Protective CD8$^+$ T Cell Responses against the Pre-erythrocytic Stages of Malaria Parasites: an Overview

J Oliveira-Ferreira$^+$, CT Daniel-Ribeiro

Laboratório de Pesquisas em Malária, Instituto Oswaldo Cruz-Fiocruz (WHO Collaborating Center for Research and Training in Immunology of Parasitic Disease), Av. Brasil 4365, 21045-900 Rio de Janeiro, RJ, Brasil

CD8+ T cells have been implicated as critical effector cells in protection against the pre-erythrocytic stage of malaria in mice and humans following irradiated sporozoite immunization. Immunization experiments in animal models by several investigators have suggested different strategies for vaccination against malaria and many of the targets from liver stage malaria antigens have been shown to be immunogenic and to protect mice from the sporozoite challenge. Several prime/boost protocols with replicating vectors, such as vaccinia/influenza, with non-replicating vectors, such as recombinant particles derived from yeast transposon (Ty-particles) and modified vaccinia virus Ankara, and DNA, significantly enhanced CD8+ T cell immunogenicity and also the protective efficacy against the circumsporosoite protein of Plasmodium berghei *and* P. yeti. *Based on these experimental results the development of a CD8+ T cell inducing vaccine has moved forward from epitope identification to planning stages of safety and immunogenicity trials of candidate vaccines.*

Key words: malaria - cytotoxic T lymphocyte (CTL) responses - CTL and helper peptides - CD8$^+$ and CD4$^+$ epitopes - recombinant vectors - Ty-virus like particles (VLP)

Malaria remains one of the major causes of disease and death in the tropics. There are an estimated 300-500 million cases of malaria each year, resulting in over 1,5 to 2,7 million deaths, mainly of children under five years old in Africa (WHO 1997). The development of an effective vaccine represents one of the most promising alternatives to control the disease and over the past decade, there has been considerable progress in the identification of candidate vaccine antigens and their genes. This overview describes recent advances in the area of cell-mediated immunity with emphasis on cytotoxic T cells directed against sporozoite and liver stage antigens of malaria parasites and their role in protection.

It is well documented that immunization of mice (Nussenzweig et al. 1967), monkeys (Nussenzweig et al. 1970) and humans (Clyde et al. 1973) with radiation attenuated sporozoites of *Plasmodium* species protects against challenge with live sporozoites, but does not protect against challenge with infected erythrocytes. Therefore, the protective immunity induced by irradiated sporozoites is stage specific and must be against either extracellular sporozoites prior to their invading the hepatocytes or parasites developing within the hepatocytes. The sera from mice (Vandenberg et al. 1969) and humans (Clyde et al. 1973) immunized with irradiated sporozoite precipitate the surface coat of sporozoites (CS protein). Because of this, for many years antibodies against sporozoites were thought to be the major immune effectors responsible for the observed protection.

However, in 1977 it was reported that µ-suppressed mice immunized with irradiated sporozoites were protected against sporozoites challenge (Chen et al. 1977) and several years later it was shown that: (a) *in vivo* depletion of CD8$^+$ T cells drastically reduced protective immunity against a challenge with live sporozoites (Schofield et al. 1987, Weiss et al. 1988); (b) passive transfer of cloned murine CD8$^+$ T cells, specific for the circumsporozoite (CS) protein or the sporozoite surface protein 2 (SSP2), conferred protection to mice (Romero et al. 1989, Rodrigues et al. 1991, Khusmith et al. 1991) and (c) treatment of irradiated sporozoite immunized mice with monoclonal antibody against IFN-γ or with an inhibitor of inducible nitric oxide synthetase also eliminated protection (Schofield et al. 1987, Seguin et al. 1994).

The CD8$^+$ T cells have been implicated as critical effectors cells in protection against the pre-erythrocytic stage of malaria. The target of these malaria specific CD8$^+$ T cells is most likely to be

The authors dedicate this publication in honor of the Instituto Oswaldo Cruz, on the occasion of the 1st Centenary of its foundation, May 25th, 1900.

$^+$Corresponding author. Fax: +55-21-280.1589. E-mail: lila@gene.dbbm. fiocruz.br

Received 26 April 2000
Accepted 18 September 2000

the infected hepatocyte, which presents, on its surface, parasite-derived epitopes in association with class I MHC molecules. The $CD8^+$ T cells subsequently secrete IFN-γ, which in turn induces the infected hepatocytes to produce nitric oxide that eliminates the infected hepatocytes or inactivates the intracellular parasite (Schofield et al. 1987, Nussler et al. 1993, Seguin et al. 1994, Klotz et al. 1995).

