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Candidacy of liver stage antigen-1 for *Plasmodium falciparum* vaccine development

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ABSTRACT

Malaria is the world's most important parasitic disease, imposing a massive health burden on people living in the tropics, often in the poorest countries. The vast majority of deaths in humans from malaria are caused by one species of the hemoprotozoan, *Plasmodium falciparum*., against which effective control measures are urgently needed. The global situation has deteriorated in recent times due to increased resistance of the anopheline mosquitoes that transmit *P. falciparum* to insecticides, and of the parasites themselves to drug therapy. An efficacious and cost-effective vaccine against this parasite is considered a public health priority. A vaccine that targets pre-erythrocytic parasites in the liver could potentially prevent clinical disease by blocking development of the pathogenic erythrocytic stage of the parasite's life cycle. Among around 40 known *P. falciparum* antigens, liver stage antigen-1 (LSA-1) is the only protein expressed exclusively by liver stage parasites. Independent studies in humans have consistently related immune responses to LSA-1 with resistance to malaria infection or disease, providing a powerful rationale for the development of liver stage vaccines based on LSA-1. By dissecting the mechanism(s) of immunity to this antigen, epitopes associated with protection can be evaluated in different delivery systems as components of a focused and coordinated multi-antigen malaria vaccine strategy.

INTRODUCTION

Malaria is caused by protozoan parasites of the genus *Plasmodium*. Each year, 2-3 million children die as a result of *P. falciparum* malaria, and up to 500 million people throughout the world suffer clinical disease (Engers and Godal 1998). Malaria thus ranks alongside acute respiratory infections, measles and diarrheal diseases as a major cause of mortality worldwide. The situation has gradually worsened in recent years because of increasing resistance of the anopheline mosquitoes that transmit malaria to insecticides, and of the parasites themselves to antimalarial chemotherapy (Ridley 2002). Thus, the rational design of a safe and broadly effective malaria vaccine represents a high priority intervention strategy to control both the transmission of infection and the impact of disease (Taylor-Robinson 1998). However, this remains an unrealized dream. Currently, only one vaccine candidate, the circumsporozoite protein (CSP)-based formulation RTS, S (Stoute et al. 1997), has shown limited efficacy in vaccine trials in sub-Saharan Africa.

THE NEED FOR A LIVER STAGE MALARIA VACCINE

The parasite's complex life cycle offers several targets in the human host and the mosquito vector (Fig. 1), and vaccines against the sporozoite, intrahepatic, asexual intraerythrocytic and sexual stages of the parasite are currently in development (Miller and Hoffman 1998). For prophylactic purposes, a pre-erythrocytic vaccine is required as it aims to prevent or reduce the acquisition of clinical infection. By preventing either invasion of hepatocytes by sporozoites or pre-erythrocytic stage development within hepatocytes, a vaccine targeting the liver would preclude both the progression of disease, since clinical symptoms of malaria manifest only during the subsequent erythrocytic stage, and parasite transmission, since no sexual stages would develop (Taylor-Robinson 2002) (Fig. 1). This would benefit individuals who either are malaria-naïve or who have lost their previously acquired immunity. It would also enhance the naturally acquired protective immune response of individuals resident in malaria-endemic countries that is achieved upon prolonged exposure, in order to either prevent blood stage infection or to reduce the numbers of parasites that emerge from the liver (Taylor-Robinson and Smith 1999).

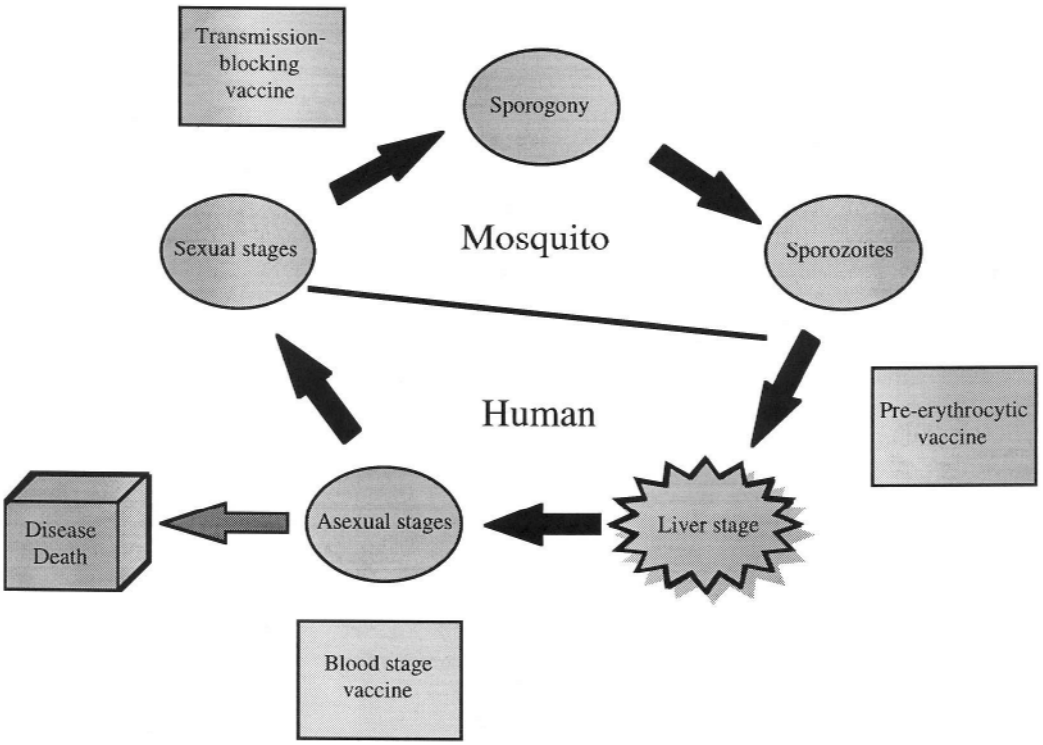


Figure 1. Schematic life cycle of the malaria parasite, *Plasmodium falciparum*, indicating where vaccination may be expected to intervene.

