Workshop Cell Panel were received from the European Collection of Animal Cell Cultures, Salisbury, UK. Isotype matched hybridoma cell supernatants of irrelevant specificity were incorporated in control wells. The APC and T cells were incubated for 30 min with an appropriate dilution of hybridoma cell culture supernatant prior to addition of the antigen.

Flow cytometry

TS-HVS was stained using standard procedures with appropriately diluted cell culture supernatants containing mouse mAb specific for HLA-class I (W6/32), HLA-DR (L243), HLA-DQ (SPV-L3), HLA-DP (B7/21) or human CD58 (Pharmingen). As a secondary antibody, appropriately diluted FITC-conjugated goat anti-mouse Ig antibody was employed (Sigma). For the analysis of TCRVβ17, TCRVβ13.1, CD4, CD80 and CD154 expression, FITC-labeled mAb (Pharmingen) were used. Ten thousand events were collected for each sample on a FACScan flow cytometer (Becton Dickinson) and analyzed using CellQuest software (Becton Dickinson).

Cytokine detection at the single cell level

T cells were stimulated with 10^{-7} M PMA plus 1 μM ionomycin (Sigma) for 4 h. Monensin (Sigma) was present during the last 2 h at 6 μM. Cells were fixed with freshly prepared 4 % paraformaldehyde in HBSS, permeabilized with HBSS containing 1 % BSA and 0.5 % saponin and stained with FITC-labeled mouse anti-human INF- γ and PE-labeled mouse anti-human IL-4 mAbs (Pharmingen). Ten thousand events were collected for each sample on a FACScan flow cytometer (Becton Dickinson) and analyzed using CellQuest software (Becton Dickinson).

Sequence analysis of T cell receptor transcripts

TCR gene expression was analyzed by RT-PCR amplification of TCR transcripts as described (Moonka and Loh, 1994) and DNA sequence analysis of PCR products was performed using an ABI Prism 310 genetic analyzer (PE Biosystems, Foster City, USA). Sequence data were collected using the ABIPrism Data collection software and further processed with the Sequence Navigator program (PE Biosystems). Sequences were analyzed using software coming from the server of IMGT (http://imgt.cines.fr:8104; initiator and coordinator: Marie-Paule Lefranc, Montpellier, France), the international ImMunoGeneTics database (Lefranc, 2001).

Results

Phenotype of HVS-transformed cells and isolation of MSP-1$^{38-58}$-specific T cell lines

A HVS-transformed T cell line named TS-HVS was generated from donor TS and analyzed for surface expression of molecules implicated in antigen presentation and T cell co-stimulation by flow cytometry. Fig. 1 demonstrates that TS-HVS expressed MHC class I, MHC-DR, -DP and -DQ molecules, the adhesion molecules ICAM 1 and CD4, and the co-stimulatory molecules CD80 and CD154. These results confirmed findings with other *H. saimiri*-transformed T cell lines (Meinl et al., 1995).

From donor TS, two T cell lines, TS-A and TS-B, were established by three rounds of *in vitro* restimulations with a peptide derived from MSP-1 of *P. falciparum* (peptide MSP-1$^{38-58}$) using autologous PBMC or TS-HVS as APC, respectively. When the specificity of the resulting T cell lines for MSP-1 $^{38-58}$ was tested in proliferation assays using PBMC and MSP-1$^{38-58}$ peptide, TS-A and TS-B displayed SI of 87 and 60, respectively. These results demonstrated that HVS-transformed T cells support the establishment of peptide-specific T cell lines *in vitro*.

Antigen-presentation by HVS-transformed T cells

In a subsequent step, we explored the capacity of TS-HVS to replace PBMC as APC for the isolation of human antigen-specific TCC by limiting dilution. Line TS-A was cloned using either autologous PBMC or TS-HVS in the presence of rhIL-2 and MSP-1 $^{38-58}$ peptide. Cells growing from wells seeded with 0.5 cells/well were expanded by regular restimulations using autologous PBMC, rhIL-2 and the MSP-1 $^{38-58}$ peptide. From 1000 culture wells each set up with PBMC or TS-HVS cells, 29 and 5 antigen-specific TCC could be recovered, respectively. All TCC expressed the surface markers CD3 and CD4 as shown by antibody staining and FACS analysis (data not shown). Activation of TCC was dose-dependent with respect to the peptide present in culture wells. For a selected group of TCC results are presented in Fig. 2.

In order to investigate whether both sets of MSP-1 $^{38-58}$-specific TCC express a comparable range of TCR, we amplified and sequenced TCR transcripts. Seven different TCR alpha/beta chain combinations were found in 12 randomly selected MSP-1 $^{38-58}$-specific clones generated with PBMC as APC. This indicates that line TS-A was composed of a range of different antigen-specific TCC at the time it was cloned.

The two TCRA - TCRB combinations found among the five clones stimulated with TS-HVS, were also found among the TCC generated with PBMC as APC. Hence, clonally related cells were recovered from cell line TS-A using two different types of APC.

A selection of TCC was analyzed in more detail for MHC class II restriction. These experiments revealed that both HLA-DR and HLA-DP restricted clones were present among the MSP-1^{38-58}-specific TCC. Restimulation of TCC with the homozygous EBV-LCL Herluf and BM9 in combination with antibody blocking experiments allowed a further confinement of the restriction elements employed. The majority of TCC were restricted by HLA-DPA1*0103/DPB1*02012, while others were restricted by HLA-DRB1*0801 (data not shown).

Isolation of vaccine-specific TCC

As a next step we established a protocol allowing for the generation and characterization of vaccine-specific TCC requiring one single blood donation of 20 ml. This analysis was conducted with the 45 amino acid encompassing peptide vaccine SPf66 (Lopez et al., 1994). T cell line TS-S was established from PBMC by two rounds of *in vitro* restimulation with autologous PBMC and SPf66. For this step about 10^7 PBMC equivalent to 10 ml of peripheral blood were required. For the subsequent cloning and expansion steps HVS- and EBV-transformed cell lines were used. These can be established with 5×10^6 PBMC equivalent to 5 ml of peripheral blood. The SPf66-specific T cell line TS-S was restricted by HLA-DR, as shown by antibody blocking experiments and displayed a SI of 21 in proliferation assays (data not shown). TCC were generated from TS-S by limiting dilution with a multiplicity of 0.5 cells/well using either PBMC or TS-HVS cells as APC. From 1000 culture wells stimulated with PBMC, 12 TCC were recovered, while from the cultures stimulated with TS-HVS cells 19 TCC could be recovered. Thus the cloning efficiencies were comparable. The probability of clonality in both groups was calculated according to the Poisson distribution as 97.9 % and 96.8 %, respectively. Once established, TCC were further expanded by regular restimulations using autologous EBV-LCL, rhIL-2 and the antigen, since EBV-LCL can in to contrast to HVS-transformed cells be cultivated without continuous supply with exogenous rhIL-2. After a phase of expansion, TCC were tested for their antigen-specificity. 6/12 (50 %) of the TCC established by stimulation with PBMC were antigen-specific while 13/19 (68%) of the TCC generated by stimulation with TS-HVS were antigen-specific. The rest of TCC were stimulated by APC in the absence of antigen. Except for one TCC (TS-S39H) all were barely stimulated when incubated with antigen alone, demonstrating a limited capacity for auto-

presentation (Table 1). All TCC expressed the CD3 and CD4 surface molecules as demonstrated by antibody staining and FACS analysis (data not shown). TCC established on TS-HVS were readily restimulated by antigen using PBMC as well as EBV-LCL as APC (Table 1). The blockage of proliferative responses of TCC ranged from 70 % to 100 % by addition of mAb specific for HLA-DR and from 1 to 13 % by inclusion of mAb specific for HLA class I molecules (Table 1). A more detailed analysis of the restriction element employed by the three clones TS-S8P, TS-S9P and TS-S11P with homozygous EBV-LCL in combination with mAb blocking suggests that TS-S8P and TS-S9P are restricted by HLA-DRB1*0801, while TS-S11P is restricted by HLA-DRB1*1201 (data not shown).

Cytokine production of vaccine-specific TCC

HVS-transformed T cells produce INF-γ due to auto-stimulation via CD2 (Biesinger et al., 1992). While irradiated PBMC produced less than 20 pg/ml of INF- γ, cell line TS-HVS produced 1000 pg/ml of INF- γ before irradiation and 750 pg/ml INF- γ after irradiation with 30 Gy in a ^{60}Co-source as measured by ELISA in tissue culture supernatants (data not shown). In order to investigate whether this difference in INF- γ concentrations present during the limiting dilution would favor the outgrowth of TCC expressing a particular cytokine pattern, the production of IL-4 and INF- γ was determined by intracellular staining. TCC established on PBMC and TS-HVS displayed comparable cytokine profiles and the INF- γ enriched environment present during cultivation did not influence the cytokine expression pattern of the established clones (Fig. 3). Except for TS-S11P, which produced IL-4 and INF- γ, all other TCC produced only IL-4.

Discussion

We propose here a widely applicable protocol for the establishment and maintenance of antigen-specific TCC starting with roughly 2 x 10^7 PBMC. Firstly, an antigen-specific T cell line is raised using autologous PBMC as APC. This cell line is cloned subsequently by limiting dilution using HVS-transformed cells as APC in the presence of rhIL-2 and antigen. Outgrowing cultures are further propagated with EBV-LCL expressing MHC class II molecules of interest. We used this approach since humans are normally not exposed to *H. saimiri*. In contrast, common exposure to EBV results eventually in the outgrowth of EBV-antigen specific T cells, when EBV-LCL are present in early steps of the isolation of TCC. Therefore, use of HVS-transformed cells as APC during the establishment of antigen-specific T cell lines and the limiting dilution step avoids *in vitro* restimulation of virus-specific T cells. EBV-LCL were employed for later restimulation of established TCC since HVS-transformed cells can be maintained only in the presence of 40 - 80 U/ ml of rhIL-2.

In contrast to mouse T cells, activated human T cells synthesize and express MHC class II molecules (Ko et al., 1979) and it has been suggested that the role of antigen presentation by activated T cells to T cells is to augment immune responses (Barnaba et al., 1994). Antigen presentation by T cells may, however, also lead to T cell anergy upon subsequent antigenic restimulation in the presence of professional APC in both human and rat T cells (LaSalle et al., 1992; Lombardi et al., 1996). However, TCC established here on TS-HVS were not anergic but were restimulated by PBMC or EBV-LCL (Table 1). When panels of TCC generated either on PBMC or TS-HVS cells were restimulated with PBMC, both groups showed comparable level of incorporation of radioactivity (Table 1).

PBMC and TS-HVS supported the outgrowth of TCC expressing identical TCR rearrangement combinations from a heterogeneous T cell line specific for MSP-1 $^{38-58}$. This indicates that both APC provide a comparable density of MHC-peptide complexes and levels of co-stimulatory signals.

It is well known that the use of alum as adjuvant is associated with the induction of a TH2 response (Bomford, 1980). Therefore, the finding that the TCC elicited by vaccination with the alum adjuvanted peptide vaccine SPf66 predominantly produced IL-4 can be explained by the adjuvant used for vaccination. Recently, it has been demonstrated that the culture of human Th2 cells from allergic individuals with allergen, IL-2 and INF- γ was able to convert the responses from Th2 to Th1 upon *in vitro* restimulation (Parronchi et al., 1999).

Therefore, we compared the cytokine production of clones established on PBMC (low INF- γ environment) with clones established using TS-HVS (high INF- γ environment). We could not detect differences in the cytokine production in the panel of TCC specific for SPf66 (Figure 3). These data support the notion that the interleukin profile of human TCC in culture is normally stable and it is not easy to reverse Th2 to Th1 cells (Coffman et al., 1999).

MSP-1 of *P. falciparum* is a promising malaria vaccine candidate (Engers and Godal, 1998; Genton et al., 2000). Our data demonstrate for the first time that the N-terminus of MSP-1 contains an epitope presented in the context of HLA-DR and HLA-DP molecules. Furthermore, these MHC-peptide complexes were stimulatory for human TCC expressing at least 7 different TCRA - TCRB rearrangement combinations. The phenomenon of different TCR recognizing the same MHC-antigen complex is known as synonymous TCR (Janeway, 1998) and has been described for other epitopes (Ding et al., 1998; Wienhold et al., 2000). Other T cell epitopes presented both by HLA-DP and HLA-DR molecules have been described (Higgins et al., 1994). It is not known whether such epitopes are particular immunogenic in human populations.

Taken together, our data demonstrate that TCC raised using either PBMC or HVS-transformed T cells as APC had comparable properties with respect to TCR repertoire, cytokine profile and responsiveness to restimulation with EBV-LCL and PBMC. During the course of clinical intervention trials, when only small volumes of peripheral blood are available, the described T cell cloning procedure will allow clonal analyses of the fine specificity, diversity and cytokine production of cellular immune responses.

Figure legends

Fig. 1. Flow cytometric analysis of the phenotype of the *H. saimiri*-transformed cell line TS-HVS. The filled graphs represent the staining with specific mAbs while the open graphs represent staining with the secondary mAb only.

Fig. 2. Stimulation of TCC depends on concentrations of antigen present in culture wells. TCC TS-M15P (●), TS-M33P (◆) and TS-M82P (▲) (2 x 10^4 cells/well) were stimulated with autologous PBMC (2 x 10^4 cells/well) and graded concentrations of MSP-1 $^{38-58}$ peptide for 3 days. During the last 18 h ^{3}H-thymidine was present. The results are given as SI.

Fig. 3. Analysis of the intracellular cytokine production of SPf66-specific TCC. Cells were stimulated with PMA and ionomycin for 4 h and INF- γ and IL-4 production was measured by intracellular staining with cytokine specific mAb. Panel A shows stainings with isotype control mAbs and panel B stainings with specific mAbs.

Table 1

MHC restriction and restimulation of SPf66-specific TCC raised on TS-HVS and PBMC. TCC (2 x 10^4 cells/well) were stimulated either with PBMC (2 x 10^4 cells/well) or autologous EBV-LCL (5 x 10^3 cells/well) and SPf66 (20 μg/ml) for 72 h. During the last 16 h 1 μCi/well ^{3}H-thymidine was present. MAb (cell culture supernatant at a 1:5 dilution) was added to certain cultures to establish MHC restriction. Mab W6/32 and L243 are specific for MHC class I and HLA-DR molecules, respectively. TCC were not stimulated by the presence of SPf66 without APC. TS-S8P depended on the presence of exogenous rhIL-2 for the last 24 h of cultivation. N.d.: not determined. The results are presented as mean cpm of triplicate cultures. SD of cpm < 10 %.

Figure 1

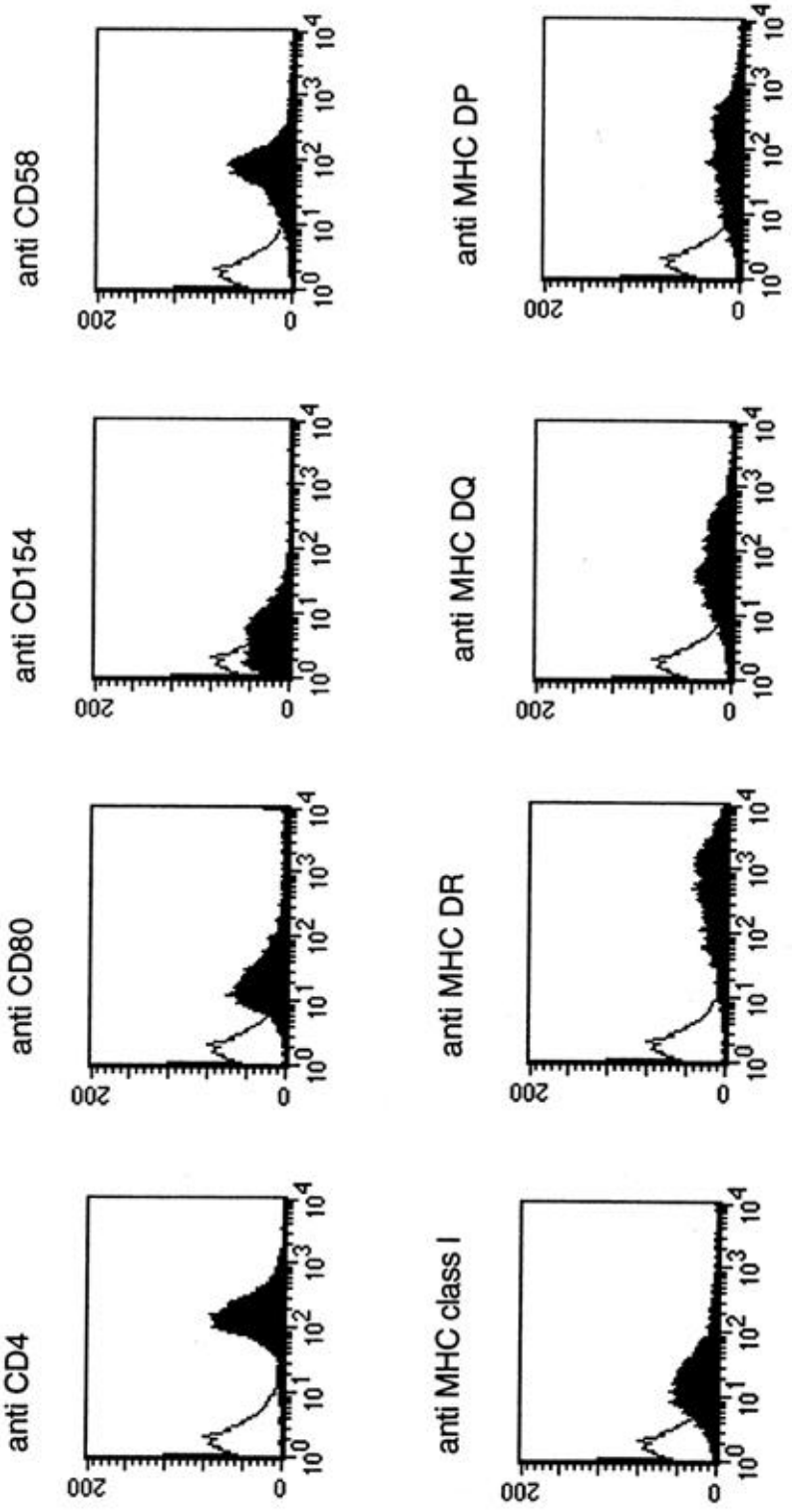

anti CD58
anti CD154
anti CD80
anti CD4
anti MHC DP
anti MHC DQ
anti MHC DR
anti MHC class I
200
0
10^0
10^1
10^2
10^3
10^4