This hypothesis is supported by studies which indicated that in the rodent model, four antigens expressed in hepatocytes have been established as targets for the protective $CD8^+$ T cell responses: the surface antigens of the sporozoites *P. berghei* (PbCSP) and *P. yoelii* (PyCSP and PySSP2) and the protein expressed on the surface of hepatocytes and erythrocytes – PyHEP17. The *P. falciparum* homologues of these antigens, PfCSP, PfSSP2/TRAP and PfExp-1, respectively, have been shown to be targets for $CD8^+$ T cells after immunization with radiation-attenuated sporozoites and after natural exposure to *P. falciparum* (Rogers et al. 1992, Doolan et al. 1996a), as well as a fourth antigen, *P. falciparum* liver stage antigen 1- LSA1 (Guerin-Marchand et al. 1987).

While several antigens appear to be involved in protection, so far, a single protective epitope has been identified in the murine *P. yoelii* and *P. berghei* systems. These epitopes are located in the CS protein and their amino acid sequences are SYVPSAEQI and SYIPSAEKI respectively (Romero et al. 1989, Weiss et al. 1990). In the *P. falciparum* CS protein, a $CD8^+$ epitope was identified using murine T cells obtained from B10.BR (H-2k) mice immunized with a vaccinia vector expressing the entire *P. falciparum* CS protein. $CD8^+$ T cells from mice immunized with *P. falciparum* sporozoites also recognized this epitope. Furthermore, the same epitope sensitized target cells for lysis by $CD8^+$ T cells derived from three out of four sporozoite-immunized volunteers (Mallik et al. 1991).

During the past decade, the major efforts on pre-erythrocytic malaria vaccine development have focused on inducing antibodies to the sporozoite surface and $CD8^+$ T cells against infected hepatocytes; however, there is now considerable interest in protective $CD4^+$ T cell responses against infected hepatocytes.

New data indicates that in several strains of mice, protection induced by irradiated sporozoites can be eliminated by treatment of mice with anti-$CD4^+$ antibodies or anti-$CD8^+$ antibodies. The $CD4^+$ T cells specific for the CS protein produced IFN-γ, IL-2 and IL-6 (Tsuji et al. 1990, Renia et al. 1991, 1993). These authors have also demonstrated that $CD4^+$ T cell clones against PyCSP eliminated infected hepatocytes from culture and conferred protection when adoptively transferred to naive animals (Renia et al. 1991). Immunization with synthetic peptides from two liver stage proteins induces consistent, genetically restricted, $CD4^+$ T cell and IFN-γ dependent protection (Wang et al. 1996).

In view of these encouraging results, the challenge for the future is to develop a pre-erythrocytic vaccine capable of inducing not only protective antibodies against sporozoites but also $CD4^+$ and $CD8^+$ T cell responses against the hepatocytes. Remarkable progress in research on these forms in recent years has led to better understanding of the biology of these forms and attempts are currently being made to induce protective anti-plasmodia T cells *in vivo*, using live and synthetic immunogens.

INDUCTION OF ANTI-MALARIA $CD8^+$ T CELLS USING LIVE RECOMBINANT BACTERIAL AND VIRAL VECTORS

Recombinant live bacteria and viruses expressing immunologically relevant antigens are attractive delivery systems, designed to elicit protective immunity against microbial pathogens. The most widely studied live virus vehicle is the recombinant vaccinia virus, which tolerates insertions of large segments of foreign DNA and replicates within the cytoplasm of the host cell. The $CD8^+$ CTL epitope in *P. falciparum* CS protein was originally defined using cells obtained from mice immunized with a recombinant vaccinia virus expressing the CS protein (Kumar et al. 1988).

In subsequent studies, live viral vectors expressing the appropriate proteins and epitopes have been used as promising vaccine candidates (Li et al. 1992, Rodrigues et al. 1994, Murata et al. 1996). In these studies, conditions which influence the $CD8^+$ T cell responses induced by immunization of mice with different recombinant viruses were also identified and analyzed. These live vectors were engineered to express either the entire sequence of the *P. yoelii* CS protein or only the H-$2K^d$ restricted epitope, SYVPSAEQI. Using this system, a certain degree of protective immunity against this parasite could be induced by a single immunization with either virus but a second dose of the same recombinant virus failed to enhance this immunity (Li et al. 1993, Rodrigues et al. 1994, Murata et al. 1996). The immunization with recombinant vaccinia virus expressing only the $CD8^+$ epitope was as efficient as with the version expressing the entire CS protein. In contrast, protective immunity induced with influenza virus could only be achieved when it expressed the CS B cell epitope (Rodrigues et al. 1994). The magnitude of these responses depended on the type of vector used for

immunization and appeared to be strictly dose dependent (Murata et al. 1996).