Figure 2

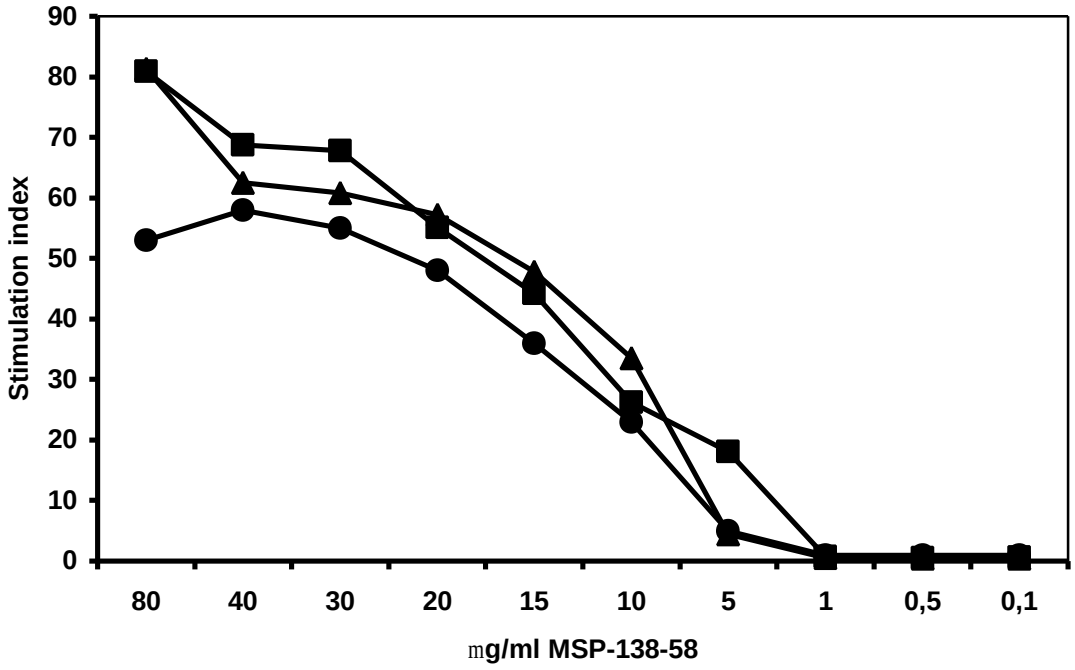
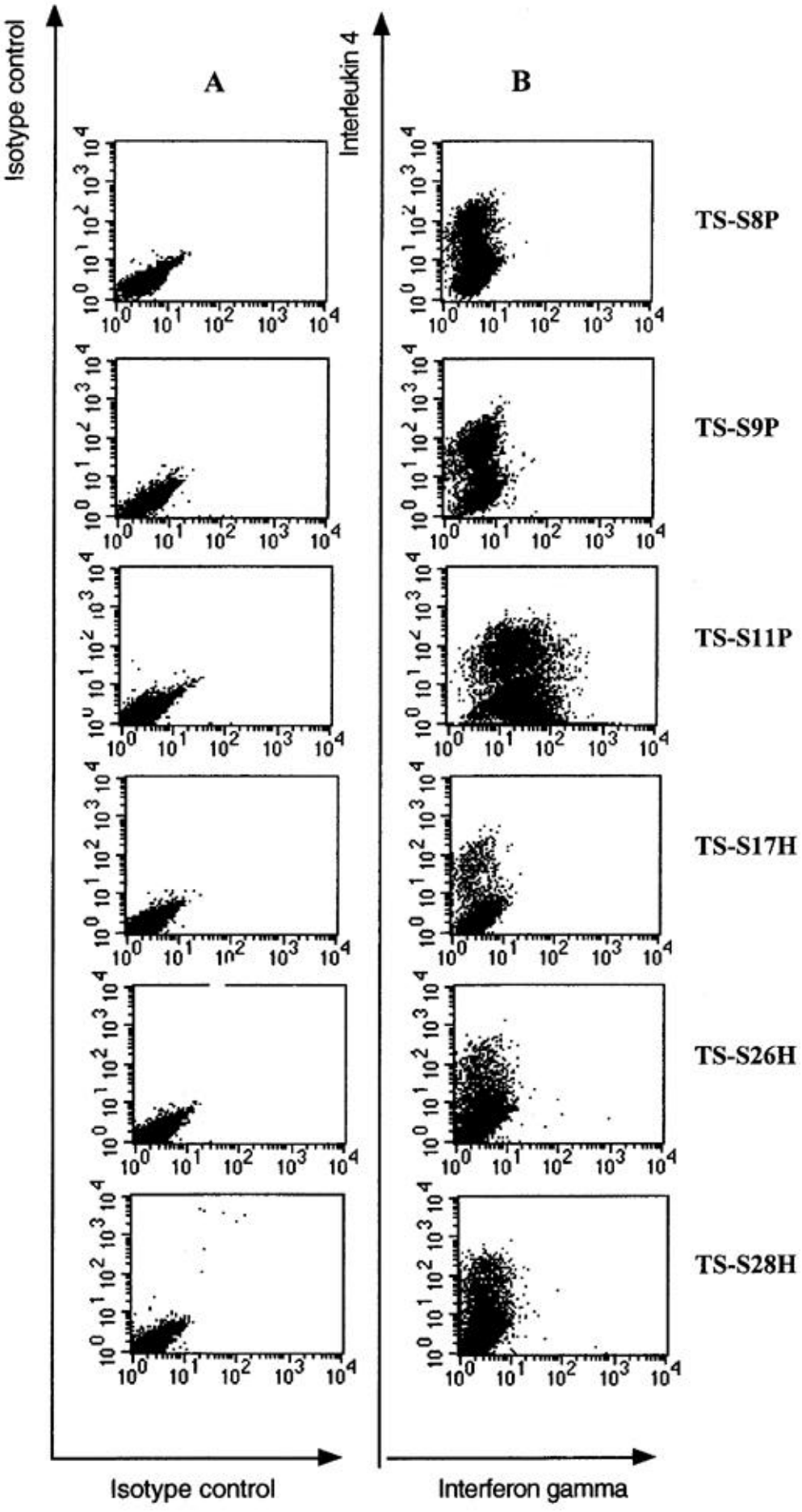


Fig. 2. Stimulation of TCC depends on concentrations of antigen present in culture wells. TCC TS-M15P (●), TS-M33P (◆) and TS-M82P (▲) (2 x 10^4 cells/well) were stimulated with autologous PBMC (2 x 10^4 cells/well) and graded concentrations of MSP-1$^{38-58}$ peptide for 3 days. TS-M15P, TS-M33P and TS-M82P remained unstimulated in the absence of APC (open symbols). During the last 18 h ^{3}H-thymidine was present. The results are given as SI.

Figure 3


A
B
Isotype control
Interleukin 4
TS-S8P
TS-S9P
TS-S11P
TS-S17H
TS-S26H
TS-S28H
Isotype control
Interferon gamma

Restimulation of TCC with	TCC							
	TS-S17H	TS-S26H	TS-S28H	TS-S39H	TS-S52H	TS-S8P	TS-S9P	TS-S11P
	mean cpm of triplicate cultures							
PBMC alone	2922	3138	1224	1159	4710	175	810	156
SPf66 alone	5400	5196	3013	14793	3729	166	796	801
SPf66 + PBMC	41448	39607	37848	29969	42218	2524	9018	14777
SPf66 + PBMC + anti-DR	13630	10764	4870	5445	3973	n.d.	n.d.	n.d.
SPf66 + PBMC + anti-class I	41047	37255	33042	29078	39669	n.d.	n.d.	n.d.
EBV-LCL alone	4444	4513	1812	2946	2337	145	508	110
SPf66 + EBV-LCL	30403	69528	31079	24263	31739	2666	2616	13773
SPf66 + EBV-LCL + anti-DR	8634	9962	1706	5015	4870	n.d.	n.d.	n.d.
SPf66 + EBV-LCL + anti-class I	28569	68505	279221	24289	29250	n.d.	n.d.	n.d.

Table 1. MHC restriction and restimulation of SPf66-specific TCC raised on TS-HVS and PBMC. TCC (2 x 10^4 cells/well) were stimulated either with PBMC (2 x 10^4 cells/well) or autologous EBV-LCL (5 x 10^3 cells/well) and SPf66 (20 µg/ml) for 72 h. During the last 16 h 1 µCi/well ^{3}H-thymidine was present. MAb (cell culture supernatant at a 1:5 dilution) was added to certain cultures to establish MHC restriction. Mab W6/32 and L243 are specific for MHC class I and HLA-DR molecules, respectively. TCC were not stimulated by the presence of SPf66 without APC. TS-S8P depended on the presence of exogenous rhIL-2 for the last 24 h of cultivation. N.d.: not determined. The results are presented as meancpm of triplicate cultures. SD of cpm < 10 %.

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6. Safety, Tolerability and Immunogenicity of New Formulations of the Plasmodium falciparum Malaria Peptide Vaccine SPf66 combined with the immunological adjuvant QS-21

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Abstract

SPf66 is a synthetic malaria peptide vaccine, which has been widely tested in combination with aluminium hydroxide (alum) as the adjuvant. Since this formulation is weakly immunogenic, we sought to improve its immunogenicity by using the saponin adjuvant QS-21. SPf66/QS-21 vaccines were evaluated for safety, tolerability and immunogenicity in healthy adults. The vaccines were found to be safe in 87/89 (97.8%) volunteers studied. However, 2 individuals developed severe vaccine allergy following the third dose of 1 of the 3 SPf66/QS-21 formulations tested. Vaccine formulations containing QS-21 induced a 45- to over 200-fold increase in anti-SPf66 IgG titres over the alum formulation after the second and third doses, respectively. Anti-SPf66 antibody from some subjects reacted against asexual blood stage parasites, as demonstrated by immunofluorescence and immunoblotting. Antibody responses generated by the QS-21 formulations were of longer duration compared to those evoked by the alum formulation. These observations demonstrate that the use of QS-21 can substantially enhance the immunogenicity of peptide vaccines, such as SPf66.

Introduction

The search for a malaria vaccine, especially one against *Plasmodium falciparum*, has been fuelled in recent years, owing to increased resistance of the parasite to anti-malarial drugs, and that of the vectors to most insecticides (1). These factors have reinforced the view that a vaccine against *P. falciparum* is an urgently required tool for the prevention and control of malaria world-wide and especially in sub-Saharan Africa (2).

The malaria vaccine SPf66 was developed by Patarroyo and co-workers (3, 4). SPf66 consists of a polymeric synthetic peptide incorporating amino acid sequences derived from three proteins isolated from *P. falciparum* infected erythrocytes. The peptide epitopes are linked by Pro-Asn-Ala-Asn-Pro (PNANP) sequences from the *P. falciparum* circumsporozoite protein (CSP) repeat unit, and all four peptide sequences are assembled into a 45-amino acid long monomer unit. The monomer has cysteine residues added at the C- and N-terminal ends to allow for polymerisation. Current SPf66 vaccine formulations use aluminium hydroxide (alum) as the adjuvant.

Several malaria vaccines are under clinic al development, and a few have reached advanced evaluation in field trials in humans (2). The SPf66 vaccine is the only candidate to have been evaluated extensively for safety, immunogenicity and efficacy in several countries, including Colombia (5-7), Ecuador (8), Venezuela (9), Tanzania (10-12), The Gambia (13), Thailand (14), and Brazil (15). Results from all these trials have shown the alum-formulated vaccine to be safe in malaria-naïve adults (10, 16), and semi-immune and immune children and adults (5-14). Efficacy evaluation of the vaccine has nevertheless led to mixed results, demonstrating some effects against *P. falciparum* malaria in some of the major trials (17), while producing no significant effect in Gambian and Tanzanian children under one year of age (12, 13) and in older children from Northern Thailand (14). Interestingly, a reduction in multiple infections was found in asymptomatic vaccine recipients compared with those in asymptomatic placebo recipients (18). This observation was corroborated in Gambian children (19).

One commonality to all SPf66 vaccine trials has been the observation that the alum-adsorbed SPf66 vaccine is poorly immunogenic in humans. For instance, induction of measurable levels of antibody against SPf66 often requires a three-dose regimen with a large antigen dose of 2 mg for each adult immunisation. Moreover, the antibody levels evoked by vaccination are generally of short duration (16), returning to baseline levels within 15–24 months following the immunisation (11,14,16). Also, SPf66-specific T cell responses tend to

be very low (20). There are a number of reasons that could explain the mixed efficacy results observed with SPf66/alum vaccines. However given the observation that modest immune responses to SPf66 resulted in promising, although partial efficacy in some studies, we hypothesised that improving the immune responses to SPf66 could result in improved and more consistent immune protection. This has provided us with an incentive for replacing alum with a more potent adjuvant, QS-21 and analysing the immunogenicity of various formulations of QS-21-adjuvanted SPf66 vaccines

QS-21 has been evaluated for adjuvant effects when combined with a variety of antigens, and has been shown to enhance immune responses to subunit vaccines in laboratory animals (21) and humans (22-26). In human challenge studies of the *P. falciparum* CSP vaccine RTS,S (24), the addition of QS-21 to a vaccine formulation markedly improved the vaccine immunogenicity, and enhanced the vaccine efficacy, resulting in 50-80% protection against experimental challenge with *P. falciparum* sporozoites (24, 27). QS-21 has also been shown to enhance the antibody responses against a *P. falciparum* CSP-derived multiple antigen peptide (MAP) vaccine in healthy adults (28). The addition of QS-21 to alum-precipitated MAP vaccines induced higher levels of IgG anti-MAP antibody compared to the alum-bound preparation. Our recent experiments in *Aotus nancymai* monkeys immunised with SPf66 vaccines formulated with either alum or QS-21 showed a higher degree of protection in animals administered QS-21-containing vaccines than those immunised with alum-bound peptide (unpublished results) , suggesting that an SPf66 vaccine formulated with QS-21 may be highly desirable for effective immunisation of individuals living in areas where malaria is endemic. These observations provided an incentive for the assessment of the safety, tolerability and immunogenicity of QS-21-adjuvanted SPf66 vaccines. We report here the results of the first phase I clinical trial of the SPf66/QS-21 formulations in healthy adults from Colombia.

Materials and methods

Volunteers

A total of 120 male volunteers aged 18 to 23 years at entry were screened for eligibility, and 99 evaluable subjects were enrolled based on inclusion and exclusion criteria specified in Study Design. All subjects were recruited by non-coercive means under a protocol approved by the Colombian armed services institutional review board (IRB).

Study Design

The study was designed as a randomised, double-blinded, placebo-controlled Phase I trial of the safety, tolerability and immunogenicity of a 2-mg dose of SPf66 formulated with either alum, (standard vaccine or group A), 50 µg of QS-21 (group B), 100 µg of QS-21 (group C) or a combination of alum and 100 µg QS-21 (group D). Subjects in the placebo group were given 50 µg of QS-21 alone (group E).

The trial's main objectives were to determine whether 3 subcutaneous doses of SPf66 vaccines were well tolerated, and whether the addition of QS-21 to the formulations would result in induction of anti-SPf66 IgG titres at least twice as high as those seen among recipients of the alum formulation. Safety and tolerability were judged by the absence of significant haematological and biochemical abnormalities, and lack of serious adverse effects.

Participants were recruited from the Colombian Navy personnel in Tumaco by non-coercive means under international scientific and ethical standards embodied in the Declaration of Helsinki (29), and International Conference on Harmonisation guidelines on technical requirements for the registration of pharmaceuticals for human use (30). Study subjects were provided verbal explanations on the goals, design, risks and conduct of the trial. Participation in the study was conditional upon voluntary acceptance and signing of an informed consent form. Subjects were excluded if they had a history of allergic reactions to vaccination, vaccination with live virus within four weeks of the beginning of the trial; had undergone splenectomy; or had a history of malaria, malaria parasitemia, or malaria vaccination, atopy, immunosuppressive drug therapy such as steroids; abnormal haematology and/or blood chemistry values. Volunteers were also excluded if they had positive markers of active auto-immunity; had cardiovascular, renal, and hepatic dysfunction or any other medical or psychiatric condition that in the opinion of the protocol chairman would compromise the patient's ability to tolerate the treatment. Furthermore, they were excluded if they were unwilling to give informed consent, or were willing to withdraw from the study. The study

protocol was approved by the appropriate IRB. The trial was conducted by Instituto de Inmunologia, Bogota and monitored by local and World Health Organisation-appointed clinical monitors. Good laboratory procedures and clinical-grade materials were used throughout the study.

Vaccine Formulations and Immunisation

All vaccines used in this study were based on clinical grade materials produced under Good Manufacturing Practices (GMP) in Colombia (SPf66) and USA (QS-21), and contained a fixed dose of SPf66 mixed with either aluminium hydroxide, 50 µg or 100 µg of QS-21 or a combination of alum and 100 µg of QS-21.

SPf66 peptide (Lot 15.5) was synthesised at Instituto de Inmunologia, Bogota, Colombia using methods reported previously (4). The finished product was characterised chemically by size exclusion chromatography, mass spectrometry, sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis, and immunologically by analysis of the antibody response against SPf66 in mice. Aluminium hydroxide (alum) was purchased from Superfos Biosector (Vedvaek, Denmark). QS-21 vials (Lot B6831, 0.5 mg/ml, 0.6 ml, phosphate-buffered saline (PBS), pH 6.8) were supplied by Aquila Biopharmaceuticals, Framingham, MA, USA.

The standard alum-bound SPf66 vaccine was supplied in ready-to-use 2-dose sterile vials (4 mg/ml on alum; 1 ml total volume). SPf66 vaccines containing QS-21 were prepared at the clinical site on the day of the immunisations. The vaccine for the SPf66/QS-21 groups (group B and C) and the SPf66/alum/QS-21 group (group D) were formulated from bottled SPf66 stocks (4 mg/ml in saline; 1 ml total volume), and ready-to-use SPf66/alum vials (4 mg/ml; 1 ml total volume), respectively. Appropriate volumes of SPf66, SPf66/alum and QS-21 were removed from the respective vials, and were added to empty sterile 2-ml borosilicate vials to prepare a 2-dose formulation (1.4 ml total volume) for each study subject in group B-D. Half millilitre and 0.7 ml of vaccine formulations were withdrawn into individual tuberculin syringes equipped with hypodermic needles, and were used for subjects in group A and groups B and C, respectively. Each vaccine dose consisted of 2 mg of SPf66 mixed with either 1.25 mg of alum (group A), 50 µg of QS-21 (group B), 100 µg of QS-21 (group C), and 1.25 mg of alum mixed with 100 µg of QS-21 (group D). The formulations were injected subcutaneously into alternate deltoid areas during dosings 1 through 3.

Subjects in group E were given 50 µg of QS-21 alone. These formulations were prepared from ready-to-use sterile QS-21 vials (0.5 mg/ml; 0.6 ml ; pH 6.8) and sterile

physiologic saline diluent. The appropriate volumes of QS-21 and saline were added to empty borosilicate vials to make 2 doses of QS-21 in 1.4 ml solution. A volume of 0.7 ml was drawn into a syringe, and was injected in the same way as the vaccines.

Three vaccinations were given on days 0, 30 and 180 as has been done routinely for SPf66 vaccine studies (7). Following each dose, all subjects remained at the clinic for a 60-minute period for assessment of local and systemic reactions, and were again examined at 24 hours, 48 hours, 72 hours, and on day 20 post vaccination.

Safety assessment

Assessment of safety included evaluation of the reactogenicity, tolerability and toxicity. Reactogenicity was assessed by observing the incidence and severity of local and systemic reactions within 1 hour, 24-48 hours, 72 hours and 20 days following the injections. Severity was rated as none, mild, moderate or severe for measurable outcome. A safety pattern for each formulation was determined by comparing the frequency and severity of the reactions between the various formulations, focusing on the comparative assessment of local reactogenicity between the standard vaccine, and the QS-21-containing vaccines. Assessment of local reactions included erythema, tenderness/induration, pain, local pruritus, arm motion limitation related to pain and presence of local adenopathy. Erythema, tenderness/induration were scored as none, mild (<30 mm in the largest diameter), moderate (30-120 mm in the largest diameter) and severe (>120 mm in the largest diameter). The limitation of the range of motion was considered a surrogate measurement of local pain, and was used to determine the severity of the local pain. Pain was scored as none or mild, if the range of motion was unchanged, and the subject could raise his arm to greater than a 90° angle, moderate if the subject could raise his arm to no more than 45- 90° angle, and severe if the subject could not raise his/her arm to a 45° angle. Systemic reactions were also rated, including allergic reactions such as rash/hives, generalised pruritus, bronchospasm, with or without hypotension, fever (<38°C or >38°), anorexia, vomiting, abdominal pain, malaise/dizziness, chills, diarrhoea, headache, hypotension, cyst/nodules, abscess, necrosis, axillary adenopathy, myalgia, arthralgia and arthritis.

Tolerability was defined as the effect of the injections on the subject's basic daily life activities such as eating, walking, lifting objects, etc. Tolerability data were entered at 4 category levels, and was rated as excellent if there was no reaction and the subject had normal physical activity; good if there were some reactions, but the subject still had normal physical activity; acceptable if there were some reactions, and the subject had reduced physical

activity; and finally minimal if there were some reactions, and the subject had severely impaired physical activity.

To monitor toxicity, blood specimens and sera were obtained from each subject at baseline, 48 hours and 20 days post vaccination for haematology and blood chemistry. The following laboratory parameters were analysed: white blood counts (WBC) with differentials, platelet counts, haemoglobin, blood urea nitrogen, creatinine, total protein, albumin, bilirubin (total, direct and indirect), alanine aminotransferase, aspartate aminotransferase.

Immunological analyses

Serum specimens for immunological analyses were collected at baseline, on day 20 after each dose, at approximately 4 months after the second dose (day 160 of the study) and 4 months after the third dose (day 300 of the study) for measurement of vaccine-induced immune responses. Serum samples obtained at baseline were also analysed in the context of a routine screening for non-malarial infections. Serological assays were performed for the detection of anti-SPf66 antibody as well as antibody against human immunodeficiency virus (HIV), hepatitis B surface antigen, hepatitis C virus, Chagas and syphilis (special consent forms and potential counselling were included). HIV, HBsAg, and HCV antibodies were detected by enzyme-linked immunosorbent assays (ELISA). Chagas antibodies were detected by indirect immunofluorescence assays (IFA). Appropriate positive and negative control sera were included in each assay.

Anti-SPf66 IgG, and IgG directed against the 83.1, 55.1 and 35.1 peptide building blocks of SPf66 were detected by Falcon Assays Screening Tests (FAST-ELISA, Becton Dickinson, New Jersey, USA) using synthetic polymeric peptides as reported elsewhere (31). Titres of SPf66-specific IgG were measured against the SPf66 lot 15.5 used for vaccination in this study. Titres were also measured against the reference SPf66 peptide lot 10.4. The antibody reactivity against these 2 lots was comparable. Therefore, only the reactivity against the peptide lot 15.5 is reported here. IFA was used to detect antibodies reactive against *P. falciparum* asexual blood stage parasites of the FCB-2 strain, and the assay was run as described previously (7). A subset of 40 sera was randomly selected and analysed by Western Blot for reactivity against parasite proteins using FCB-2 lysates as described (31). All the immunological analyses were conducted on samples obtained at baseline, on day 20 after each dose and at approximately 4 months after the second and third doses, respectively.

For a detailed analysis of cellular immune responses, blood specimens were obtained from four subjects (subjects 21, 29, 34 and 89) four months after the third immunisation .