On the other hand, immunization with recombinant vaccinia virus after priming with influenza virus significantly increased the protective immunity (Li et al. 1993, Rodrigues et al. 1994, Murata et al. 1996). Furthermore, with this sequence of immunization, a strong inhibition (more than 90%) of parasite development in the liver was observed and approximately 50 to 70% of immunized mice failed to display parasitemia (Li et al. 1992, Rodrigues et al. 1994, Murata et al. 1996).

More recently, studies on evaluating the immunogenicity of a replication-defective recombinant adenovirus expressing the CS protein of *P. yoelii* showed that a single immunizing dose of 109 plaque forming units (pfus) of this recombinant virus induced a large number of CS-specific CD8+ and CD4+ T cells in the spleen of immunized mice and also induced high titers of anti-sporozoite antibodies, particularly when given by the subcutaneous or intramuscular route. Most importantly, a single dose of the recombinant virus resulted in a 93% inhibition of the liver stage development of the parasite, as measured by the level of parasite rRNA present in the liver of immunized mice, after sporozoite challenge. Furthermore, 40% of the immunized mice displayed complete resistance to infection. CD8+ T cells were the principal mediators of this protective effect, as shown by a greatly reduced level protective immunity following the depletion of this T cell subset (Rodrigues et al. 1997).

A highly attenuated strain of vaccinia virus (NYVAC) has been used to develop a multistage vaccine for malaria by including genes encoding seven *P. falciparum* antigens derived from sporozoite, liver, blood and ookinete stages. This vaccine designated NYVAC-Pf7 elicited high titers of specific antibodies in Rhesus monkey (Tine et al. 1996). In Phase I and II trials in humans, the vaccine proved to be safe and well tolerated but presented variable immunogenicity. Most of the vaccinated individuals presented a delayed time to onset of parasitemia but only 1 out of 35 failed to develop infection (Ockenhouse et al. 1998).

Bacterial vectors have also been used to induce CS specific $CD8^+$ CTL in immunized mice. Initial studies using an attenuated strain of *Salmonella typhimurium* transformed with plasmid expressing the CS gene of *P. berghei* and *P. yoelii* demonstrated the capacity of this vector to induce $CD8^+$ T cell dependent immune protection in 55%-70% of immunized BALB/c mice (Sadoff et al. 1988, Aggarwall et al.1990, Flyn et al. 1990). This approach was recently evaluated in humans immunized orally with recombinant *S. typhi* expressing the CS protein of *P. falciparum.* In that study, only 3 out of 10 volunteers mounted an antibody response and 1 produced CSP specific $CD8^+$ T cells (Gonzales et al. 1994).

The studies described above indicate that the use of live recombinant viruses is an attractive system for vaccine design based on the induction of $CD8^+$ T cells against intracellular pathogens. However there are restrictions with regard to the induction of secondary $CD8^+$ T cells or the *in vivo* expansion of antigen specific $CD8^+$ T cells, since this can only be achieved under very specific conditions of immunization. In addition, such vectors are difficult to modify and also have the potential to cause diseases in immuno-compromised hosts, or even in healthy individuals if the attenuated vector reverts to a virulent phenotype.

INDUCTION OF $CD8^+$ T CELLS USING SYNTHETIC, NON-INFECTIVE IMMUNOGENS

The use of simple proteins or synthetic peptides to induce CTL responses has also been explored. Free short peptides are poor immunogens for CTL and several approaches have been used to overcome this difficulty including the use of adjuvants, the chemical modification of peptides and the addition of helper epitopes.

A peptide containing a $CD8^+$ T cell T epitope of the *P. berghei* CS protein, when modified by the addition of a palmitoyl-cys-ser lipid tail, induced murine $CD8^+$ T cells specific for parasites (Deres et al. 1989). However, high cell densities of responding T cells by sensitization either *in vivo* or *in vitro* with a short peptide could not easily be achieved (Widmann et al. 1992, Valmori et al. 1994, Allsopp et al. 1996).

The induction of $CD8^+$ T cells could be achieved by immunization with a synthetic peptide corresponding to the CTL epitope of the *P. yoelii* and *P. berghei* CS protein, however the number of antigen specific $CD8^+$ T cells induced, was relatively modest. This response could be enhanced by the administration of this peptide together with peptides corresponding to $CD4^+$ T cell epitopes (Romero et al. 1992, Widmann et al. 1992, Valmori et al. 1994).