These subjects were from group B (SPf66/50 μg of QS-21), and were selected based on high IgG titres in the presence (subjects 21 and 34) or absence (subjects 29 and 89) of allergic reactions. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized peripheral blood by Ficoll-Hypaque gradient centrifugation and cryopreserved. Limiting dilution microcultures were set up from cryopreserved PBMC in 96-well round-bottomed plates in doubling dilutions starting from 3 x 10^4 cells/well in 36 replicate wells with or without SPf66 (20 μg/ml). Culture medium consisted of RPMI 1640 supplemented with 10% heat-inactivated pooled human AB serum, 2 mM glutamine, 10 mM HEPES, and 100 U penicillin/streptomycin. Limiting dilution cultures were incubated for 7 days at 37 ^{0} C in a humidified atmosphere containing 5% CO_2. On day 7, proliferation of individual cultures was determined by addition of 1 μCi/well [^{3}H]thymidine (Amersham) for an additional 16 hours. The amount of radioisotope incorporated into cultures was determined by scintillation counting. The frequencies of proliferating cells were calculated according to the Poisson distribution relationship between responding cells seeded per well and the fraction of non-responding wells. Individual wells were considered positive only if their counts per minute exceeded the mean of 36 control wells containing PBMC without antigen by at least 3 standard deviations. Frequencies with $p<0.05$ were accepted as accurate.

T cell lines reactive to SPf66 were established from subjects 21, 29, 34 and 89 as follows: PBMC were adjusted to 1 x 10^6 /ml in RPMI 1640 supplemented with 10% heat-inactivated pooled human AB serum, 2 mM glutamine, 10 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco/Life technologies, Rockville, MD 20850). Cells were cultured in 24-well plates in the presence of 20 μg/ml of SPf66 for 8 days at 37^0 C, 5% CO_2. Blastoid cells were recovered and re-stimulated in complete medium at 1 x 10^6 cells/well in 24-well plates in the presence of 20 μg/ml SPf66 and 1 x 10^6 irradiated (30 Gy) autologous PBMC for additional 8 days. On day 2, interleukin-2 (IL-2) was added at a final concentration of 20 IU IL-2/ml. Antigen specificity of the resulting cell lines was assessed in a proliferation assay. 2 x 10^4 blastoid cells were co-cultured with 2 x 10^4 irradiated autologous PBMC with 20 μg/ml SPf66 for 3 days in 96-well round-bottom plates. For the last 16 h 1 μCi/well of [^{3}H] thymidine was added and incorporated radioactivity was measured with scintillation counting. Mean counts per minute (cpm) values of triplicate cultures were used for data analysis.

Surface phenotype of cell lines was tested by FACScan analysis using phycoerythrin (PE)-labelled anti-CD3 and fluorescein-isothiocyanate (FITC)-labelled anti-CD4 or anti-CD8

monoclonal antibodies (BD Pharmingen, Franklin Lakes, NJ 07417).

Statistical methods

All data were entered using Epi Info 6 software. The records were entered twice on an IBM compatible computer, and were checked for accuracy and consistency. The command merge of Epi info was used to compare databases and correct mistakes. Statistical analyses were performed by means of the Stata version 6.0 and Epi info version 6.02b CDC. Data were summarised by tabulation of the number of subjects (n), mean, median, standard deviation, and range (for continuous variables), and by tabulation of the number of subjects, and proportion (categorical data) by study groups, by dose, and by time point of collection. Geometric mean titres (GMT) of antibody raised against the vaccines were rounded to 2 significant figures. For those samples with undetectable antibody at 1:100 dilution, a titre of 1 was assigned. Because of the reduction of the sample size at various time points, the Barrett's test of homogeneity was performed to ensure that the sample sizes did not deviate unacceptably. A p value <0.05 was considered significant.

Results

Study groups and enrolment

One hundred-twenty subjects were screened for eligibility, and 99 were enrolled. Table 1 shows study groups, enrolment and baseline characteristics of the study subjects. All subjects were males, aged 18 to 23 years. The mean age for the groups was approximately 19 years. The mean haemoglobin, WBC and lymphocyte counts ranged from 12.84 to 13.93 g per dL; 7.95 to 8.43 x 10^3/µl, and 34.5 to 41.47%, respectively (Table 1). There were no statistical differences between the mean values of the various groups. Subjects' accountability and disposition appear in Table 2. Of the 99 enrolled subjects, 89 received the first dose, 85 subjects received the second dose, and 42 subjects were administered the third dose. Because of very high antibody titres observed in most subjects following the second dose of vaccine, and the potential for greater reactogenicity at the third dose of vaccine, it was agreed that only half of the subjects would initially be given the third dose, and that all immunisations would be discontinued should 2 or more serious adverse events occur. Two (both from group B (SPf66+50 µg of QS-21)) of the 42 subjects administered the third dose developed severe allergic reactions. Therefore, immunisations were discontinued for the remaining subjects. An additional 14 subjects enrolled in the trial were discontinued at various time points as shown in Table 2.

Safety assessment

Assessment of the vaccine safety included local and systemic reactogenicity, tolerability and toxicity. No serious adverse events were noted after the first two doses as shown in Table 3 and Table 4.

At the first dose, 30 of 89 subjects from all groups (33.7%) experienced mild to moderate local erythema. Of these 30 subjects, 25 had pain, and 11 had induration at 24-48 hours (Table 4). Induration was slightly more prevalent among recipients of alum-formulated vaccines than vaccines formulated with QS-21 alone. Indeed, induration was observed in 4/17 (23.5%) subjects immunised with SPf66 on alum (group A), and 5/20 (25%) recipients of SPf66 mixed with alum and 100 µg of QS-21 (group D) versus 1/20 (5%) subjects administered SPf66 plus 50 µg of QS-21 (group B), and 1/19 (5.3%) subjects administered the peptide mixed with 100 µg of QS-21 (group C) as shown in Table 4. There were no marked differences in the rate and severity of adverse events between the various formulations.

At the second dose, there were more reports of mild to moderate erythema and

induration in all groups, except for the adjuvant alone group (group E) (Table 4). Forty-eight of the 87 (55.2%) subjects developed erythema. Of these 48 subjects, 25 (52.1%) had induration, and 32 (66.7%) had pain. Again, no marked differences in reactogenicity were noted between the various groups. One subject administered SPf66/100 µg of QS-21 (group C) developed severe erythema and induration at 24-48 hours. The reaction resolved within 2 days without sequelae.

The third dose of QS-21-containing vaccines produced more local reactions and more severe systemic adverse events within the first hour following the vaccination than seen with the previous doses. Sixteen subjects from the various groups experienced mild to moderate induration. Of these 16 subjects, 3 (18.7%) had developed erythema within the first hour of the immunisation (Table 3). Five of 11 (45.5%) subjects in group B, 1/6 (16.7%) subjects in group C, 2/8 (25%) subjects in group D, and 1/9 (11.1%) subjects in group E developed contralateral induration. Four of the 5 subjects who had a contralateral reaction also developed mild localised pruritus. More than half of the subjects administered SPf66 preparations formulated only with QS-21 [(group B: 6/11 (54.5%) subjects; group C: 5/6 (83.3%) subjects)] developed some induration at 60 minutes after vaccination. In contrast, only 25% of subjects administered the alum-precipitated vaccine with or without QS-21 developed such reactions (Table 3).

Other local reactions such as nodules, adenopathy and limitation of the range of motion were infrequent. Subcutaneous nodules were detected at the injection site on day 20 following immunisation with alum-precipitated vaccines only. Indeed, a total of 13 subjects (4 from group A, and 9 from group D), had small nodules at the site of injection on day 20 following the first vaccination. At the second dose, 3 subjects each from group A and group D developed nodules at the injection site. One subject from group A had palpable nodules after the third dose. The nodules resolved completely within 72 hours in most subjects. Small adenopathies (<1 cm) were also infrequent, and were detected only in subjects administered QS-21-containing vaccines (groups B, C and D). These were noted at 24-48 hours in 1 subject after dose 1, 3 subjects after dose 2, and 1 subject after dose 3. The adenopathy resolved within 24-48 hours for most subjects, and waned completely by day 20-post vaccination.

Systemic symptoms were infrequent after the first 2 doses (Table 3 and Table 4), and consisted mainly of headache and myalgia. No systemic adverse event was noted following the first vaccination. At the second dose, 2 recipients of vaccines formulated with QS-21, one each from group B (1/11 subjects), and group C (1/6 subjects) experienced low-grade fever with chills with or without myalgia within 24-48 hours of the vaccinations (Table 4). The

reactions were short-lived and resolved within 24 hours. There were more severe systemic reactions noted within the first 60 minutes of the third dose administration than seen with the first 2 doses. Five subjects from group B (4/11; 36.4%) and group C (1/6; 16.7%) developed generalised itching, mainly without rash (4/5 subjects). Of the 4 subjects from group B who had generalised pruritus, one developed mild bronchospasm, and another one had hypotension and minor facial angioedema (Table 3). These 2 volunteers were treated with intravenous fluids, corticosteroids and antihistamines. They recovered within one hour, and returned to their normal activities. Because of the 2 allergic events, a decision was made to stop further immunisations after the first 42 volunteers had received the third dose.

Analysis of the vaccine tolerability showed all study formulations, with the exception of the SPf66/50 µg QS-21 preparation (group B), to be well tolerated by all subjects as shown in Table 5. Analysis of the toxicity results obtained after each dose showed no significant alteration in haematological and serum chemistry parameters.

Immunogenicity

Sera were obtained at baseline, on day 20 following each immunisation, and at 4 months after the second and third doses, respectively. The antibody response against SPf66 has been characterised in previous reports based on end-point titres, using 2-fold increments, as low/no response (ELISA titres $\leq$100), intermediate (ELISA titre: 200-800) or high (ELISA titre: =1600) (6). According to these criteria, all subjects, but one volunteer each from groups A (SPf66/alum) and C (SPf66/100 µg QS-21), had no pre-vaccination antibody titre (Table 6). After dose 1, 4/14 (28.6%) subjects from group A seroconverted (intermediate responses), while 8/17 (47.1%) subjects of group B (SPf66/50 µg QS-21) and 10/18 (55.6%) subjects of group C seroconverted with antibody responses characterised as intermediate or high (Table 6). Interestingly, the SPf66 vaccine formulated with both alum and QS-21 (group D) induced the highest seroconversion rate after a single dose (15/17 subjects). Nearly all recipients of QS-21 formulations seroconverted, and had a high antibody response after dose 2 (94.6 to 100%) and dose 3 (100%). In the alum group, the proportion of high responders after the second (53.4%) and third doses (37.5%) was lower than that observed with QS-21-containing vaccines after the second (53.4%) and third doses (37.5%).

Table 7 shows GMT of IgG against SPf66 in the various groups. Vaccines formulated with QS-21 (groups B-D) evoked IgG titres that were significantly higher than those achieved by the standard alum-precipitated vaccine (group A) after the second and third doses. GMT of anti-SPf66 IgG were 20,400 and 31,300 for formulations containing 50 µg and 100 µg of QS-

21, respectively, and only 1600 for the alum-based vaccine after the second dose ($p<0.001$). Levels of anti-SPf66 IgG peaked after the second dose for 3 of the 4 vaccine formulations (SPf66/alum; SPf66/50 µg QS-21, and SPf66/alum+100 µg QS-21), and could not be boosted further (Table 7). Moreover, formulations containing QS-21 induced long-lasting antibody responses that could be detected at high titres in 83.3 to 94.6% of subjects 4 months after the second dose (Table 6). Overall, QS-21-containing vaccines induced IgG titres that were at least 2 logs higher than those evoked by the standard alum vaccine after doses 2 and 3 (Figures 1 and 2).

To determine the specificity of the anti-SPf66 response for 3 of the 4 building blocks included in SPf66, ELISA designed to detect the reactivity against the 35.1, 55.1, and the 83.1 peptide sequences was performed. The anti-35.1, -55.1 and -83.1 IgG responses induced by 3 doses of the SPf66/alum (group A) formulation, and the SPf66 peptide mixed with 100 µg of QS-21 (group C) are shown in Figures 3, 4 and 5, respectively. Compared to alum, QS-21 enhanced significantly the titres of antibody against all 3 peptides, and induced a 1- to 2-log increase in anti-peptide titres after 2 or 3 vaccinations. Administration of the third dose boosted effectively the anti-peptide antibody response for both the SPf66/alum and the SPf66/100 µg QS-21 formulations.

In contrast to the antibody response against the immunogen, titres of merozoite-reactive antibody as determined by IFA were low in all groups (Maximum IFA titre: 320). Comparative analyses of the IFA-reactive antibodies between groups showed no differences after doses 1 and 2. However, when considered as a group after the third dose, sera from subjects administered SPf66/QS-21 formulations demonstrated more reactivity against *P. falciparum* FCB-2 blood stage parasites than sera from subjects given SPf66/alum formulations. Indeed, 9 of 24 (37.5%) subjects immunised with QS-21-containing formulations (groups B through D) were IFA-positive, as opposed to only 1 of 8 (12.5%) subjects given the alum-precipitated standard vaccine (group A) (Table 8).

Immunoblot analysis was performed on a subset of 40 subjects chosen at random from all groups, and focused mainly on demonstration of reactivity against the merozoite surface protein-1 (MSP-1). Previous studies using a monoclonal antibody against the *P. falciparum* MSP-1 protein as a control, have shown reactivity against the 195-kD *P. falciparum* MSP-1 protein in some recipients of the standard SPf66/alum vaccine (31). Similar reactivity with the 195-kD *P. falciparum* MSP-1 protein and its cleavage products were observed with sera of some of the recipients of the different vaccine formulations evaluated here (Fig. 6). Specificity for MSP-1 was demonstrated by pre-incubation of sera with a 45-amino acid long

synthetic peptide derived from the 83-kD fragment of MSP-1, which completely abrogated the reactivity against the 195-kD and 83-kD bands as shown in Figure 6. The immunoblot reactivity against these *P. falciparum* protein bands was lower in sera from subjects given the standard alum vaccine as compared to recipients of QS-21-containing formulations (data not shown). In addition to reactivity against MSP-1, staining of additional protein bands was observed with some sera.

Four subjects from group B (SPf66/50 µg QS-21) were selected for an analysis of the magnitude and nature of the vaccine-induced T cell responses against SPf66. These include the two individuals (subjects 21 and 34) with allergic events after the third immunisation and two other individuals from group B (subjects 29 and 89) who had developed comparatively high anti-SPf66 IgG responses, but no systemic symptoms after the third vaccination. PBMC were collected four months after the third immunisation and the frequency of SPf66-reactive T cells was determined by limiting dilution analysis. High frequencies (1/3753 and 1/6115) were found in both subjects with allergic events (Table 9). A comparatively high frequency (1/3038) was also observed in one of the 2 other subjects, while the frequency of SPf66-reactive T cells was low (<1/20000) in the fourth subject analysed (Table 9). These precursor frequencies were higher than those observed with the standard SPf66/alum vaccine in a previous trial in Switzerland (unpublished results).

For a preliminary analysis of the nature of the elicited cellular immune responses, T cell lines were generated from the 4 study subjects by repeated stimulation with SPf66 in the presence of autologous irradiated PBMC as antigen-presenting cells. A characterisation by flow cytometry revealed mixed populations of CD4+ and CD8+ T cells in all four subjects (Figure 7). The relative proportion of these T cell subtypes differed from line to line ranging from a clear dominance of CD8+ T cells in a line generated from PBMC of one of the subjects (subject 21) with allergic reactions to a dominance of CD4+ T cells in a line from the other subject (subject 34) with allergic reactions. Cell lines from the two individuals (subjects 29 and 89) who had not developed allergic reactions after vaccination exhibited an intermediate CD4+/CD8+ composition. The finding that the SPf66/QS-21 formulation has the potential to elicit SPf66-specific CD8+ T cell responses, is in contrast to our finding that vaccination with the standard SPf66/alum vaccine induced a predominantly CD4+ T cell response of the Th2 subtype in malaria-naïve Caucasians and semi-immune individuals from Africa (unpublished results).

Discussion

Previously studied SPf66 vaccines were formulated with alum, a generally weak adjuvant that acts primarily by favouring antigen deposition at the injection site, and by stimulating mainly a CD4-dependent Th-2 response (32). To induce measurable levels of antibody against SPf66, a large amount of antigen (2 mg) and a 3-dose regimen have generally been used with these alum-formulated vaccines. Clinical trials of SPf66/alum vaccines have established the safety of the vaccines in children and adults (7, 10, 12, 16). Most subjects given the alum formulations develop only mild local reactions (pain, erythema, and induration) that are self-limited. Unusual local side effects such as contralateral inflammation with or without pruritus have been observed in less than 1% of subjects enrolled in the macroTumaco field trial in Colombia (6), 3 of 15 subjects vaccinated with a US-manufactured peptide in the USA (16), and approximately 4% of immune children from Tanzania (11) and Thailand (14). These reactions have generally been observed after administration of the third dose of vaccine, and very rarely after that of alum alone (10).

Our results confirm these previous observations on SPf66/alum vaccines. The vaccines formulated with QS-21 were generally well tolerated by most of the subjects (Tables 3-5) and had a local safety profile comparable to that of the alum formulation, except for a slight increase in the incidence of contralateral inflammation after the third dose. These reactions occurred mainly among recipients of the SPf66/50 µgQS-21 vaccine (4/6 volunteers). Similar local reactions were reported in trials of other malaria peptide vaccines (Robert Edelman, personal communication), and do appear to be associated with certain malaria parasite epitopes. However, evidence points also to parasite-unrelated mechanisms since recipients of adjuvant alone formulations in previous SPf66/alum trials have experienced contralateral inflammation (6, 10). In this study 1 out of 11 subjects given QS-21 alone also developed contralateral inflammation. The mechanism of these reactions is not yet fully understood. The rapid onset of the reaction (within 5-15 minutes of the injection) and the frequent association with pruritus suggest an IgE-mediated immediate type I hypersensitivity. The finding of CSP-specific IgE in 2 subjects with contralateral inflammation and generalised pruritus following vaccination with *P. falciparum* CSP MAP vaccine lends support to a possible role for specific IgE (Edelman, personal communication). However, other mechanisms may be involved, especially when a contralateral reaction is not accompanied by pruritus, or when it occurs at the first dose, at the time when specific IgE has not been induced. Occasional reports of

contralateral reaction occurring after administration of adjuvant alone also suggest an antibody-independent mechanism.

In the study reported here, systemic reactions were infrequent, and the incidence and severity of the reactions were similar to those reported in previous trials of the SPf66/alum vaccine (6-14), and trials of other synthetic or genetically-engineered vaccines (33). However, 2 recipients of the SPf66/50 µg QS-21 vaccine formulation developed severe allergic reactions after the third dose. There were no such allergic reactions in subjects administered SPf66/ 100 µg of QS-21. The reactions were characterised by mild bronchospasm, generalised pruritus (one subject), facial angioedema with rash and hypotension (one subject). The symptoms resolved very quickly following systemic therapy with epinephrine and anti-allergy medication. Generalised pruritus, bronchospasm and hypotension were also reported in 0.28 % of subjects in another SPf66/alum trial in Colombia (6). The reactions associated with the alum-formulations were nevertheless milder than those seen in this study. Systemic allergies have been observed in association with other vaccines (34). Grotto et. al (35) have reviewed the literature on adverse events associated with yeast-derived HBV vaccines, and found evidence for local and systemic reactions such as anaphylaxis in a few subjects. Systemic allergic reactions have also been observed following administration of Japanese encephalitis virus (JEV) vaccines (36). The causal agents for the reactions are not yet known for SPf66, but have been well documented for both HBV and JEV vaccines (35). Gelatine was shown to induce anti-gelatine IgE antibody in 6 children who developed systemic allergic reactions, including one mild anaphylaxis following JEV vaccination in Japan (36). Preservatives such as thimerosal have been implicated in vaccine-induced allergies following administration of HBV vaccines (35). Both gelatine and thimerosal have not been used in association with SPf66 vaccines. Preliminary analyses of the cellular immune responses in the 2 subjects, who had developed severe allergy after 3 doses of SPf66/50 µg QS-21 (group B) revealed no consistent and unique feature, as compared to 2 other study group B volunteers without allergic reactions. However, 4 months after the third dose, frequencies of SPf66-specific T cells were still remarkably high in 3 of these 4 individuals analysed. Phenotypic characterisation of T cell lines from these 4 analyzed subjects suggests that QS-21 can support the stimulation both of CD4+ and CD8+ T cells in humans. In contrast to alum, this adjuvant has previously been shown to have a strong potential to support CD8+ cytotoxic T cell responses in mice (22).

Overall, the SPf66/alum, SPf66/100 µg QS-21, and SPf66/alum/100 µg QS-21 vaccine formulations were all well tolerated whereas the SPf66/ 50 µg QS-21 vaccine was not.