Protection against malaria by a synthetic peptide vaccine designed to induce $CD8^+$ CTL response against a single class I restricted epitope has been reported in few studies but the best result could only protect 40% of the challenged animals (Migliorini et al. 1993, Franke et al. 1997). More importantly, in peptide-primed animals, secondary $CD8^+$ T cell responses could not be induced by a second dose of synthetic peptides or by the use of a recombinant vaccinia virus expressing the CS protein (Murata et al. 1996, Oliveira-Ferreira et al. 2000).

Recently, a new semi-synthetic system in which proteins self-assembled in particles, such as the particle-forming p1 protein encoded by the TYA gene of the yeast retrotransposon Ty, has been developed (Adams et al. 1987, Kingsman & Kingsman 1988). When this protein is expressed in yeast, high levels of virus like particles (Ty-VLP) are produced and proteins of interest can be fused to the C-terminus of the p1 protein, resulting in a hybrid Ty-VLP. In this system, defined protein sequences can be expressed without loss of their particulate structure, which has been reported to facilitate the generation of high levels of antibody and T cell responses to a number of antigens (Griffiths et al. 1991, Harris et al. 1993, Layton et al. 1993). Since protein sequences can be easily inserted and expressed by Ty-VLP, this system is very attractive for the eventual development of vaccines using non-replicating subunits.

Immunization with Ty-VLP carrying a $CD8^+$ T cell epitope of the *P. berghei* CS protein (TyPb) resulted in an immune response (Allsopp et al. 1996). This study compared four adjuvants designed for the use with peptide, lipopeptides, recombinant salmonella, vaccinia virus and an attenuated vaccinia, plasmid DNA and Ty-VLPs. Only the lipopeptide and Ty-VLPs were able to stimulate CTL in all immunized mice. Subcutaneous immunization with Ty-VLPs without any adjuvant resulted in high levels of CTL and these CTL T cells were still present ten weeks after a single immunization.

Although Ty-VLP particles carrying a $CD8^+$ T cell epitope of the CS protein of *P. berghei* and *P. yoelii* can induce CS specific $CD8^+$ T cells, protection has only been achieved when mice were primed with the particles and boosted with vaccinia recombinant expressing the whole CS protein. This sequence of immunization induced high numbers of specific $CD8^+$ T cells, inhibited the liver stage development (94%) and protected 62-95% of the mice from sporozoites infection (Plebanski et al. 1998, Oliveira-Ferreira et al. 2000).

These data suggest that particulate antigens in the form of hybrid Ty-VLP are potent immunogens and have the potential to constitute a multistage vaccine against malaria. Since protein sequences can be easily inserted and expressed by Ty-VLP, this system is very attractive for the eventual development of vaccines using non-replicating subunits.

INDUCTION OF $CD8^+$ T CELLS USING DNA PLASMID

The demonstration that plasmid DNA can induce antigen encoded specific immune responses following intramuscular or intradermal injection or following administration of DNA in gold particles has suggested a different approach to vaccine development. The simplicity of the DNA approach implies that it is possible to combine many DNA sequences encoding different antigens and thereby broaden the immune response. By incorporating a complete antigen rather than few epitopes, DNA immunization may circumvent the problem of genetic restriction of the protective immune response to target epitopes.

Several research groups are working on the production of DNA vaccines against malaria and the results obtained so far, using animal models of malaria, are encouraging. It was first reported that BALB/c mice are protected against sporozoite challenge by intramuscular immunization with plasmid DNA encoding the PyCS gene; 56% of mice were protected against challenge with sporozoites and the levels of induced CTL were significantly higher than the levels induced by protective immunization with irradiated sporozoites (Sedegah et al. 1994). However, it was demonstrated that this protective immunity was genetically restricted; 86% of BALB/c mice were protected while only 20% of the other four strains of mice studied were protected (Doolan et al. 1996a).

In contrast, immunization with plasmid DNA expressing the hepatocyte erythrocyte protein 17 (PyHEP17) gave more than 20% (up to 86%) of protection for three of five strains of inbred mice. Immunization with a mixture of the two plasmids circumvented some of the genetic restriction of protection, which, otherwise, was dependent on $CD8^+$ T cells, IFN-γ and nitric oxide (Doolan et al. 1996b).

Recently sequential immunization with plasmid DNA encoding *P. yoelii* CS protein and with a recombinant vaccinia virus expressing the CS protein was found to generate a high $CD8^+$ T cell response and to contribute to a substantial protection in mice (Schneider et al. 1998, Plebanski et al. 1998).

In the non-human primate model, vaccination using four plasmids encoding various malaria genes has been found to be immunogenic. In addition, administration of each plasmid alone was as effective at inducing $CD8^+$ T cells as was the administration of a mixture of the four plasmids (Wang et al. 1998).

The first human vaccination trials with DNA plasmid encoding the CS protein of *P. falciparum* showed that malaria naive volunteers developed specific $CD8^+$ T cells directed against ten peptides tested and these responses were genetically restricted by multiple HLA alleles (Wang et al. 1998). However, the magnitude of the CTL response seen

in some of the volunteers was considerably higher than the response seen in humans exposed to irradiated sporozoites or natural infection (Mallik et al. 1991, Sedegah et al. 1992, Doolan et al. 1993).

Although DNA vaccines are promising, the issue of long-term safety has not yet been assessed. In addition, vaccination in mice with plasmid DNA led to the generation of anti-DNA antibodies, although these antibodies did not initiate or accelerate the course of an autoimmune disease (Mor et al. 1997). Therefore, even if immunogenicity can be improved it will take several years before the mass vaccination of children with DNA can be achieved, owing to safety concerns.

CONCLUDING REMARKS

Studies in murine systems have shown that it is possible to induce the number and quality of $CD8^+$ T cells, both of which appear to be necessary to stop the progression of malaria infection beyond the liver stages. These findings provide the rationale and the experimental basis for the development of immunogens capable of inducing both, humoral and T cell immune responses. This combination of immune effector mechanisms is likely to be much more efficient in protecting against infection with malaria parasites. We expect that studies using these systems will help to further advance investigations aimed at characterizing the protective anti-malaria immune mechanisms in humans and will eventually contribute to the development of a safe and efficient vaccine.

REFERENCES

Adams SE, Dawson KM, Gull K, Kingsman SM, Kingsman AJ 1987. The expression of hybrid HIV:Ty virus like particles in yeast. *Nature 329*: 68-70.

Aggarwal A, Kumar S, Jaffe R, Hone D, Gross M, Sadoff J 1990. Oral *Salmonella*: malaria circumsporozoite recombinants induce specific $CD8^+$ cytotoxic T cells. *J Exp Medicine 172*: 1083-1090.

Allsopp CEM, Plebanski M, Gilbert S, Sinden RE, Harris S, Frankel G, Dougan G, Hioe C, Nixon D, Paoletti E, Layton G, Hill AVS 1996. Comparison of numerous delivery systems for the induction of cytotoxic T lymphocytes by immunization. *Eur J Immunol 26:* 1951-1959.

Chen DH, Tigelaar RE, Weinbaum FI 1977. Immunity to sporozoite-induced malaria infection in mice. I the effect of immunization of T and B cell deficient mice. *J Immunol 118*: 1322-1327.

Clyde DF, Most H, McCarthy VC, Vanderberg JP 1973. Immunization of man against sporozoite-induced falciparum malaria. *Am J Med Science 266:* 169-177.

Deres K, Schild H, Weismuller KH, Jung G, Rammensee HG 1989. *In vivo* priming of virus-specific cytotoxic T lymphocytes with synthetic lipopeptides vaccine. *Nature 342:* 561-563

Doolan DL, Hedstrom RC, Rogers W, Charoenvit Y, Rogers W, de la Vega M, Hoffman SL 1996a. Identification and characterization of the protective hepatocyte erythrocyte protein 17kDa gene of *Plasmodium yoelii*, homologous of *Plasmodium falciparum* exported protein 1. *J Biol Chem 271*: 17861-17868.

Doolan DL, Khanboonruang C, Beck HP, Houghten RA, Good MF 1993. Cytotoxic lymphocyte T cell low-responsiveness to the *Plasmodium falciparum* circumsporozoite protein in naturally exposed endemic population: analysis of human CTL response to most known variants. *Int Immunol 5:* 37-46.

Doolan DL, Sedegah M, Hedstrom RC, Hobart P, Charoenvit Y, Hoffman SL 1996b. Circumventing genetic restriction of protection against malaria with multi-gene DNA immunization: $CD8^+$ T cell, interferon-γ and nitric oxide dependent immunity. *J Exp Med 183*: 1739-1746.

Flynn JL, Weiss WR, Norris KA, Seifer HS, Kumar S, So M 1990. Generation of cytotoxic T-lymphocyte response using a *Salmonella* antigen-delivery system. *Mol Microbiol 4*: 2111-2118.

Franke ED, Corradin G, Hoffman SL 1997. Induction of protective CTL response against the *Plasmodium yoelii* circumsporozoite protein immunization with peptides. *J Immunol 159:* 3424-3433.