A main objective of this trial was to determine whether adding QS-21 to SPf66 formulations would improve the antibody response to SPf66. We measured anti-SPf66 IgG titres, and compared the titres between alum and QS-21 formulations. Our results show that QS-21 significantly enhances anti-SPf66 IgG titres, and the enhancement is seen with all QS-21 formulations, including the vaccine formulated with both alum and QS-21. Anti-SPf66 IgG titres were at least 2 logs higher among recipients of QS-21 formulations compared to subjects vaccinated with the alum formulation, after the second (GMT of SPf66/Alum: 1,600; GMT of SPf66/QS-21: 20,400 to 31,300), and the third doses (GMT of SPf66/Alum: 530; GMT of SPf66/QS-21: 25,700 to 31,300). The antibody titres after 3 doses SPf66/QS-21 were the highest titres ever seen with SPf66 formulations, and were at least 2 logs higher than GMT reported in previous trials in Colombia (GMT: 538) (unpublished results), and Tanzania (GMT: 2783) (11).

To determine the specificity of the anti-SPf66 response for 3 of the 4 SPf66 peptide building blocks, IgG titres against the 35.1, 55.1, and the 83.1 peptide sequences were analysed. Again, SPf66/QS-21 induced significantly higher anti-peptide IgG titres than the alum formulation after 2 and 3 doses. IgG titres against the 55.1 and the 83.1 peptides were higher than those against the 35.1 peptide. All vaccine formulations elicited little IFA reactivity against the *P. falciparum* FCB-2 blood stages after the first 2 doses. After the third dose, 9 of 24 (37.5%) subjects given SPf66/QS-21 formulations developed detectable IFA titres. Only 1 of the 8 subjects administered the SPf66/alum formulation had such antibody reactivity. The low IFA reactivity elicited by SPf66/alum vaccines has also been observed by Gordon et al (16), who reported that only 1 out of 15 subject administered SPf66/alum vaccine manufactured in the US developed anti- *P. falciparum* asexual blood stage antibodies. The incidence of IFA-reactive antibody has not been studied extensively in other SPf66 vaccine trials. Thus, evaluation of SPf66 vaccine performance based on IFA reactivity is difficult. We have performed immunoblot analyses to investigate further the vaccine-induced antibody reactivity with parasite proteins. We found that both alum and QS-21 formulations induced antibody reactive against several parasite proteins, including MSP-1. Specificity for MSP-1 was demonstrated by immunoadsorption with a soluble MSP-1 derived peptide. There was increased recognition of MSP-1 in subjects vaccinated with SPf66/QS-21 compared to SPf66/alum recipients. These results confirm earlier findings that SPf66 can evoke genuine anti-*P. falciparum* asexual blood stage antibodies in some vaccinated subjects (16), and establish the superior adjuvanticity of QS-21 over alum.

Another important goal for this trial was to examine the safety and immunogenicity of the new formulations in order to select a single formulation for further studies. Comparison of the immunogenicity of the 2 QS-21 doses (50 and 100 µg) show similar effects on antibody response after 2 doses (GMT: 24,550 vs. 25,000, respectively). However, after 3 doses, the 100-µg dose induced a significantly higher anti-SPf66 IgG titre (GMT: 16,980 vs. 38,900, respectively), and a more frequent IFA reactivity than the 50-µg dose. A further under performance of the 50-µg QS-21 was its lower level of tolerability when compared to the 100-µg QS-21 dose. Thus, the SPf66 formulation containing 100 µg of QS-21 appears to be more suitable for induction of humoral responses to SPf66. Concerning the dose regimen, it is important to determine whether 2 rather than 3 doses of vaccine would be optimal for generation of an effective immune response. Our data suggest that a 2-dose regimen would be ideal since high anti-SPf66 titres can be evoked with minimum risk of reactogenicity. Studies on antigen dose were not undertaken in this work. Such studies will be critical since they will help determine whether the immune response induced by 2 doses of SPf66 vaccines containing 100 µg of QS-21 could be optimised further by reducing the amount of antigen in the preparation. Results of recent studies of HIV-1MN gp120 vaccines combined with QS-21 in healthy uninfected adults have shown that reducing the antigen dose from 30-600 µg to 3 µg resulted in the generation of the highest neutralising antibody titre ever seen with a range of adjuvants tested (37). Indeed, HIV-1-specific proliferative T cell responses, V3 loop-specific and fusion inhibiting antibodies at a 3- µg antigen dose were equal or superior to the responses seen with formulations containing 30-600 µg of antigen.

A central question for this trial is whether or not improvement in anti-SPf66 titres has a predictive value on the efficacy of the new formulation. Although critical, such a question can only be answered in a Phase III efficacy trial setting. Knowledge of protective immunity in malaria is still incomplete. It is generally believed that induction of malaria-specific antibodies is critical, but perhaps not sufficient for an effective control of human malarial infection (38, 39). An effective malaria vaccine is likely to require the induction of antibody and cellular responses against key parasite antigen targets. Three peptide sequences derived from proteins isolated from infected erythrocytes and one CSP-derived epitope are built into the SPf66 vaccine. Anti-SPf66 antibodies induced by QS-21-containing formulations have specificity for all 3 blood stage antigen epitopes, and may have the potential to interfere with

parasite entry into erythrocytes (40, 41). Studies in *Aotus* monkeys vaccinated with SPf66/QS-21 formulations showed that these vaccines were more effective against experimental challenge with *P. falciparum* blood stage parasites than both SPf66/alum and SPf66/Freund's adjuvant vaccine formulations (unpublished results). This demonstrates that QS-21 has a large potential as an adjuvant for the delivery of peptide-based vaccines in humans.

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Figure legends

Figure 1. Kinetics of anti-SPf66 IgG responses among subjects administered 2 doses of vaccines or placebo. Subjects were immunised subcutaneously on days 0 and 30 with 2 mg of peptide bound onto alum (◊), or mixed with 50 µg of QS-21 (▼), 100 µg of QS-21 (■), or a combination of alum and 100 µg of QS-21 (∇). Subjects in the placebo group (□) were given 2 doses of formulations containing 50 µg of QS-21, and no peptide. Geometric mean titres (GMT) of IgG against SPf66 were determined at baseline, on day 20 following each vaccination, and 4 months after the second dose.

Figure 2. Kinetics of anti-SPf66 IgG responses among subjects administered 3 doses of vaccines or placebo. Subjects were immunised subcutaneously on days 0, 30 and 180 with 2 mg of peptide bound onto alum (◊), or mixed with 50 µg of QS-21 (▼), 100 µg of QS-21 (■), or a combination of alum and 100 µg of QS-21 (∇). Subjects in the placebo group (□) were given 2 doses of formulations containing 50 µg of QS-21, and no peptide. GMT of IgG against SPf66 were determined at baseline, on day 20 following each vaccination, and 4 months after the second and third doses.

Figure 3. Kinetics of anti-35.1 peptide IgG responses. Subjects were immunised twice (days 0 and 30) with 2 mg of SPf66 bound onto alum (□), or mixed with 100 µg of QS-21 (◆), or three times (days 0, 30 and 180) with alum (▲) or 100 µg of QS-21 (∇).

Figure 4. Kinetics of anti-55.1 peptide IgG responses. Subjects were immunised twice (days 0 and 30) with 2 mg of SPf66 bound onto alum (□), or mixed with 100 µg of QS-21 (◆), or three times (days 0, 30 and 180) with alum (▲) or 100 µg of QS-21 (∇).

Figure 5. Kinetics of anti-83.1 peptide IgG responses. Subjects were immunised twice (days 0 and 30) with 2 mg of SPf66 bound onto alum (□), or mixed with 100 µg of QS-21 (◆), or three times (days 0, 30 and 180) with alum (▲) or 100 µg of QS-21 (∇).

Figure 6. Immunoblot reactivity against *P. falciparum* FCB-2 proteins. This figure is representative of *P. falciparum* immunoblot-reactive serum specimens. Sera obtained from subjects 13 (SPf66/alum), 91 (SPf66/ 50 µg QS-21) and 59 (SPf66/100 µg QS-21) were

diluted individually at 1:100 in the presence (+) or absence (-) of an MSP-1-derived synthetic peptide, and were reacted with *P. falciparum* FCB-2 lysates. PI: pre-immune. II: 20 days after the second dose. III: 20 days after the third dose. Neg.: Negative control sera. H: hyperimmune sera from a malaria-experienced subject.

Figure 7. Two-colour flow cytometric analysis of T cell lines generated by repeated stimulation of PBMC with SPf66. Cells were stained with phycoerythrin- (PE-) labelled anti-CD3 and fluorescein-isothiocyanate (FITC-) labelled anti-CD4 or anti-CD8 monoclonal antibodies. Controls were stained with PE- and FITC- labelled IgG isotype controls. In addition to cell lines obtained from the 4 volunteers 21, 34, 29 and 89 of study group B (SPf66/ 50 µg QS-21), typical results with a cell line from a volunteer immunised with SPf66/alum (TS) in a previous trial are shown.

Table 1. ***Background and demographic characteristics of study subjects.***

Parameter	Statistic	**Study Groups**				
		A SPf66/Alum	B SPf66/50 µg QS-21	C SPf66/100 µg QS-21	D SPf66/Alum 100µg QS-21	E 50µg QS-21
	N	17	20	19	20	13
Age	Mean	19	20	19	19	19
	SD	1	1	1	1	1
Weight (Kg)	Mean	62.0	60.4	60.9	61.5	63.2
	SD	4.9	6.9	5.6	8.5	8.9
Height (m)	Mean	1.73	1.70	1.70	1.72	1.74
	SD	0.06	0.06	0.05	0.07	0.07
Haemoglobin (g/dL)	Mean	13.85	13.65	12.84	13.59	13.93
	SD	1.01	0.96	1.01	1.17	0.67
WBC (x1000/µl)	Mean	8.40	8.29	5.53	8.43	7.95
	SD	2.22	2.18	1.66	2.47	1.36
Lymphocytes (%)	Mean	36.90	41.47	36.56	34.55	37.49

N= number of subjects. SD: standard deviation.

Table 2. *Subjects accountability and disposition*

Parameter	Study groups as n (%)					
	A SPf66/ Alum	B SPf66/ 50 μg QS-21	C SPf66/ 100 μg QS-21	D SPf66/ Alum 50 μg QS-21	E 50 μg QS-21	All
Total number of enrolled volunteers	20	20	20	20	19	99
Number of subjects who received dose 1.	17	20	19	20	13	89
Number of subjects who received dose 2.	17	18	19	18	13	85
Number of subjects who received dose 3.	8	11	6	8	9	42
Number of subjects discontinued	12	9	14	12	10	57
Reasons for discontinuation:						
Non-compliance with protocol	2	0	1	0	6	9
Intercurrent illness	1	0	0	0	0	1
Withdrew consent	0	0	0	0	0	0
Did not return to follow-up	0	2	0	2	0	4
Treatment discontinued[a]	9	7	13	10	4	43

[a]Treatment withdrawn due to occurrence of serious adverse events at the third administration.

Table 3. *Incidence of local and systemic reactions within one hour of vaccine administration.*

Adverse events	Dose 1					Dose 2					Dose 3				
	Study group					Study group					Study group				
A. Local reaction	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E
N=	17	20	19	20	13	17	18	19	18	13	8	11	6	8	9
Erythema	0	0	0	0	0	1	1	1	1	1	0	1	1	1	0
Oedema/Induration	0	0	1	0	0	0	0	0	1	0	2	6	5	2	1
Pain	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
Contralateral inflammation	0	0	0	0	0	0	0	0	0	0	0	4	1	2	1
B. Systemic reaction															
Fever	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Headache	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0
Malaise	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0
Hypotension	0	0	0	0	0	0	0	0	0	0	0	1[a]	0	0	0
Bronchospasm	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
Generalised Pruritus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Rash	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0

A: SPf66/Alum; B: SPf66/50 µg of QS-21; C: SPf66/100 µg of QS-21; D: SPf66/Alum + 100 µg of QS-21; E: 50 µg of QS-21

[a]with minor facial angioedema.

Table 4. *Incidence of local and systemic reactions within 24-48 hours of vaccine administration.*

Adverse Events		Dose 1					Dose 2					Dose 3				
		Study Group					Study Group					Study Group				
A. Local reaction		A	B	C	D	E	A	B	C	D	E	A	B	C	D	E
	N=	17	20	19	20	13	17	18	19	18	13	8	11	6	8	9
Erythema		6	4	7	9	4	10	12	12	9	5	4	6	3	7	5
Oedema/Induration		4	1	1	5	0	4	6	8	5	3	1	5	5	5	0
Pain		5	1	9	6	4	6	5	9	9	3	2	5	1	3	0
Contralateral inflammation		0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
B. Systemic reaction																
Fever		0	0	0	0	0	0	0	1	1	0	0	0	0	0	0
Headache		0	2	1	1	1	0	2	1	2	1	0	0	0	0	0
Malaise		0	0	1	2	0	0	0	1	2	0	0	1	0	0	0
Hypotension		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bronchospasm		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Generalised pruritus		0	0	0	0	0	0	0	0	0	0	0	4	1	0	0
Rash		0	0	0	0	0	0	0	0	0	0	0	1	0	0	0

A: SPf66/Alum; B: SPf66/50 µg of QS-21; C: SPf66/100 µg of QS-21; D: SPf66/Alum + 100 µg of QS-21; E: 50 µg of QS-21.

Table 5. *Tolerability following each dosing in study subjects.*

Time Post Dosing	Tolerability	Group A			Group B			Group C			Group D			Group E		
		Dose			Dose			Dose			Dose			Dose		
		1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
1 hr	Excellent	17	16	6	20	17	4	19	18	1	20	17	5	13	13	8
	Good		1	2		1	5		1	5		1	3			1
	Acceptable						1									
	Minimal						1									
24-48 hrs	Excellent	9	3	1	17	2	4	7	2	1	8	1	0	8	5	3
	Good	7	12	7	3	15	6	12	15	5	9	14	8	5	7	6
	Acceptable	1	2			1	1		2		3	3			1	
	Minimal															
72 hrs	Excellent	16	17	8	20	18	11	19	19	6	15	16	8	13	13	9
	Good	1									5	0				
	Acceptable											2				
	Minimal															
20 days	Excellent	17	17	8	20	18	11	19	19	6	20	18	8	13	13	9
	Good															
	Acceptable															
	Minimal															

A: SPf66/Alum; B: SPf66/50 µg of QS-21; C: SPf66/100 µg of QS-21; D: SPf66/Alum + 100 µg of QS-21; E: 50 µg of QS-21

Excellent = No reaction / Normal physical activities; Good = Some reaction / Normal physical activities; Acceptable = Some reaction / Reduced physical activity; Minimal = Some reaction / Impaired physical activity.

Table 6. *Serum IgG response against SPf66 after vaccinations with SPf66 in various adjuvants.*

Time points	Antibody response	**Study group**				
		A SPf66/Alum	B SPf66/50 µg QS-21	C SPf66/100 µg QS-21	D SPf66/Alum 50 µg QS-21	E 50 µg QS-21
Pre-Immune	N	16	17	18	17	13
	Low	15 (93.8)	17 (100)	17 (94.4)	17 (100)	13 (100)
	Medium	1 (6.2)	0	1 (5.6)	0	0
	High	0	0	0	0	0
Dose 1	N	14	17	18	17	13
	Low	10 (71.4)	9 (52.9)	8 (44.4)	(2 (11.8)	0
	Medium	4 (28.6)	7 (41.2)	7 (38.9)	2 (11.8)	0
	High	0	1 (5.1)	3 (16.7)	13 (76.4)	0
Dose 2	N	15	15	17	16	12
	Low	2 (13.3)	0	1 (5.9)	0	12 (100)
	Medium	5 (33.3)	0	0	0	0
	High	8 (53.4)	15 (100)	16 (94.6)	16 (100)	0
4 months post Dose 2	N	12	16	17	12	12
	Low	7 (58.3)	0	0	0	12 (100)
	Medium	5 (41.7)	2 (12.5)	1 (5.9)	2 (16.7)	0
	High	0	14 (87.5)	16 (94.6)	10 (83.3)	0
Dose 3	N	8	10	5	8	8
	Low	2 (20)	0	0	0	8 (100)
	Medium	3 (37.5)	0	0	0	0
	High	3 (37.5)	10 (100)	5 (100)	8 (100)	0

N= number of subjects. SD: standard deviation

Table: 7A.

Geometric Mean Titres of IgG against SPf66 after two vaccinations with SPf66 in various adjuvants

Study Group	Pre-immune	20 Days after Dose 1 (Day 20 of Study)	20 Days after Dose 2 (Day 50 of Study)	4 Months after Dose 2 (Day 160 of Study)	Day 200 of Study	Day 300 of Study
A	2	20	1,600	240	150	20
B	0	6	20,400	8,130	6,460	3,210
C	0	20	31,330	3,920	3,220	2,090
D	0	2,400	22,390	2,510	1,580	1,100
E	0	0	0	0	0	0

A: SPf66/Alum; B: SPf66/50 µg of QS-21; C: SPf66/100 µg of QS-21; D: SPf66/Alum + 100 µg of QS-21; E: 50 µg of QS-21.

Table 7B.

Geometric Mean Titres of IgG against SPf66 after three vaccinations with SPf66 in various adjuvants

Study group	Pre-immune	20 Days after Dose 1 (Day 20 of Study)	20 Days after Dose 2 (Day 50 of Study)	4 Months after Dose 2 (Day 160 of Study)	20 Days after Dose 3 (Day 200 of Study)	4 Months after Dose 2 (Day 300 of Study)
A	0	20	530	20	530	190
B	0	100	37,760	4,050	25,700	4,390
C	3	400	51,290	11,220	44,670	12,890
D	0	830	38,100	5,100	31,330	5,850
E	0	0	0	0	0	0

A: SPf66/Alum; B: SPf66/50 µg of QS-21; C: SPf66/100 µg of QS-21; D: SPf66/Alum + 100 µg of QS-21; E: 50 µg of QS-21.

Table 8.

P. falciparum FCB-2 IFA-reactive antibody following vaccination with SPf66 in various adjuvants.

Time-points (20 days after)	Study group				
	A SPf66/Alum	B SPf66/50 µg QS-21	C SPf66/100 µg QS-21	D SPf66/Alum 50 µg QS-21	E 50 µg QS-21
Dose 1	1/16	0/17	1/18	1/17	0/13
Dose 2	1/16	0/17	1/18	1/17	0/13
Dose 3	1/8	3/11	3/5	3/8	0/8

Table 9.

Frequencies of SPf66-specific T cells in PBMC of volunteers from group B four months after receiving the third dose of SPf66/50 µg QS-21.