Gonzalez C, Hone D, Noriega FR, Tacket CO, Davis JR, Losonsky G, Nataro JP, Hoffman S, Malik A, Nardin E, Sztein MB, Heppner DG, Fouts TR, Isibasi A, Levine MM 1994. *Salmonella typhimurium* vaccine strain CVD 908 expressing the circumsporozoite protein of *Plasmodium falciparum*: strain construction safety and immunogenicity in humans. *J Infec Dis 169*: 927-931.

Griffiths JC, Berrie EL, Holdsworth L, Moore JP, Harris SJ, Senior JM, Kingsman AJ, Adams SE 1991. Induction of high-titer neutralizing antibodies, using hybrid human immunodeficient virus V-3:Ty virus like particles in a clinically relevant adjuvant. *J Virol 65*: 450-456.

Guerin-Marchand C, Druilhe P, Galey B, Londono A, Patarapotikut Beaudoin RL, Dubeaux C, Tartar A, Mercereau-Puijalon O, Langsley G 1987. A liver-stage antigen of *Plasmodium falciparum* characterized by gene cloning. *Nature (London) 360*: 434-439.

Harris SE, Gearing AJH, Layton GT, Adams SE, Kingsman SM, Kingsman AJ 1993. Enhanced proliferative cellular responses to HIV-1 V3 peptide and gp120 following immunization with V3:Ty virus-like particles. *Immunology 77*: 135-321.

Khusmith S, Charoenvit Y, Kumar S, Sedegah M, Beaudoin RL, Hoffman SL 1991. Protection against malaria by vaccination with sporozoite surface protein 2 plus CS protein. *Science 252*: 715-718.

Kingsman SM, Kingsman AJ 1988. Polyvalent recombinant antigens: a new vaccine strategy. *Vaccine 6*: 304.

Klotz FW, Scheller LF, Seguin MC, Kumar N, Marletta MA, Green SJ, Azad AF 1995. Co-localization of inducible-nitric oxide synthase and *Plasmodium*

berghei in hepatocytes from rats immunized with irradiated sporozoites. *J Immunol 154*: 3391-3395.

Kumar S, Miller L, Quakyi I, Houghten R, Maloy W, Moss B, Berzofsky J, Good M 1988. Cytotoxic T cells specific for the circumsporozoites protein of *Plasmodium falciparum. Nature 334:* 258-160.

Layton GT, Harris SJ, Gearing AJH, Hill-Perkins M, Cole JS, Griffiths JC, Burns NR, Kingsman AJ, Adams SE 1993. Induction of HIV-specific cytotoxic T lymphocytes are primed *in vivo* with hybrid HIV-1 V3:Ty-virus-like particles. *J Immunol 151*: 1097-1107.

Li S, Rodrigues M, Rodrigues D, Rodrigues JR, Esteban M, Palese P, Nussenzweig RS, Zavala F 1993. Priming with recombinant influenza virus followed by administration of recombinant vaccinia virus induces $CD8^+$ T-cell-mediated protective immunity against malaria. *Proc Natl Acad Sci USA 90:* 5214.-5218

Li S, Schulman JL, Moran T, Bona C, Palese P 1992. Influenza A virus transfectants with chimeric hemagglutinins containing epitopes from different subtypes. *J Virol 66:*399-404.

Mallik A, Egan JE, Houghten R, Sadoff JC, Hoffman SL 1991. Human cytotoxic T lymphocytes against the *Plasmodium falciparum* circumsporozoite protein. *Proc Natl Acad Sci USA 88:* 3300-3304.

Migliorini P, Betschart B, Corradin G 1993. Malaria vaccine: immunization of mice with synthetic T cell helper alone leads to protective immunity. *Eur J Immunol 23*: 582-584.

Mor G, Singla M, Steinberg A, Hoffman S, Okuda L, Klinman D 1997. Do DNA vaccines induce autoimmunity disease? *Hum Gene Ther 8*: 293-300.

Murata K, Garcia-Sastre A, Tsuji M, Rodrigues M, Rodriguez D, Rodriguez JR, Nussenzweig RS, Palese P, Esteban M, Zavala F 1996. Characterization of *in vivo* primary and secondary $CD8^+$ T cell responses induced by recombinant influenza and vaccinia viruses. *Cel Immunol 173*: 96-107.

Nussenzweig RS, Vanderberg J, Most H, Orton C 1967. Protective immunity produced by the injection of X-irradiated sporozoites of *Plasmodium berghei*. *Nature 216:* 160-162.

Nussenzweig RS, Vanderberg J, Most H, Orton C 1970. Immunity in simian malaria induced by irradiated sporozoites . *J Parasitol 56*: 252-257.

Nussler AK, Renia L, Pasquetto V, Miltgen F, Matile H, Mazier D 1993. *In vivo* induction of the nitric oxide pathway in hepatocytes after injection with irradiated sporozoites, malaria blood parasites or adjuvants. *Eur J Immunol 23*: 882-887.

Ockenhouse CF, Sun PF, Lanar DE, Wellde BT, Hall BT, Kester K, Stoute JA, Magill A, Krzych U, Farley L, Wirtz RA, Sadoff JC, Kaslow DC, Kumar S, Church LW, Crutcher JM, Wizel B, Hoffman S, Lalvani A, Hill AV, Tine JA, Guito KP, de Taisne C, Anders R, Ballou WR 1998. Phase I / IIa safety, immunogenicity and efficacy trial of NYVAC-Pf7, a pox-vectored multiantigen, multistage vaccine candidate for *Plasmodium falciparum* malaria. *J Infect Dis 177:* 1664-1673.

Oliveira-Ferreira J, Myahira Y, Layton GT, Savage N, Esteban M, Rodriguez D, Rodriguez JR, Nussenzweig RS, Zavala F 2000. Immunogenicity of Ty-VLP bearing a $CD8^+$ T cell epitope of the CS protein of *P. yoelii*: enhanced memory response by boosting with recombinant vaccinia virus. *Vaccine 18:* 1863-1869.

Plebanski M, Gilbert SC, Schneider J, Hannan CM, Layton G, Blanchard T, Becker M, Smith G, Butcher G, Sinden RE, Hill A 1998. Protection from *Plasmodium berghei* infection by priming and boosting T cells to a single class I restricted epitope with recombinant carriers suitable for human use. *Eur J Immunol 28:* 4345-4355.

Renia L, Grillot D, Marussig M, Corradin G, Miltgen F, Lambert PH 1993. Effector function of circumsporozoite peptide primed $CD4^+$ T cell clones against *Plasmodium yoelii* liver stages. *J Immunol 150*: 1471-1478.

Renia L, Marussig MS, Grillot D, Pied S, Corradin G, Miltgen F, Del Giudice G, Mazier D 1991. *In vitro* activity of $CD4^+$ and $CD8^+$ T lymphocytes from mice immunized with synthetic malaria peptides. *Proc Natl Acad Sci USA 88*: 7963-7967.

Rodrigues M, Cordey AS, Arreaza G, Corradin G, Romero P, Maryanski JL, Nussenzweig RS, Zavala F 1991. $CD8^+$ cytolitic T cell clones derived against the *Plasmodium yoelii* circumsporozoite protein protect against malaria. *Int Immunol 3*: 579-585.

Rodrigues M, Li S, Murata K, Rodriguez D, Rodriguez JR, Bacik I, Bennink J R, Yewdell J W, Garcia-Sastre A, Nussenzweig RS, Esteban M, Palese P, Zavala F 1994. Influenza and vaccinia viruses expressing malaria $CD8^+$ T cell and B cell epitopes. *J Immunol 153*: 4636-4648.

Rodrigues EG, Zavala F, Eichinger D, Wilson JM, Tsuji M 1997. Single immunizing dose of recombinant adenovirus efficiently induces $CD8^+$ T cell-mediated protective immunity against malaria. *J Immunol 158:* 1268-1274.

Rogers WO, Malik A, Melouk S, Nakamura K, Rogers MD, Szarfman A, Gordon MD, Nussler AK, Aikawa M, Hoffman SL 1992. Characterization of *Plasmodium falciparum* sporozoite surface protein 2. *Proc Natl Acad Sci USA 89*: 579-585.

Romero P, Eberl G, Casanova JL, Cordey AS, Widmann C, Luescher I, Corradin G, Maryanski J 1992. Immunization with synthetic peptides containing a defined malaria epitope induces a highly diverse cytotoxic T lymphocyte response. *J Immunol 143:* 4263-4266.

Romero P, Maryanski JL, Corradin G, Nussenzweig RS, Nussenzweig V, Zavala F 1989. Cloned cytotoxic T cells recognized an epitope in the circumsporozoite protein protect against malaria. *Nature 341*: 323-325.

Sadoff J, Ballou W, Baron L, Majarian W, Brey R, Hockmeyer W, Cryz S, Ou J, Lowell G, Chulay J 1988. *Salmonella typhimurium* vaccine expressing circumsporozoite protein protects against malaria. *Science 240:* 336-338.