Volunteer ID	21	34	29	89
Reactogenicity	Systemic allergic reaction	Systemic allergic reaction	none	local contralateral
Frequency	1 / 3753	1 / 6115	< 1 / 20 000	1 / 3038
Confidence interval (p >0.05)	1 / 2993 – 1 / 5029	1 / 4732 – 1 / 8638		1 / 2507 – 1 / 3855

Figure 1

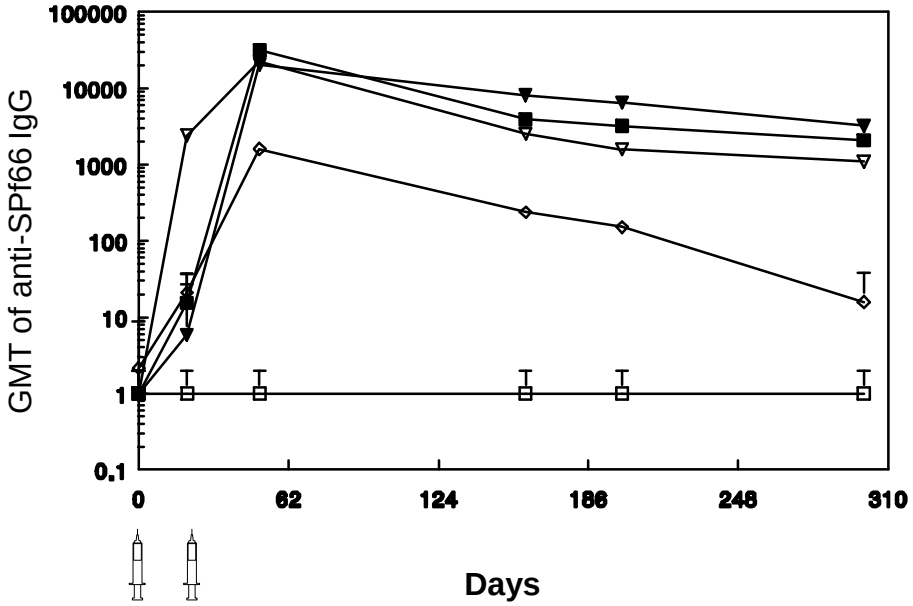


Figure 2

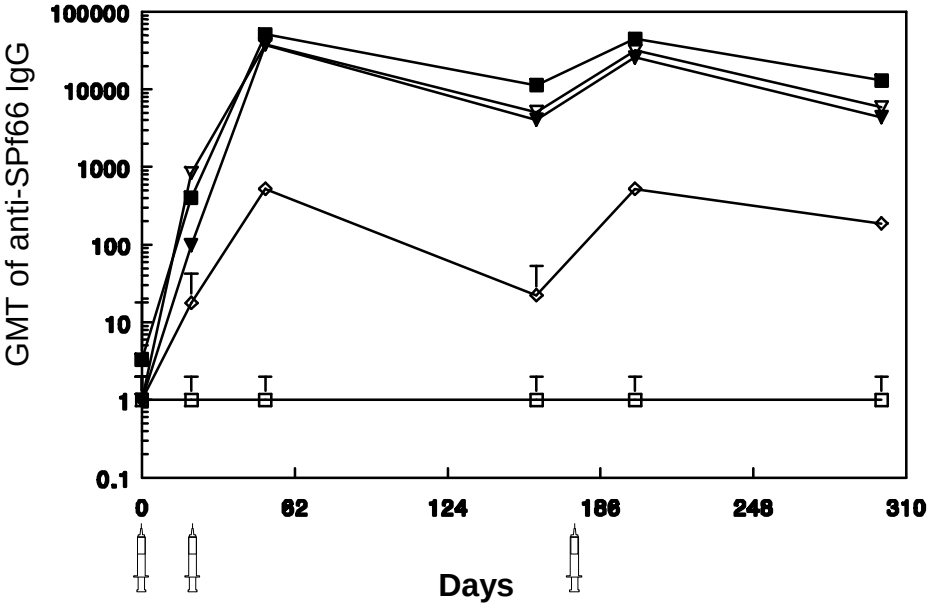


Figure 3

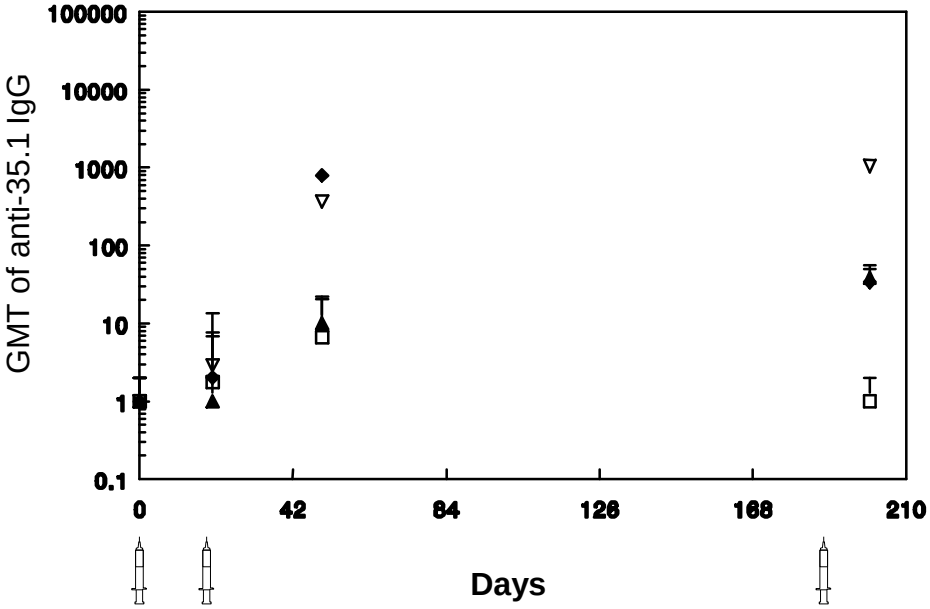


Figure 4

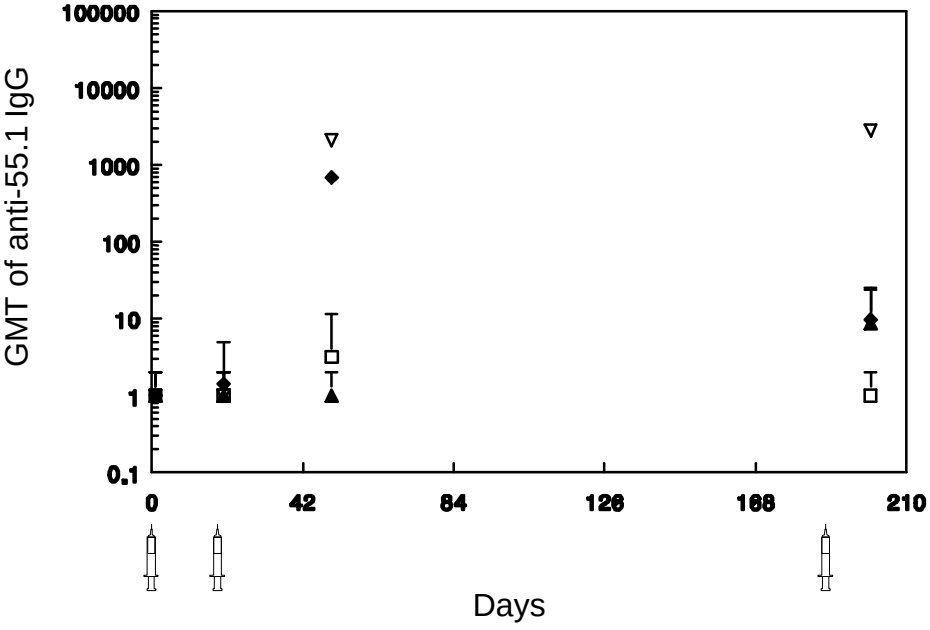


Figure 5

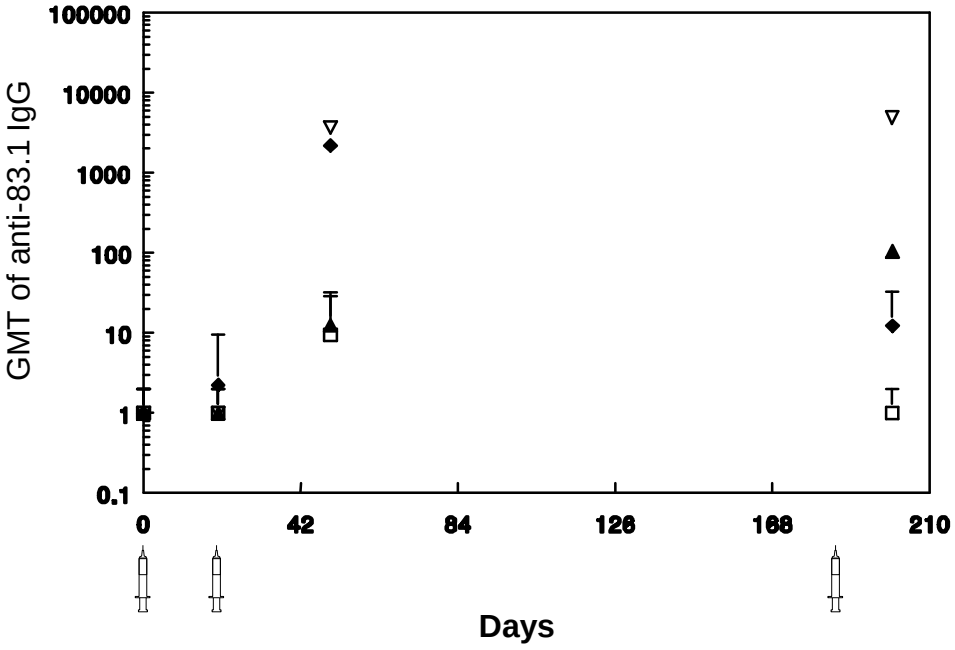


Figure 6

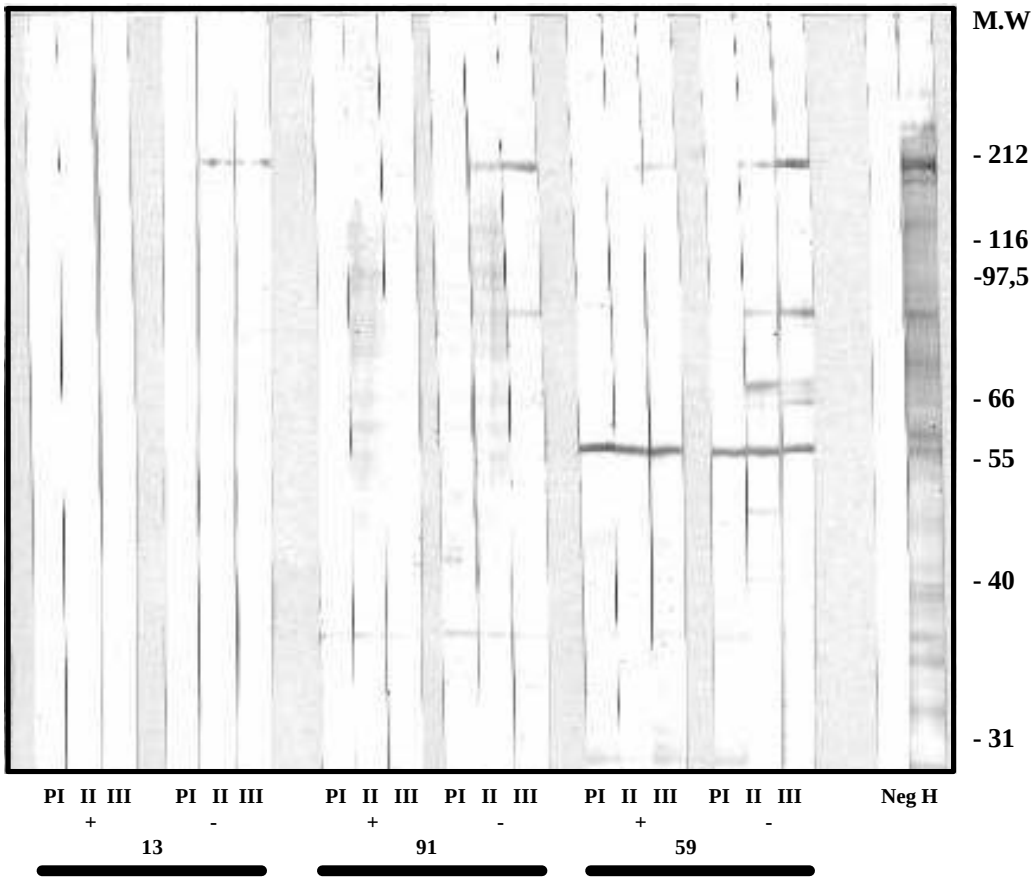
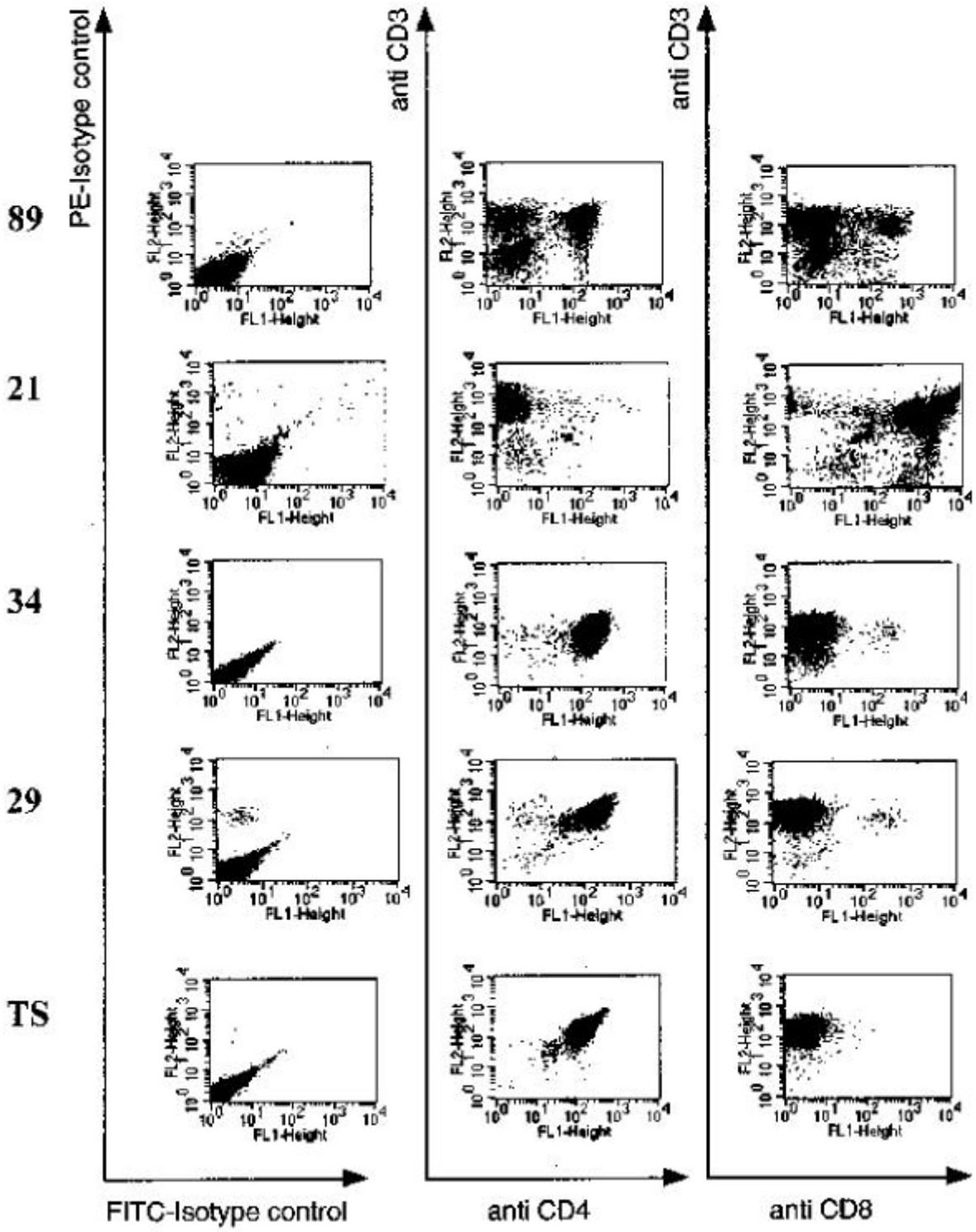


Figure 7


89
21
34
29
TS
PE-Isotype control
anti CD3
anti CD3
FITC-Isotype control
anti CD4
anti CD8
FL2-Height
FL1-Height

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7. Malaria Vaccines: General Discussion

Malaria remains one of the leading causes of morbidity and mortality in the tropics. There are an estimated 300-500 million new cases of malaria each year, resulting in over 1 million deaths, mainly of children under five years old in Africa (World Health Organisation 1995). The development of an effective malaria vaccine represents one of the most important goals to support currently available malaria control strategies, which have have been challenged by the spread of resistance of the parasite to various drugs. Despite the intensive research during the last twenty years, there is still no effective vaccine available (Facer and Tanner 1997). The complexity of the parasite life cycle, imperfect tools to assess the efficacy of immune responses and limited knowledge of the factors that determine the outcome of an infection are still the main obstacles in developing such a vaccine. However, several observations justify the continued search for a malaria vaccine as a realistic option : (1) Following continuous exposure for several years individuals living in endemic areas acquire non-sterile concomittant immunity. Passive transfer experiments showed that immunoglobulins of such semi-immune individuals can protect against clinical malaria (Cohen et al. 1961; McGregor 1964). (2) Specific antibodies directed against a number of parasite derived proteins suppress growth and multiplication of malaria parasites *in vitro* (Bouharoun-Tayoun et al. 1990; Harnyuttanakorn et al. 1992; Lozano et al. 1998; Moreno et al. 2001). And (3), immunization studies with irradiated sporozoites of *Plasmodium falciparum* and *Plasmodium vivax* induced sterile protective immunity against sporozoite challenge in humans (Clyde 1975;Edelman et al. 1993; Egan et al. 1993; Herrington et al. 1991; Rieckmann et al. 1974).

7.1 Strategies for malaria vaccine development

There are two different strategies to developing a malaria vaccine. One is to elucidate the mechanism of natural immunity and to attempt to induce its development. The other is to induce an immune response that does not normally develop, but nevertheless is protective. In both cases several stages of the parasite life cycle should be targeted.

The first strategy is essentially restricted to developing live attenuated malaria vaccines, which in the case of irradiated sporozoites has been highly effective. However, production of sufficient quantities for mass vaccination purposes is not feasible. Studying the mechanisms that lead to natural immunity may contribute substantially to the development of subunit

vaccines. Immune responses that may be active against different life cycle stages of the malaria parasite are summarized in Table 1.

Table 1. *Immune responses that may be active against different stages of the malaria life cycle*[a]

Life cycle stage	Immune response
Sporozoite	• Antibodies (Ab) that block sporozoite invasion of hepatocytes or mediate killing of sporozoites, via opsonization, complement activation
Infected hepatocyte	• CD8+ or CD4+ CTL that directly kill the infected hepatocyte • CD4+ T cells that provide help for the activation and differentiation of CTL precursors • CD8+ or CD4+ T cells that indirectly kill/inactivate the intracellular parasite, via cytokines or other factors • Ab that mediate killing of the infected hepatocyte or the intrahepatic parasite, either directly, with complement or via antibody-dependent cellular cytotoxicity (ADCC)
Asexual erythrocytic	• Cytokines released by activated CD4+ T cells or secreted by reticuloendothelial cells, that directly kill the infected erythrocyte or the intraerythrocytic parasite • Ab that agglutinate the merozoites at rupture of mature schizonts or block merozoite invasion of erythrocytes • Ab that mediate killing of the infected erythrocyte via opsonization and subsequent phagocytosis or via ADCC • Ab that prevent infected erythrocytes from adhering to endothelial cells (cytoadherence) by blocking receptor/ligand interactions • Ab that prevent the release of, or neutralize, harmful soluble parasite toxins
Sexual erythrocytic	• Cytokines and Ab that kill/inactivate gametocytes within the infected erythrocyte and Ab that interfere with fertilization

[a](Adapted from Doolan and Hoffman, 1997)

All strategies based on subunit vaccine candidates fall into the second category since exposing the immune system to only a single or a limited number of malaria antigens must induce a focussed immune response. The quantity and quality of antibody and helper T cells induced by such vaccination will possibly differ from what develops following natural exposure.

The search for protective antigens to be included into subunit vaccines has generated a long list of potential vaccine candidates. The nature of antigens ranges from recombinant proteins and synthetic peptides to recombinant virus and naked parasite DNA. Different approaches have led to different kinds of vaccines, which have been tested in animal models, and some of them in human trials. Although studies of immunogenicity and protection in animal models and the results of *in vitro* growth inhibition experiments have been promising for many vaccine candidates, efficacy of protection in field trials has not been satisfactory. To date, there is consensus that a highly effective malaria vaccine will require a combination of key antigens and/or epitopes (multi-component) from different stages of the life cycle (multi-

stage), and that induction of both humoral and cellular immunity is required for optimal efficacy (Engers and Godal 1998; Holder 1999). In contrast to inclusion of whole antigens, epitope focussed vaccines can advantageously exclude unwanted immune responses to undesirable epitopes (Delves et al. 1997) or can focus the immune response on crucial protective epitopes which normally remain cryptic in the intact protein (Wrightsman et al. 1994). To circumvent genetic restriction of the immune responses, "promiscuous" T cell epitopes should be included which bind to a broad range of different MHC allelic variants. B cell epitopes, which are frequently discontinuous, can be mimicked by conformationally constrained mimetics, like cyclic peptidomimetics (Bisang et al. 1998; Lioy et al. 2001) or modified non-natural peptides like reduced amide pseudopeptide analogues (Lozano et al. 1998). The development of suitable and save delivery systems for such antigens to evoke sufficient immune responses represents another major field in malaria vaccine design. In the following chapter some examples will be given for different types of vaccine candidates.

7.2 Multi-component vaccine candidates

The first human trial with a genetically engineered virus against malaria (**NYVAC-Pf7**) was a multicomponent, multistage vaccine comprising seven genes of *P. falciparum* (sporozoite stage: CSP, SSP2/TRAP; liver stage: LSA-1; asexual blood stage: MSP-1, AMA-1, SERA; sexual stage: Pfs25). These were combined and administered within an attenuated vaccinia virus as vehicle but did not result in any significant protection against malaria episodes. In a phase I/IIa trial, cellular immune responses were detected in >90% of volunteers, while antibody responses were generally poor. However, there was a significant delay in the onset of parasitemia among vaccinated individuals when compared to controls (Ockenhouse et al. 1998). Delay in the prepatent period did not correlate with antibody titers, CTL activity, or antigen-specific lymphoproliferative responses. The administration of genetic material in combination with virus particles or directly as naked DNA may have many advantages compared to recombinant or peptide vaccines. Antigens expressed in their native forms can lead to improved processing by the immune system and thereby lead to induction of antigen specific T cell responses. DNA-based vaccines are easy to produce, purify, modify, and to combine, they may induce long-term immunity due to long persistence in the body and may require only low numbers of doses. On the other hand there are disadvantages which should carefully be considered, such as potential incorporation of foreign DNA into host

chromosomes or even germ-line cells. Introduction of foreign DNA could also stimulate anti-DNA antibodies and thus autoimmune reactions.

More recently, results with a recombinant hepatitis B surface antigen vaccine incorporating repeat and non-repeat CSP sequences (**RTS,S**) have been very encouraging. When RTS,S was formulated in an oil-in-water emulsion containing the saponin QS-21, it has not only shown its potency in inducing effective cellular and humoral immune responses but also promising efficacy against artificial and natural challenges (Stoute et al. 1997; Tanner 2001). Although the C-terminal CSP sequence included into RTS ,S contained T helper and cytotoxic T cell epitopes, no cytotoxic T cell activity could be demonstrated in peripheral T cell populations from immunized subjects (Stoute et al. 1997). No correlation between antibody or T cell responses and protection could be observed supporting the idea that immunization might induce more than one potentially protective mechanism. Nevertheless it is surprising that no cytotoxic T cell response could be elicited by the RTS ,S/QS-21 formulation despite the inclusion of cytotoxic T cell epitopes. In contrast to alum, the saponin adjuvant QS-21 has been shown to support the induction of CD8+ cytotoxic T cell responses (Kensil 1996; Newman et al. 1997). We have confirmed this observation in our studies by the generation of antigen specific CD8+/CD4+ T cell lines from SPf66/QS-21 vaccinated volunteers (chapter 6).

Combination B, a vaccine candidate combining three recombinant merozoite proteins, MSP-1, MSP-2 and RESA in a water-in oil emulsion with Montanide ISA70, has been tested in a recent trial in Papua New Guinea among school-aged children (Genton et al. 2000). In one subgroup, Combination B showed an efficacy of 62% in reducing parasite density, which was the primary endpoint tested in this trial. The vaccine components and immune mechanisms, which are responsible for the effect on parasite densities, will need to be investigated in further studies.

Sequences of two components which are also present in Combination B, MSP-1 and MSP-2, are present in a multi-component, epitope-focussed, recombinant vaccine candidate (**CDC/NIIMALVAC-1**) where 12 B cell and 9 T cell epitopes derived from 9 stage-specific antigens were expressed in a baculovirus system (Shi et al. 1999). Immunization of rabbits with the purified protein generated antibody responses that recognized 7 of 12 B cell epitopes and 6 of 10 T cell epitopes. Furthermore, the choice of adjuvants had a significant influence

on the induction of antibodies that cross-react ed to parasites. The sequential order of T cell and B cell epitopes in a multi-epitope peptide construct can significantly affect their immunogenicity (Chatterjee et al. 1995; Denton et al. 1994) and should carefully be chosen. Our results (chapter 3) demonstrate the influence of epitope flanking sequences on T cell stimulation. T cell clones recognizing the same core epitope within the N-terminal part of MSP-1 needed different flanking regions for activation. The order of the assembled epitopes in CDC/NIIMALVAC-1 could explain why only some epitopes were immunogenic. Unfortunately, cellular responses to the antigen were not investigated. The antibody responses of rabbits can only give preliminary insights into how a human population might respond to the antigen. Clearly, more data with an animal model for *P. falciparum* infection and blood-stage development, like *Aotus* monkeys, are needed.

SPf66 was the first multivalent, multistage, synthetic peptide vaccine extensively tested in several human phase III trials (Patarroyo et al. 1987; Patarroyo and Amador 1999). The chemically synthesized vaccine contained a portion of each of a 35-kDa, a 55-kDa, and a 83-kDa protein isolated from *P. falciparum* infected erythrocytes. These peptide building blocks were linked by NANP sequence units from the CSP. The three peptide building blocks (35.1, 55.1, 83.1) included in SPf66 conferred partial protection against *P. falciparum* blood-stage infection in *Aotus* monkey studies and elicited parasite-binding antibody titers in humans. Although SPf66/alum showed promising results in early trials (Patarroyo et al. 1988), large-scale human trials in the Gambia, Thailand and in Tanzania revealed that it provided only limited protection (D'Alessandro et al. 1995; Graves et al. 1998; Graves and Gelband 2000). Again, no correlation between antibody titers and protection could be observed. To get more insights into the elicited immune response, we have investigated T cell responses of SPf66-vaccinated volunteers to the 83.1 epitope. This epitope represents part of the semi-conserved N-terminal region of MSP-1 of *P. falciparum* and has been shown to be relevant for the binding of malaria parasites to red blood cells (Lioy et al. 2001; Lozano et al. 1998). In contrast to naïve blood donors, SPf66/alum vaccinated volunteers exhibited T cell responses specific for the MSP-1^{38-58} sequence and were mainly of Th2 type (chapter 2 and 3). This confirms observations, that alum as adjuvant is associated with the induction of CD4+ Th2 responses (Bomford 1980). Although aluminum-based mineral salts are poor adjuvants for cell-mediated immunity (Gupta 1998), they are the only adjuvants currently approved for use in humans (Singh and O'Hagan 1999). In order to accelerate immunogenicity, new formulations of SPf66 combined with the saponin adjuvant QS-21 have been tested in a safety

and immunogenicity trial (Kashala et al. 2001). In the course of this study we detected mixed populations of CD4+ helper T cells and CD8+ cytotoxic T cells in peripheral blood cells of SPf66/QS-21-vaccinated volunteers (chapter 6). Beside the accelerated cellular responses, anti-SPf66 IgG titers were up to 100 fold higher, compared to vaccination with alum. Our results confirm that the saponin QS-21 is a powerful adjuvant with the potential to support humoral and cellular immune responses, CD8+ cytotoxic T cell responses in particular (Kensil 1996; Newman et al. 1997). Whether this immune response, induced by SPf66/QS-21 is protective, remains to be investigated.

7.3 Adjuvants and delivery systems

Adjuvants are compounds or formulations that help antigens to elicit an early, strong and long-lasting immune response. Since adjuvants can mediate a depot-like effect the total quantity of applied antigen in combination with adjuvant can be reduced. In recent years, adjuvants received much attention because of the development of subunit and synthetic vaccines, which are poor immunogens and require improved adjuvants to evoke the immune response. Depending on the selected adjuvant, immune responses can be preferentially modulated to MHC class I or MHC class II restriction and Th1 or Th2 type (Gupta et al. 1996).

The most common adjuvants for human use today are still aluminum hydroxide and aluminum phosphate (alum), although calcium phosphate and oil emulsions also have some use in human vaccinations (Aguado et al. 1999). As alum is known to be a weak adjuvant for antibody induction and a poor adjuvant for cell-mediated immunity, much progress has been made on development, isolation and chemical synthesis of alternative adjuvants (Singh and O'Hagan 1999). Several adjuvant formulations have been tested in human trials, such as derivatives of muramyl dipeptide, monophosphoryl lipid A (MPL), liposomes, saponins like QS-21, MF-59 and immunostimmulating complexes (ISCOMS). Other areas in adjuvant research focus on the controlled release of vaccine antigens using biodegradable polymer microsphers, which should target antigens on mucosal surfaces, and reciprocal enhanced immunogenicity of protein-polysacharide conjugates, which will be useful for the development of combination vaccines (Gupta and Siber 1995). A promising antigen delivery system already applied successfully for hepatitis A and influenza vaccines consists of immunopotentiating reconstituted influenza virosomes (IRIV) (Gluck 1999). Such virosomes

represent suitable delivery systems for linear peptides as T cell epitopes (Poltl-Frank et al. 1999) and conformationally constrained peptidometrics as B cell epitopes.

7.4 SPf66 as a model for epitope-focused vaccines

While B cells recognize conformational or sequential epitopes on the surface of native proteins, T cells typically recognize peptides derived from the processing of antigens by antigen-presenting cells (APCs) and presented in association with MHC molecules. There are major differences between the recognition of antigens by CD4+ and CD8+ T cells, both in terms of the cellular site were the MHC-bound peptides are generated and the nature of the MHC molecules required for recognition. CD4+ T cells recognize peptides from exogenous molecules, which are internalized by APCs, processed and presented on MHC class II molecules. CD8+ T cells usually recognize peptides of cytosolic origin, which are presented on MHC class I molecules.

A major prerequisite for the development of vaccines including T cell epitopes is the identification of promiscuous T cell epitopes derived from target-organism-proteins. SPf66 consists of three peptide sequences derived from proteins isolated from infected human erythrocytes which were chemically synthesized as a continuous peptide linked by PNANP spacer-sequences.

7.5 Implications of this work

In this PhD thesis, the synthetic peptide vaccine SPf66 was used as a model system to investigate human cellular immune responses to a parasite derived epitope. The 83.1 epitope which had been included into SPf66 is part of the semi-conserved block 1 of MSP-1, a major surface protein of asexual blood stage malaria parasites. This region of MSP-1 has originally been shown to provide protection against homologous experimental infection with *P. falciparum* in *Aotus* monkeys (Patarroyo et al. 1987) and was therefore incorporated into the multi-epitope vaccine SPf66. In the course of human vaccine trials, the antibody response to the different peptide components of SPf66 has been investigated which revealed that the 83.1 epitope represented a suitable human B cell epitope (Molano et al. 1992).

The main issue of this PhD thesis was to investigate whether the same region of MSP-1 also represented a suitable human T cell epitope and if so, to analyze the fine-specificity of

the MSP-1 specific T cell response. For this purpose human T cells were isolated from PBMC of SPf66-vaccinated volunteers and analyzed for their specificity. Due to the short length (11 amino acids) of the 83.1 epitope (MSP-1$^{43-53}$) as single peptide it did not exhibit a significant *in vitro* T cell stimulatory activity. Therefore the MSP-1 $^{38-58}$ peptide which is C- and N-terminally extended by 5 amino acids was used as antigen. The present work deals on one side with the antigen-specific T cell response of a parasite derived antigen, with important implications for future malaria vaccine development, and on the other side with basic immunological issues concerning peptide presentation to MHC class II-restricted T cells in general.

MSP-1$^{38-58}$ contains promiscuous T cell epitopes

The use of peptides as MHC epitopes to induce protective T cell responses is of particular interest in the development of epitope-focused subunit vaccines. However, a potential obstacle to the development of a vaccine designed to induce T cell-mediated immunity is the large degree of polymorphism of human HLA molecules. Therefore the incorporation of "promiscuous" T cell epitopes which are able to bind to multiple HLA alleles (Delves et al. 1997; Hammer et al. 1993) is prerequisite for the effectiveness of a vaccine within a population of immunogenetically diverse individuals.

In the course of this study, we were able to generate MSP-1$^{38-58}$ specific T cell lines from eight SPf66/alum-vaccinated volunteers (chapter 2, 5 and 8) with different genetic backgrounds. CD4+ T cell responses were either MHC-DR or –DP restricted. Volunteers immunized with a SPf66/QS-21 formulation developed in addition a class I restricted CD8+ T cell response (chapter 8). Generation of MSP-1 specific T cell lines from blood samples before vaccination or from un-vaccinated volunteers failed, indicating that antigen-specific T cells were elicited by vaccination. The fact that the MSP-1 epitope was presented by multiple MHC alleles and different types of MHC class II molecules, namely HLA-DR and HLA-DP, demonstrates that this region contains "promiscuous" T cell epitopes.

The T cell response to MSP-1$^{38-58}$ is variant- specific

A closer look at the clonal level of the MSP-1 specific response revealed that the elicited T cell response was strictly specific for the particular MSP-1 sequence variant that had been included into SPf66. Four other naturally occurring sequence variants of this semi-conserved epitope, exhibiting dimorphism at only three defined positions (S ^{44}/ G^{44}, Q^{47}/H^{47} and

$V^{52}/I^{52}/L^{52}$) (Jiang et al. 2000; Miller et al. 1993), were not stimulatory for the MSP-1^{38-58} specific T cell clones (chapter 2).

Single amino acid substitutions of anchor positions within peptides have been shown to dramatically influence the binding affinity to MHC class II (De Oliveira et al. 2000; Doherty et al. 1998; Gautier et al. 1996). On the other hand, T cell recognition is based on interactions of the TCR with the MHC and with solvent -exposed peptide residues, which are located in positions p2/3, p5 and p7/8 between MHC anchor residues (Dessen et al. 1997; Fremont et al. 1996; Ghosh et al. 1995; Hennecke et al. 2000). Substitution of amino acid positions within this TCR contact region of the peptide can completely abolish T cell stimulation without interfering with peptide binding (De Oliveira et al. 2000). In order to address the reason for the loss of stimulatory activity of naturally occurring sequence variants of MSP-1^{38-58} peptide, we have measured the binding affinities of all sequence variants to purified DRA/DRB1*0801 complexes, which represent the restriction element emp loyed by the DR-restricted clones from volunteer TS (chapter 2). Our experiments demonstrate that all sequence variants of the MSP-1 epitope were able to compete for binding to DR8 molecules. Therefore, dimorphism at these positions seems to affect TCR contact residues, leading to complete loss of T cell activation if variants are presented to the T cell.

Dimorphism as a strategy for immune evasion?

It has been shown that altered peptide ligands (APL) exhibiting single amino acid substitutions within the TCR contact region can downregulate T cell responses by the induction of anergy or apoptosis (Combadiere et al. 1998; Pingel et al. 1999; Verhoef and Lamb 2000). Several immune evasion mechanisms might be operating in malaria infections. *Plasmodium* infection can lead to anergy and deletion of parasite-specific CD4+ T cells in mice (Hirunpetcharat and Good 1998). Interference with the priming of human T cell responses by naturally occurring variants was demonstrated for a cytotoxic T cell epitope of CSP (Plebanski et al. 1999). A similar mechanism of immune interference may also be applied by malaria blood-stage parasites. The dimorphism of block 1 of MSP-1 could provide APL which suppress antigen -specific T cell responses directed against other sequence variants. We failed to detect proliferative responses to MSP-1^{38-58} in PBMC from blood donors living in malaria hyperendemic regions. It remains to be investigated whether the frequently observed presence of parasites with different sequence variants of the MSP-1 epitope in endemic areas is indeed associated with immunological antagonism.

On the other hand, the spacing between dimorphic positions within the MSP-1 epitope (position 44, 47 and 52) fit into the peptide binding motive used by most MHC class II-DR molecules to bind peptides with anchors at positions p1, p4 and p9 (Rammensee 2001). Therefore, variation at these positions could prevent binding to MHC class II molecules and by this avoid peptide presentation to T cells. In the case of DRA/DRB1*0801 complexes we could demonstrate that all sequence variants of the MSP-1 $^{38-58}$ peptide bind to the MHC molecules and are therefore most likely presented to the DR-restricted T cells.

To elucidate, whether dimorphism is a strategy to prevent peptide binding to other MHC class II alleles, more peptide binding-data with other purified MHC-DR and –DP allelic variants are needed.

MSP-1 peptides do not bind by a typical DR-binding motive to HLA-DRB1*0801

Although a variety of peptides with HLA-DR8 binding capacity have been reported, no allele-specific binding motive for DR8 molecules could be assigned yet (Calvo-Calle et al. 1997; Chicz et al. 1993; Doolan et al. 2000; Gautier et al. 1996; Rammensee 2001). It thus seems that peptide binding to the DRB1*0801 allele is not associated with a typical HLA-DR binding motive, bearing an aromatic or hydrophobic residue at p1 and additional anchors at p4, p6 and p9. To determine residues which interact with the HLA-DR8 molecule, we have analyzed binding affinities of glycine-substituted MSP-1 $^{38-55}$ analogues to purified DRA/DRB1*0801 complexes (chapter 3). We found that the 3 residues L45, E49 and V52 within the MSP-1 epitope interfered most significantly with DR8 binding, since single substitution of these positions decreased binding affinity of the peptides dramatically. These results support the assumption that peptides are not bound by a typical DR-binding anchor constellation to HLA-DRB1*0801. Computational modeling of the DR8 binding pocket with prediction of binding feasibility of MSP-1 $^{38-58}$ peptide could give more insight into the binding motive.

Flanking residues are important for T cell activation

Once having generated MSP-1 $^{38-58}$ specific human T cell clones, we were also interested in the structural basis of MHC class II-restricted T cell stimulation. In chapter 3 of this thesis we demonstrate the important role of residues that flank the core peptide associated with the class II binding groove for T cell activation. T cell clones using the same restriction element required flanking regions of different length for activation although the same peptide core sequence was used for TCR contact, as confirmed with single position substituted analogues.

Flaking residues can influence the conformation of the MHC-peptide complex and thereby affect T cell stimulation. These results are in accordance with the finding that flanking regions which are not in direct contact with the TCR can significantly modulate the immunogenicity of a peptide (Moudgil et al. 1998). Crystal structure data of MHC-peptide-TCR complexes revealed that maximally nine residues of the presented peptide are in contact with the bound TCR (Hennecke et al. 2000; Reinherz et al. 1999). We could demonstrate that, for T cell activation, a subset of only 8 to 10 amino acid residues was "read out" by the bound TCR. Single amino acid replacements by alanine or glycine within this core region of the peptide were not tolerated without loss of stimulatory activity. A variable number of additional flanking region residues turned out to be essential, but could be replaced by glycine or alanine residues without loss of stimulatory activity.

Peptide-backbone modifications can enhance or decrease T cell activation

A major drawback of synthetic peptide vaccines is the poor immunogenicity of short peptides, their short serum half-life and their rapid degradation by proteolytic enzymes (Bona et al. 1998). This could be overcome by making the peptides resistant to proteolysis by either substitution of critical amino acids, or by modification of the peptide backbone. Non-natural peptides, including pseudopeptides with reduced amide backbone modifications exhibit increased resistance to proteolytic degradation (Calbo et al. 2000; Lozano et al. 1998; Stemmer et al. 1999) and have therefore been proposed to advantageously replace natural peptides in immunoprophylactic and therapeutic strategies (Kieber-Emmons et al. 1997). Such pseudopeptides with reduced $\Psi[CH_2\text{-}NH]$ bonds in the backbone have been successfully applied as strong B cell epitopes (Lozano et al. 1998). Since Th cell activation is essential for the induction of protective immune responses this has raised the question whether pseudopeptides can serve both as B cell as well as T cell epitopes in human vaccines. In chapter 4 of this thesis we demonstrate that pseudopeptide analogues of MSP-$1^{38\text{-}58}$ and MSP-$1^{42\text{-}61}$ containing single reduced $\Psi[CH_2\text{-}NH]$ bonds represent strong epitopes for human MSP-1 specific T cell clones. In the case of an HLA-DR restricted T cell clone, two out of eight backbone modifications studied resulted in improved stimulation of both proliferation and cytokine expression, demonstrating that pseudopeptides can even be better T cell activators than their unmodified peptide homologues. The reason for this increased stimulatory property could be a more favorable interaction of the TCR with important side chains of the peptide or with the backbone itself as a result of the introduction of an additional degree of freedom into the peptide. Modification at another position had little effect on either T cell stimulatory

property or HLA-DR8 binding, while three other modifications impeded both HLA binding and T cell stimulation. Loss of HLA binding is consistent with results of X-ray crystallographic studies which demonstrated that interactions of certain carbonyl groups with conserved residues along the length of the peptide binding groove of MHC class II molecules contribute significantly to peptide binding (Madden 1995; Reinherz et al. 1999). However, two pseudopeptides were good HLA-DR binders but failed to stimulate the HLA-DR restricted T cells indicating that TCR triggering can be strongly modified by the introduction of the single $\Psi[CH_2\text{-}NH]$ modification. Our data demonstrate that particular peptide backbone carbonyls contribute critically to MHC binding or TCR triggering of Th cells.

Taken together, the demonstration that pseudopeptides can be more efficient stimulators of human malaria antigen-specific Th cells encourage attempts to profile such altered ligands as elements of synthetic epitope-focused vaccines. However, the fact that pseudopeptides can also loose HLA-DR binding properties and T cell stimulatory activity should lead to a careful selection of the positions to be modified within a certain epitope.

Why are there no MSP-1$^{38\text{-}58}$ specific T cell reactivities in semi-immune individuals?

It has been proposed that determinants capable of binding to many allelic products of a single MHC locus or to different MHC molecules in the same individual tend to be immunodominant (Sercarz et al. 1993). This raised the question whether the 83.1 epitope of MSP-1, which has been demonstrated to be "promiscuous" in binding to MHC molecules represents an immunodominant epitope in naturally acquired premuniton. Contrary to this hypothesis, we were not able to prove any MSP-1$^{38\text{-}58}$ -specific T cell stimulation in blood samples of semi-immune individuals living in malaria endemic areas of Ghana and Tanzania (data not shown).

What is the reason for this lack of naturally acquired T cell responsiveness? The dimorphism of this region of MSP-1 may help the parasite to evade host immunity by preventing presentation of native antigen on MHC class II molecules or by induction of T cell anergy (Plebanski et al. 1997; Ramasamy 1998). On the other hand, the region could remain cryptic under natural processing conditions of the whole MSP-1 protein and would therefore not be presented efficiently to the immune system (Sercarz et al. 1993). In this case, however, variation at defined amino acid positions would represent no benefit for the parasite under immunological selection pressure. Peptide elution from surface MHC class II molecules of professional APCs after phagocytosis of whole MSP-1 protein could give insights which products are generated upon processing of the native antigen. Since our attempts failed to

stimulate MSP-1$^{38-58}$ specific T cell clones *in vitro* with purified MAD20 or FVO merozoites or with infected red blood cells from malaria cultures (data not shown), inefficient presentation of MSP-1 derived naturally processed peptides may in fact be an explanation for the lack of naturally acquired T cell reactivities. Another reason for missing stimulation of MSP-1 specific T cells with merozoites could be that the *in vitro* system with PBMC as APCs is not optimal. The lack of sufficient numbers of professional APCs like dendritic cells, which could take up parasites by phagocytosis and process antigens, could explain why T cell stimulation was only possible with synthetic MSP-1 peptides.