Schneider J, Gilbert SC, Blanchard TJ, Hanke T, Robson KJ, Hannan CM, Backer M, Siden R, Smith GL, Hill AVS 1998. Enhanced immunogenicity for CTL induction and complete protective efficacy of ma-

laria DNA vaccination by boosting with modified vaccinia virus Ankara. *Nature Med 4*: 397-402.

Schofield L, Villaquiran J, Ferreira A, Schellekens H, Nussenzweig RS, Nussenzweig V 1987. γ interferon $CD8^+$ cells and antibodies required for immunity to malaria sporozoites. *Nature 330*: 664-666.

Sedegah M, Hedstrom RC, Hobart P, Hoffman SL 1994. Protection against malaria by immunization with plasmid DNA encoding circumsporozoite protein. *Proc Natl Acad Sci USA 88:* 2726-2730.

Sedegah M, Sim BKL, Mason C, Nutman T, Mallik A, Roberts C, Johnson A, Ochola J, Koech D, Were B, Hoffman S 1992. Naturally acquired $CD8^+$ cytotoxic T lymphocytes against *Plasmodium falciparum* circumsporozoite protein. *J Immunol 149*: 966-971.

Seguin MC, Klotz FW, Scheneider I, Weir JP, Goodbary M, Slayter M, Raney JJ, Aniagolu JU, Green SJ 1994. Induction of nitric oxide synthetase protects against malaria in mice exposed to irradiated *P. berghei* infected mosquitoes involvement of interferon-gamma and $CD8^+$ T cells. *J Exp Med 180*: 353-358.

Tine JA, Lanar DE, Smith DM, Wellde BT, Schultheiss P, Ware LA, Kauffman EB, Wirtz RA, De Taisne C, Hui GS, Chang SP, Church P, Hollingdale MR, Kaslow DC, Hoffman S, Guito KP, Ballou WR, Sadoff JC, Paoletti E 1996. NYVAC-pf7: a poxvirus-vectored, multiantigen, multistage vaccine candidate for *Plasmodium falciparum* malaria. *Infec Immun 64*: 3833-3844.

Tsuji M, Romero PJ, Nussenzweig RS, Zavala F 1990. CD4+ cytolitic T cell clone confers protection against murine malaria. *J Exp Med 172*: 1353-1358.

Valmori D, Romero JF, Men Y, Maryanki JL, Romero P, Corradin G 1994. Induction of a cytotoxic T cell response by coinjection of a helper peptide and cytotoxic T lymphocyte peptide in incomplete Freund's adjuvant (IFA): further enhancement by pre-injection of IFA alone. *J Immunol 24:* 1458-1462.

Vandenberg J, Nussenzweig RS, Most H 1969. Protective immunity produced by injection of x-irradiated sporozoite of *P. berghei*. V. *In vitro* effects of immune serum on sporozoites. *Mil Med 134*: 1183-1187.

Wang R, Charoenvit Y, Corradin G, De La Veja P, Franke ED, Hoffman SL 1996. Protection against malaria by *Plasmodium yoelii* sporozoite surface protein 2 linear peptide induction of $CD4^+$ T cell and IFN-gamma-dependent elimination of infected hepatocytes. *J Immunol 157*: 4061-4067.

Wang R, Doolan DL, Le TP, Hedstrom RC, Coonan KM, Charoenvit Y, Jones TR, Hobart P, Margalith M, Ng J, Weiss WR, Sedegah M, Taisne C, Norman JA, Hoffman SL 1998. Induction of antigen-specific cytotoxic T lymphocytes in humans by malaria DNA vaccine. *Science 282*: 476-480.

Weiss WR, Mellouk S, Houghten R, Sedegah M, Kumar S, Good MF, Berzofsky JA, Miller LH, Hoffman SL 1990. Cytotoxic T cells recongnize a peptide from the circumsporozoite protein on malaria-infected hepatocytes. *J Exp Med 171*: 763-773.

Weiss WR, Sedegah M, Beaudoin RL, Miller LH, Good MF 1988. $CD8^+$ T cells (cytotoxic/suppressor) are required for protection in mice immunized with malaria sporozoites. *Proc Natl Acad Sci USA 85*: 573-576.

WHO-World Health Organization 1997. World malaria situation in 1994. *Weekly Epidemiol Rec 72*: 269-274.

Widmann C, Romero P, Maryanski JL, Corradin G, Valmori D 1992. T helper epitopes enhance the cytotoxic response of mice immunized with MHC class I-restricted malaria peptides. *J Immunol Meth 155*: 95-99.