7.6 Can epitope focused vaccines elicit protective T cell responses?

One major question remains to be elucidated, whether MSP-1$^{38-58}$ specific T cells which were elicited by SPf66 vaccination can also recognize MSP-1 peptides derived by natural processing.

If anti MSP-1 responses are important for the protective efficacy of the SPf66 vaccine, our results may give indications why SPf66 vaccination yielded unsatisfactory results in clinical field trials (Graves and Gelband 2000) while vaccine efficacy was significant in the early *Aotus* and human challenge experiments. The FVO strain used for challenge experiments of *Aotus* monkeys exhibited the same sequence variant of the 83.1 epitope (Moreno 2001) as the one present in the vaccine (Patarroyo et al. 1987). Provided that the MSP-1 specific T cell response plays an important role in protection it is not surprising that these early experiments in which exactly the same sequence variant of MSP-1 was present in the pathogen like in the vaccine showed a high degree of protection. The malaria wild type strain used for human challenge experiments upon SPf66 vaccination (Patarroyo et al. 1988) was not clearly specified, but was presumably a strain with the same 83.1 sequence variant. Vaccinated volunteers were challenged with only one single strain and therefore the immune system was not faced with several variants of the semi-conserved region of block 1 of MSP-1. All following human phase III field trials were carried out in malaria endemic areas exhibiting a broad diversity of naturally occurring *P. falciparum* strains as demonstrated for the trial site in Tanzania (Jiang et al. 2000). The immune system of vaccinated volunteers living in endemic areas was therefore confronted with several different sequence variants of the 83.1 epitope at the same time. Assuming that immune evasion by APL antagonism for this region of MSP-1 exists, no protective T cell response can be build up in vaccinated individuals

which are naturally challenged with strains harboring different MSP-1 alleles. The high specificity of the MSP-1 specific T cell response elicited by SPf66 demonstrated here may indicate that SPf66 has significant protecting efficacy against homologous challenge but that the incorporation of semi-conserved epitopes is not a suitable choice for an epitope-focused vaccine. For future malaria vaccine development, it remains to be investigated whether dimorphism of the 83.1 epitope really provides APL antagonism. If semi-conserved epitopes are used for a vaccine, protection should be tested by challenge experiments including different malaria stains exhibiting different sequence variants of the selected epitope.

A main task for future epitope-focused malaria vaccine development will be the characterization of additional parasite derived epitopes, ideally conserved ones, which are able to provide protection. Essential parameters of host-pathogen interactions that either prevent or evoke protective immune responses have to be defined. The use of MHC-binding motifs required for peptides to form peptide-MHC complexes combined with the ease of large-scale peptide synthesis will make computational approaches very promising (Hagmann 2000). Positional scanning of synthetic combinatorial peptide libraries can be used to identify peptides capable of binding to MHC class II molecules and stimulating CD4+ T cells. The prediction of MHC class II restricted T cell epitopes based on allele specific binding motives in combination with available data of the malaria genome will be the most challenging task for future epitope focused vaccine development.

7.7 References

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8. Appendix

8.1 Preparation of an Antibody-Protein A Matrix

Cells and Antibodies

As source of human HLA class II DR and DP molecules, the EBV-transformed homozygous cell line BM-9 (DRB1*0801, DPA1*0103/DPB1*02012) was used. The hybridoma cell lines producing mAb L243 (anti-human HLA-DR), B7/21 (anti-human HLA-DP), and SPV-L3 (anti-human-DQ) were purchased from American Type Culture Collection (ATCC). All cell lines were grown under standard conditions in RPMI 1640 medium, supplemented with 10% heat-inactivated foetal calf serum, 100 units of penicillin/streptomycin, 2 mM glutamine, 10 mM HEPES and 46 µM 2-mercaptoethanol at 37 °C in a humidified atmosphere containing 5% CO_2. Hybridoma cell lines were propagated in a technomouse run over 2 months, supernatants with high antibody concentrations were harvested every other day for further antibody-purification.

Affinity purification of monoclonal antibodies

Monoclonal antibodies were purified by affinity chromatography on a rat-anti-mouse IgG-Sepharose 4B column. Hybridoma supernatants from technomouse runs were filtered through a 0.22 µm filter (Millipore), applied on a Sephadex G-50 pre-column and subsequently passed over a rat-anti-mouse IgG-Protein A-Sepharose 4B column, pre-equilibrated with phosphate buffered saline, pH 7.0 (PBS). The affinity column was then washed with 10-column volumes of PBS and monoclonal mouse antibodies were subsequently eluted with 0.1 M glycine, pH 2.7. A 1/25 volume of 2.0 M Tris, pH 9.5 was added immediately to the eluate to neutralise the pH. The eluate was dialysed against PBS, concentrated within dialysis tubes with aid of polyethylenglycol (PEG 35.000), again dialysed against PBS and stored at 4 °C until further use.

Preparation of Antibody-Protein A Matrix

MHC II-specific monoclonal antibodies were covalently coupled to Protein A-Sepharose CL-4B (Pharmacia) as described (Harlow and Lane, 1999, Using Antibodies manual). Protein A-Sepharose CL-4B beads were washed three times with 10 volumes of 0.2 M sodium borate, pH 9.0 by centrifugation at 3000g for 5 min. Monoclonal antibody was bound to Protein A-

Sepharose CL-4B in a ratio of 5 mg per milliliter of wet beads for 1 hour at room temperature with gentle rocking in 0.2 M sodium borate, pH 9.0. After two wash steps with 0.2 M sodium borate, pH 9.0, beads were resuspended in 10 volumes of the same buffer and an equivalent of 10 µl beads was removed for later check of coupling-efficiency on SDS-PAGE. To the rest of resuspended beads, solid dimethyl pimelimidate (DMP) was added to bring the final concentration to 20 mM. A pH above pH 8.3 is necessary for efficient coupling with DMP. After 30 min of incubation at room temperature, an equivalent of 10 µl beads was removed again for later check of cross-linking. Reaction was stopped by washing the beads once in 0.2 M ethanolamine, pH 8.0 and further incubation for 2 hours at room temperature in 0.2 M ethanolamine, pH 8.0 with gentle mixing. Finally beads were resuspended in phosphate buffered saline (PBS), pH 7.0 and packed into a column. The column was stored at 4 °C in PBS, 0.02% NaN_3 until further use.

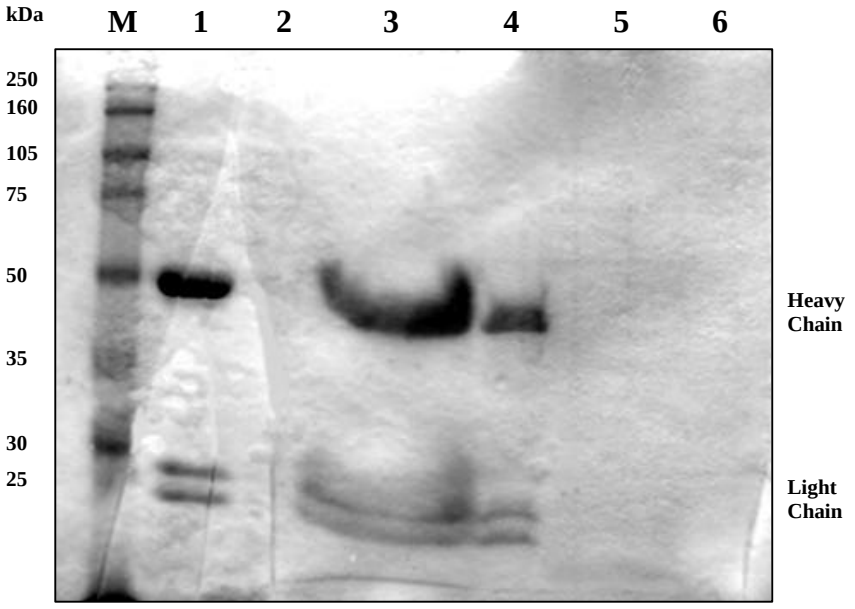


Figure 8.1.1. Check of coupling efficiency. Samples of beads taken before and after coupling were boiled in Laemmli sample buffer, run on a 10% SDS- polyacrylamide gel and stained with Coomassie blue. **M:** marker, **1:** purified mAb L-243 (10 µg), **2:** supernatant after Ab-binding to beads (20 µl), **3** and **4:** equivalents of 9 µl and 1 µl beads, respectively, before coupling, **5** and **6:** equivalents of 9 µl and 1 µl beads, respectively, after coupling. Cloned L-243 cells express two light chains with different molecular weights.

8.2 Cytokine production of DP-restricted MSP-1$^{38-58}$ specific T cell clones upon stimulation with pseudopeptide analogues (chapter 4)

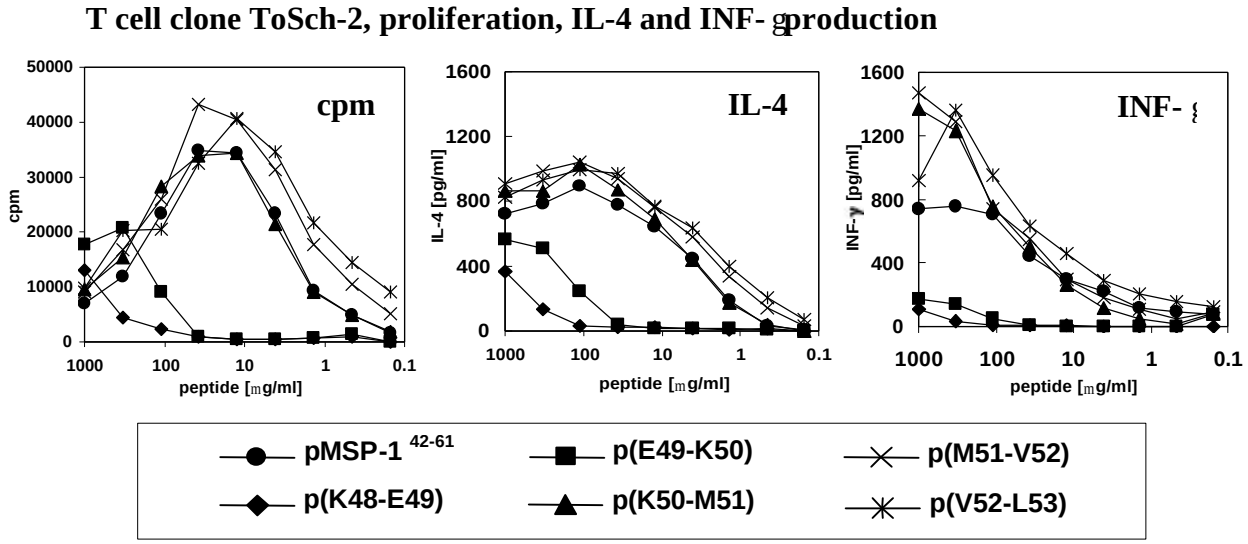


Figure 8.2.1. Proliferation (cpm), IL-4 and INF-γ production of the HLA-DP restricted T cell clone ToSch-2. T cells were stimulated with different concentrations of pMSP-1 $^{42-61}$ and its pseudopeptide derivatives. All assays were carried out in triplicates and were repeated twice. Mean values of one representative experiment are shown.

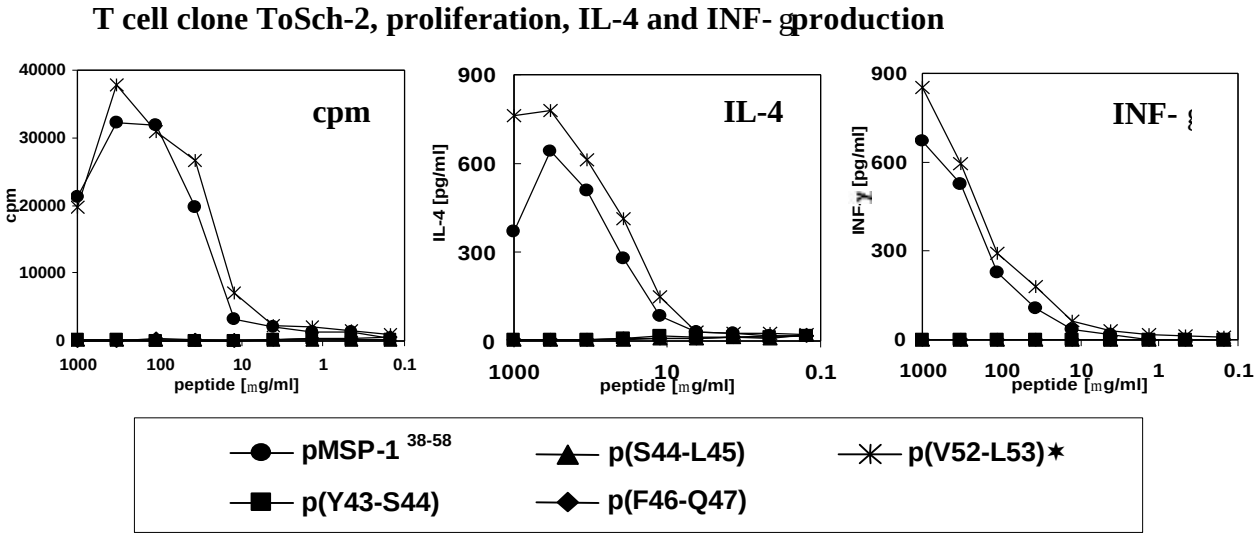


Figure 8.2.2. Proliferation (cpm), IL-4 and INF-γ production of the HLA-DP restricted T cell clone ToSch-2. T cells were stimulated with different concentrations of pMSP-1 $^{38-58}$ and its pseudopeptide derivatives. All assays were carried out in triplicates and were repeated twice. Mean values of one representative experiment are shown.

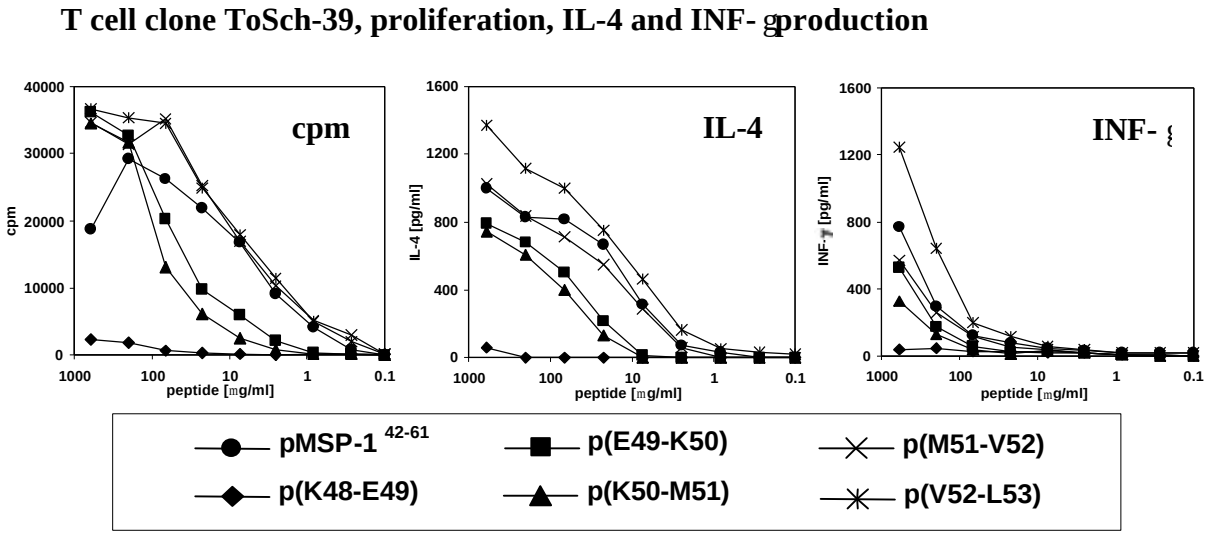


Figure 8.2.3. Proliferation (cpm), IL-4 and INF-γ production of the HLA-DP restricted T cell clone ToSch-39. T cells were stimulated with different concentrations of pMSP-1 $^{42-61}$ and its pseudopeptide derivatives. All assays were carried out in triplicates and were repeated twice. Mean values of one representative experiment are shown.

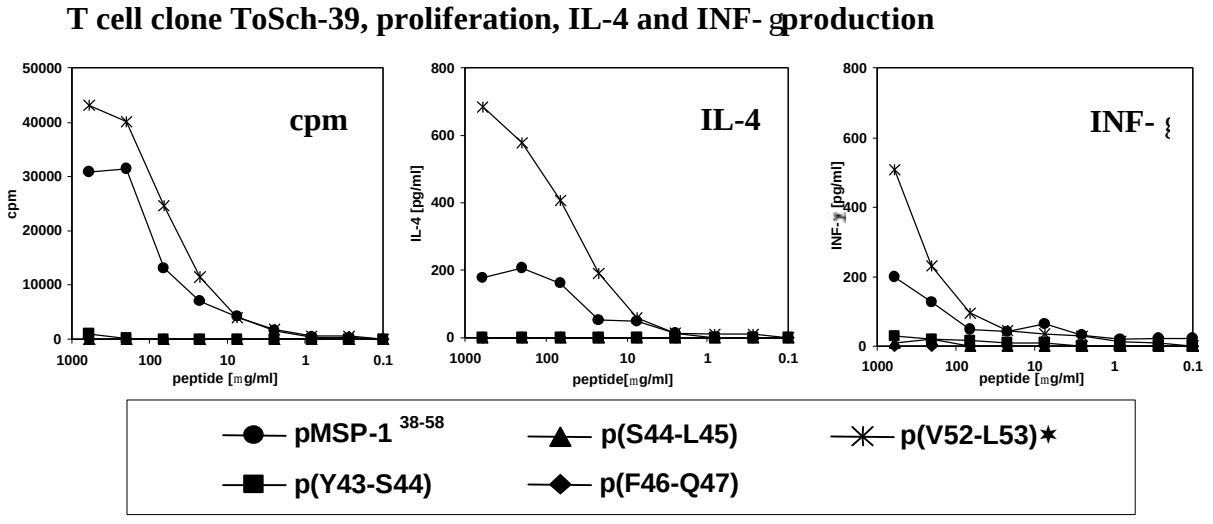


Figure 8.2.4. Proliferation (cpm), IL-4 and INF-γ production of the HLA-DP restricted T cell clone ToSch-39. T cells were stimulated with different concentrations of pMSP-1 $^{38-58}$ and its pseudopeptide derivatives. All assays were carried out in triplicates and were repeated twice. Mean values of one representative experiment are shown.

8.3 MSP-1 $^{38-58}$- and SPf66-specific T cell lines of SPf66/QS-21-vaccinated volunteers (chapter 6)

MSP-1$^{38-58}$- and SPf66-specific T cell lines of four SPf66/QS-21-vaccinated volunteers (#21, #29, #34 and #89) were generated and restimulated as described in chapter 6. Specificity was measured by proliferation assays after several rounds of peptide-specific restimulation.

Table 8.3.1. *Proliferative responses of MSP-1$^{38-58}$ specific T cell lines to MSP-1 peptides and SPf66 after **2** rounds of restimulation with MSP-1$^{38-58}$ peptide*

		MSP-1$^{38-58}$ specific T cell lines [a]			
peptide	**amino acid sequence**	**#21**	**#29**	**#34**	**#89**
(no peptide)		390)156 (1)	3333)1306 (1)	475)156 (1)	357)99 (1)
MSP-1$^{38-58}$	AVLTG**YSLFQKEKMVL**NEGTS	**65551**)1855 (**168**)	**8735**)781 (**3**)	**12764**)381 (**27**)	**13270**)721 (**37**)
83.1PNA	PNANP**YSLFQKEKMVL**PNANP	**75338**)6415 (**193**)	6057)567 (2)	**5269**)683 (**11**)	**10199**)1353 (**29**)
SPf66	-PNANP**YSLFQKEKMVL**PNANP-	**39313**)6036 (**101**)	6249)1456 (2)	**11982**)227 (**25**)	**12769**)3344 (**36**)

[a] Proliferation of T cells is expressed by cpm)SD (incorporated [^{3}H]-thyminine) and stimulation index (SI) in parentheses, which is the mean value of cpm in the presence of antigen divided by the mean value of cpm in the absence of antigen. T cells (2 x 10^4) were incubated with 2 x 10^4 irradiated autologous PBMC in the presence of 20 µg/ml antigen. All assays were performed in triplicates. SI values of 3 or higher were regarded as positive proliferation (bold letters).

Table 8.3.2. *Proliferative responses of MSP-1$^{38-58}$ specific T cell lines to MSP-1 peptides and SPf66 after **4** rounds of restimulation with MSP-1$^{38-58}$ peptide*

		MSP-1$^{38-58}$ specific T cell lines [a]			
peptide	**amino acid sequence**	**#21**	**#29**	**#34**	**#89**
(no peptide)		219)194 (1)	n.d.	28)4 (1)	n.d.
MSP-1$^{38-58}$	AVLTG**YSLFQKEKMVL**NEGTS	**46333**)10303 (**212**)	n.d.	**126**)38 (**5**)	n.d.
83.1PNA	PNANP**YSLFQKEKMVL**PNANP	**49148**)13309 (**224**)	n.d.	54)9 (2)	n.d.
SPf66	-PNANP**YSLFQKEKMVL**PNANP-	**35010**)9837 (**160**)	n.d.	**474**)11 (**17**)	n.d.

[a] Proliferation of T cells is expressed by cpm)SD (incorporated [^{3}H]-thyminine) and stimulation index (SI) in parentheses, which is the mean value of cpm in the presence of antigen divided by the mean value of cpm in the absence of antigen. T cells (2 x 10^4) were incubated with 2 x 10^4 irradiated autologous PBMC in the presence of 20 µg/ml antigen. All assays were performed in triplicates. SI values of 3 or higher were regarded as positive proliferation (bold letters). n.d. = not determined.

Table 8.3.3. *Proliferative responses of SPf66 specific T cell lines to MSP-1 peptides and SPf66 after **2** rounds of restimulation with SPf66*

peptide	amino acid sequence	SPf66 specific T cell lines [a] #21	#29	#34	#89
(no peptide)		174)45 (1)	233)38 (1)	247)74 (1)	166)61 (1)
MSP-1$^{38-58}$	AVLTG**YSLFQKEKMVL**NEGTS	**16048**)2440 (**92**)	**10586**)3024 (**45**)	**1328**)434 (**5**)	**441**)87 (**3**)
83.1PNA	PNANP**YSLFQKEKMVL**PNANP	**30962**)3833 (**178**)	**3394**)304 (**15**)	**982**)165 (**4**)	**2031**)644 (**12**)
SPf66	-PNANP**YSLFQKEKMVL**PNANP-	**22017**)15962 (**127**)	**13005**)510 (**56**)	**3923**)238 (**16**)	**4098**)779 (**25**)

[a] Proliferation of T cells is expressed by cpm)SD (incorporated [^{3}H]-thyminine) and stimulation index (SI) in parentheses, which is the mean value of cpm in the presence of antigen divided by the mean value of cpm in the absence of antigen. T cells (2 x 10^4) were incubated with 2 x 10^4 irradiated autologous PBMC in the presence of 20 µg/ml antigen. All assays were performed in triplicates. SI values of 3 or higher were regarded as positive proliferation (bold letters).

Table 8.3.4. *Proliferative responses of SPf66 specific T cell lines to MSP-1 peptides and SPf66 after **4** rounds of restimulation with SPf66*

peptide	amino acid sequence	SPf66 specific T cell lines [a] #21	#29	#34	#89
(no peptide)		209)98 (1)	140)54 (1)	44)9 (1)	n.d.
MSP-1$^{38-58}$	AVLTG**YSLFQKEKMVL**NEGTS	101)28 (0.5)	338)64 (2)	**78**)29 (2)	n.d.
83.1PNA	PNANP**YSLFQKEKMVL**PNANP	263)116 (1)	211)62 (2)	78)36 (2)	n.d.
SPf66	-PNANP**YSLFQKEKMVL**PNANP-	**4378**)899 (**21**)	**7423**)1766 (**53**)	**8446**)1443 (**192**)	n.d.

[a] Proliferation of T cells is expressed by cpm)SD (incorporated [^{3}H]-thyminine) and stimulation index (SI) in parentheses, which is the mean value of cpm in the presence of antigen divided by the mean value of cpm in the absence of antigen. T cells (2 x 10^4) were incubated with 2 x 10^4 irradiated autologous PBMC in the presence of 20 µg/ml antigen. All assays were performed in triplicates. SI values of 3 or higher were regarded as positive proliferation (bold letters). n.d. = not determined.

After 4 rounds of restimulation with MSP-1 $^{38-58}$ peptide or SPf66, respectively, T cell lines of volunteers #21, #29 and #34 exhibited a stong cytotoxic activity. Microscopic analysis of cell cultures and proliferation assays (Tables 8.3.1-8.3.4) revealed, that cytotoxic activity upon stimulation with MSP-1 $^{38-58}$ peptide was stronger for SPf66 specific T cell lines than for MSP-1$^{38-58}$ specific lines.

FACS analysis of MSP-1$^{38-58}$- and SPf66-specific T cell lines revealed that lines consisted of mixed populations of CD4+ and CD8+ T cells. Figure 8.3.1 shows FACS data of antigen specific T cell lines of volunteer #21. After three rounds of restimulation, the MSP-1 $^{38-58}$ specific line comprised 52% CD4+ and 26% CD8+ T cells (Figure 8.3.1.A) and the SPf66 specific T cell line contained up to 88% of CD8+ T cells (Figure 8.3.1.B).

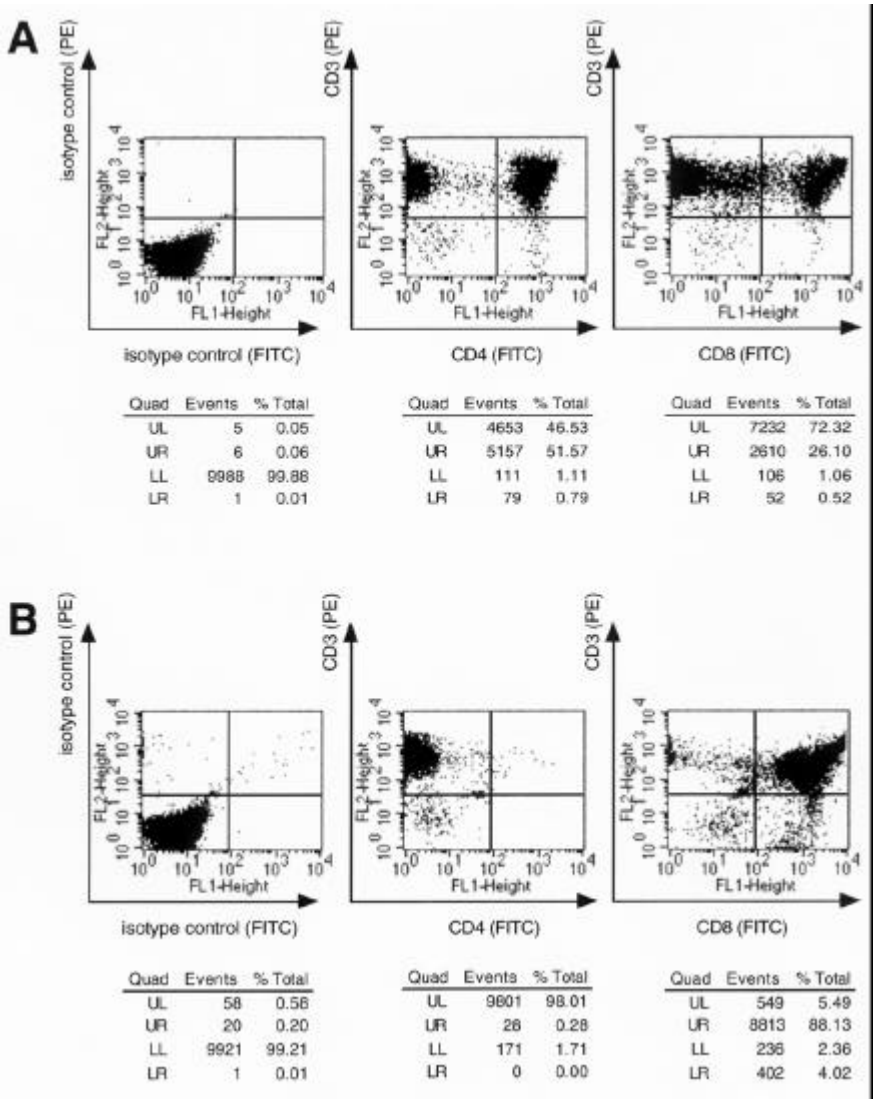
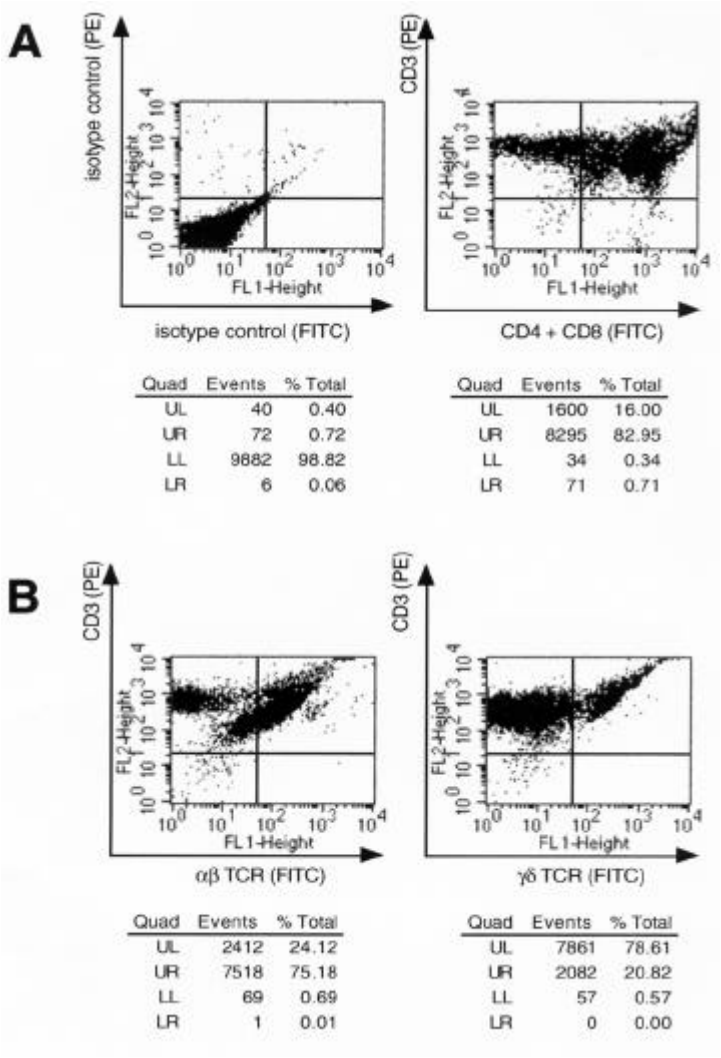


Figure 8.3.1. Flow cytometric analysis of MSP-1$^{38-58}$ specific T cell line (**A**) and SPf66 specific T cell line (**B**) of volunteer #21. Cells were stained with phycoerythrin- (PE-) labeled anti-CD3 and fluorescein-isothiocyanate (FITC-) labeled anti-CD4 or anti-CD8 monoclonal antibodies. Controls were stained with PE- and FITC- labeled IgG isotype controls. Quadrant statistics are shown for each corresponding plot including counted events and % of total cells.

The MSP-1$^{38-58}$ specific T cell line of volunteer #21 was analyzed in more detail. Double staining with anti-CD3 and anti-CD4+ CD8 antibodies showed that 16% of cells were double negative for CD4/CD8, but CD3 positive (Figure 8.3.2.A). Specific TCR-staining revealed that 75% of cells were αβTCR+ and 20% were γδTCR+ (Figure 8.3.2.B).



Quad	Events	% Total
UL	40	0.40
UR	72	0.72
LL	9882	98.82
LR	6	0.06

Quad	Events	% Total
UL	1600	16.00
UR	8295	82.95
LL	34	0.34
LR	71	0.71

Quad	Events	% Total
UL	2412	24.12
UR	7518	75.18
LL	69	0.69
LR	1	0.01

Quad	Events	% Total
UL	7861	78.61
UR	2082	20.82
LL	57	0.57
LR	0	0.00

Figure 8.3.2. Flow cytometric analysis of MSP-1$^{38-58}$ specific T cell line of volunteer #21. **A:** Cells were stained with phycoerythrin- (PE-) labeled anti-CD3 and fluorescein-isothiocyanate (FITC-) labeled anti-CD4 and anti-CD8 monoclonal antibodies. As control, cells were stained with PE- and FITC- labeled IgG isotype controls. **B:** Cells were stained with phycoerythrin- (PE-) labeled anti-CD3 and fluorescein-isothiocyanate (FITC-) labeled anti-αβTCR or anti-γδTCR monoclonal antibodies. Quadrant statistics are shown for each corresponding plot including counted events and % of total cells.

DNA fragmentation assay (JAM test) for measuring CTL activity

Cytotoxic activity of the MSP-1^{38-58} specific T cell line #21 (Figure 8.3.3) was measured by the JAM-assay essentially as described (Matzinger 1991; Tretiakova et al. 2000). As target cells, EBV-transformed B cells from volunteer #21 were labeled o/n with 5 µCi/ml [^{3}H] thymidine at 37 °C, 5% CO_2 in complete RPMI 1640 medium containing 10% inactivated FCS, 10 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 50 µM 2-mercaptoethanol. Cells were washed to stop labeling and resuspended to 5 x 10^5/ml in assay medium (complete RPMI 1640 medium with 10 % heat-inactivated human AB serum instead of FCS) with 20 µg/ml SPf66 as antigen. After 3 h of peptide loading, EBVs were washed again and resuspended to 1 x 10^5/ml in assay medium. T cells were washed once, resuspended to 2 x 10^6/ml in assay medium and plated into 96-well (200 µl) round bottomed microwells. T cells were serially 3-fold diluted in final volumes of 100 µl per well before addition of 100 µl (1 x 10^4) ^{3}H-labeled targets (T). CTL response of effector T cells (E) was assayed at E:T ratios of 20:1, 6.6:1, 2.2:1, 0.74:1 and 0.25:1 for 4.5 h at 37°C and harvested using a cell harvester. The percentage of specific killing was determined using the formula: % specific cytotoxicity = [(S-E)/S]x100, where S is the amount of the DNA retained by the target cells in the absence of effector cells (spontaneous-release in cpm) and E is the cpm value obtained with effector and target cells together.

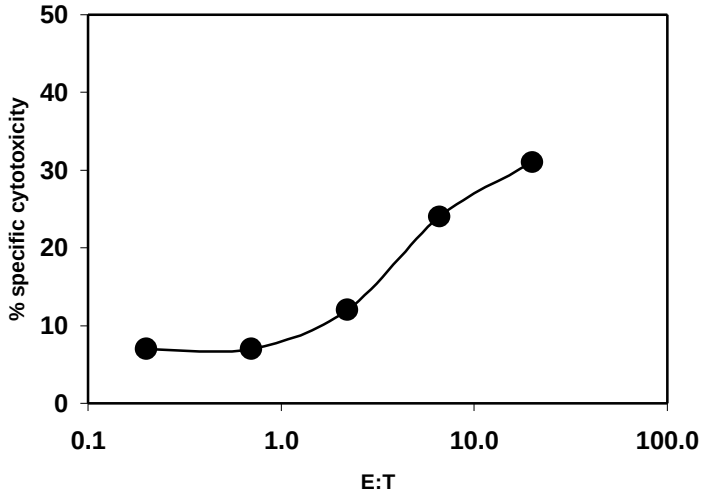


Figure 8.3.3. CTL assay of MSP-1^{38-58} specific T cell line #21. Cytotoxic activity was measured at different E:T ratios (X-axis). Nonspecific spontaneous lysis of targets was 12%.

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1989 – 1991	Medical-Laboratory-Technician at the University of Freiburg i.Br., Division of Rheumatology and Immunology, PD Dr. B. Lang. Research work on: *The Transfection of a Mouse-Macrophage Cell-line with specific Mutations of HLA-DR-genes to analyse Cell-type specific Antigen-processing and presentation*.
1995 – 1996	M.Sc. Thesis at the Swiss Tropical Institute, Division of Medical Parasitology, PD Dr. R. Kaminsky. *The Effect of (S)-9-(3-hydroxy-2-phosphonyl- methoxypropyl)-adenine [HPMPA] on DNA Synthesis of African Trypanosomes.*

1996 – 1997	Biologist at the Swiss Tropical Institute, Division of Medical Parasitology, Prof. Dr. L. Jenni. *In vitro Metacyclogenesis of African Trypanosomes.*
1997 - 2001	PhD Thesis at the Swiss Tropical Institute, Division of Molecular Immunology, PD Dr. G. Pluschke. *Human T-Cell responses to a semi-conserved sequence of the malaria vaccine candidate antigen MSP-1.*

Work abroad:

1997	Lab work and training of local staff at the Livestock Health Research Institute, Tororo, Uganda,
2000	Lab work at the Navrongo Health Research Center, Navrongo, Ghana.

Courses Attended:

1992	Trainee and Practical course at CIBA-GEIGY, Basel, Division of Pharmacological Analysis / Biotechnology, Dr. Günther. *Optimization of an ELISA for a Recombinant Protein and Western-Blot-Analysis.*
1994	Course of Light- and Electron-microscopy at the Interdepartmental Electron Microscopy and Maurice E. Müller Institute, Biozentrum, Basel.

Thesis committee:	PD Dr. G. Pluschke, Swiss Tropical Institute Prof. Dr. T. Bickle, Biozentrum Basel Prof. Dr. N. Weiss, Swiss Tropical Institute Prof. Dr. L. Jenni, MGU

PUBLICATIONS

Nickel B (1995) The Effect of (S)-9-(3-hydroxy-2-phosphonyl-methoxypropyl)-adenine [HPMPA] on DNA Synthesis of African Trypanosomes. MSc Thesis, University of Basel, Switzerland.

Daubenberger C; Lang B, Nickel B, Willcox N and Melchers I (1996) Antigen Processing and Presentation by a Mouse Macrophage-like Cell Line expressing Human HLA Class II Molecules. *Internat. Immunology* 8, 307-315.

Kaminsky R, Nickel B, Holy A (1998) Arrest *of Trypanosoma brucei rhodesiense* and *Trypanosoma brucei brucei* in the S -Phase of the Cell Cycle by (S)-9-(3-hydroxy-2 phosphonylmethoxy- propyl)adenine [(S)- HPMPA]. *Mol. Biochem. Parasitol.* 93, 91-100.

Daubenberger C, Nickel B, Hübner B, Siegler U, Meinl E and Pluschke G (2001) *Herpesvirus saimiri* transformed T cells and peripheral blood mononuclear cells restimulate identical antigen specific human T cell clones. *J Immunol Meth,* 254, 99-108.

Kashala O, Amador R, Valero MV, Moreno A, Barbosa A, Nickel B, Daubenberger C, Guzman F, Pluschke G and Patarroyo ME, Safety and immunogenicity of new formulations of the *Plasmodium falciparum* malaria peptide vaccine SPf66 combined with the immunological adjuvant QS-21. Accepted at *Vaccine.*

INVITED SEMINARS

Nickel B, Daubenberger C, Siegler U, Pluschke G, Clonal Analysis of the Human T-Cell Response to a Malaria Antigen, 11th Meeting of the Swiss Immunology PhD-Students, Ermatingen, March 1999.

Nickel B, Human T-Cell Response to a Malaria Antigen, Open-Seminar at the STI, Basel, December 1999.

Nickel B, Clonal Analysis of MSP-1 specific T cells, Navrongo Health Research Centre, Navrongo, Ghana, March 2000.

Nickel B, Siegler U, Daubenberger C, Rossi L, Lozano JM, Pluschke G, Fine-Specificity of Human MSP-1 reactive T-Cells, Meeting of PhD Students in Parasitology and Tropical Medicine, Münchenwiler, August 2000.

Nickel B, Human T-cell response to MSP-1, Invitation to the Institute of Biochemistry, Prof. Dr. J. Louis, University of Lausanne, September 2000.

Nickel B, Daubenberger C, Siegler U, Rossi L, Hübner B, Lioy E, Poeltl-Frank F, Pluschke G, Clonal Analysis of the Human T-cell response to the Malaria Antigen MSP-1, Annual Congress of the Swiss Society of Tropical Medicine and Parasitology, Fribourg, November 2000.

Nickel B, Functional Analysis of human T cell responses specific for a semi-conserved sequence of MSP-1 of *P. falciparum*, Invitation to the Institute of Virology and Immunoprophylaxis, Dr. A. Summerfield, Mittelhäusern, April 2001.

Nickel B, Anti-Parasitic Strategies: Targeting DNA-Synthesis of Trypanosomes and Surface Proteins of Malaria Parasites, Invitation to Morphochem, Dr. W. Keck, Basel, July 2001.

SYMPOSIUM CONTRIBUTIONS

Nickel B, Schmid C, Holy A and Kaminsky R; HPMPA arrests Trypanosomes in the S-Phase of the Cell Cycle, 23rd Meeting of the Federation of European Biochemical Societies (FEBS) Basel, August 1995, poster.

Nickel B, Kaminsky R and Jenni L; Metacyclogenesis of *Trypanosoma brucei* in vitro, 14th Swiss Trypanosomatid Meeting, Les Diablerets, February 1997, poster.

Nickel B, Daubenberger C, Siegler U, Pluschke G, T-cell response to the N-terminus of the Merozoite Surface Protein 1 of *Plasmodium falciparum*, 10th Meeting of the Swiss Immunology PhD-Students, Ermatingen, March 1998, poster.

Poeltl-Frank F, Nickel B, Daubenberger C, Lioy E, Huebner B, Pluschke G, Fine-specificity of *Plasmodium falciparum* MSP-1 specific Human T-cell Clones, Annual Congress of the Swiss Society of Tropical Medicine and Parasitology, Fribourg, November 2000, poster.