The liver plays a crucial role in the *Plasmodium* life cycle, as hepatocytes are an obligatory site for schizogony, a process of amplification and molecular changes for the parasite. Although only a small number of extrace11u1ar sporozoites invade the liver, schizogony increases the parasite load and gives rise to a richer pool of antigens. Moreover, in addition to liver stage-specific proteins, intrahepatic parasites produce antigens that belong to sporozoite and erythrocytic stages of infection (Fig. 2). This singles out the liver as a major depot of malarial antigens, which appears to be needed for the induction and maintenance of protective immune responses. Hence, infection of hepatocytes is not only pivotal for survival of the parasite, but also acts as a significant arena for the induction of antimalarial protective immunity.

Targeting intrahepatic parasites for vaccine development has specific advantages, including the low parasite burden at this stage of infection, the putative endogenous processing and

presentation of antigen by liver cell major histocompatibility complex (MHC) class I molecules, and an array of potential effecter mechanisms against liver stage antigens (Hollingdale et al. 1998). Unique among known *P. falciparum* antigens, liver stage antigen-1 (LSA-1) is expressed by the parasite solely during its development in the liver (Guérin-Marchand et al. 1987) (Fig. 2). The immunological significance of LSA-1 has been established only in human studies, as no homologue in mouse or non-human primate malarias has been identified. Based on these studies, LSA-1 is a focus of vaccine development in collaborative research involving laboratories in the EU and the USA.

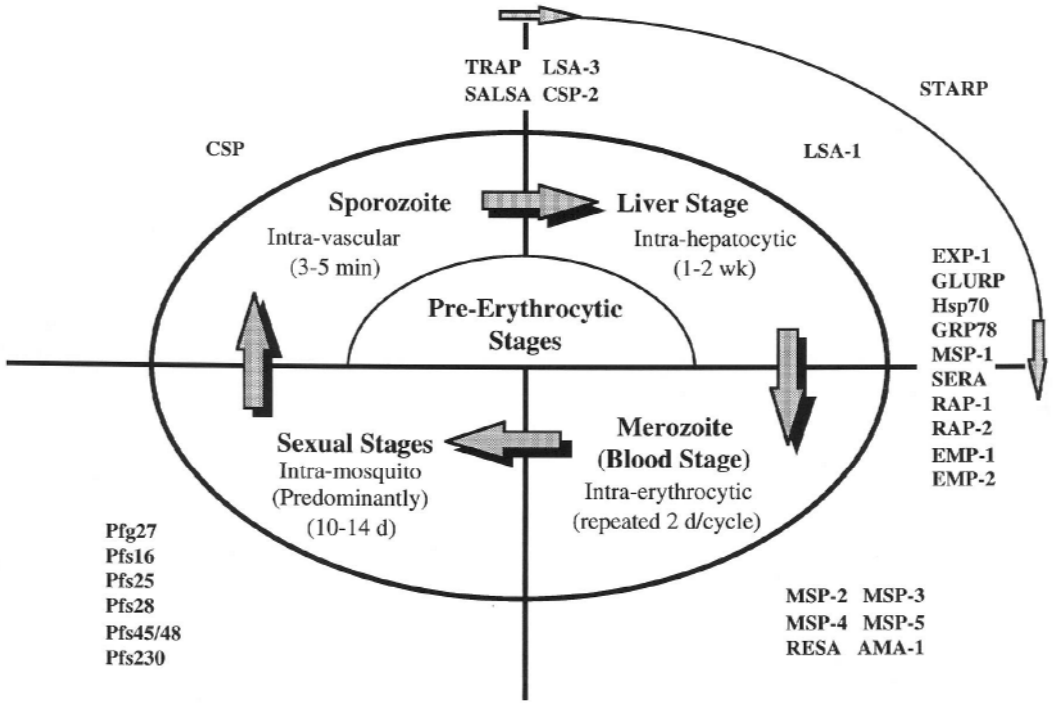
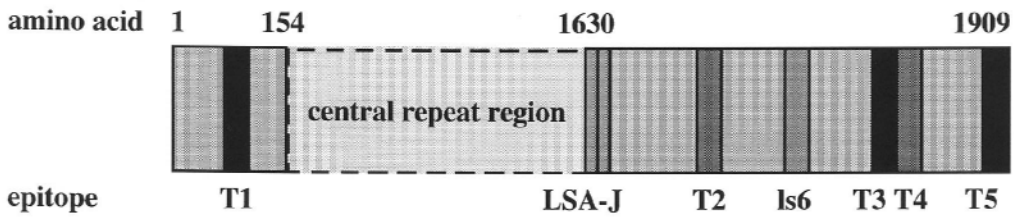


Figure 2. Schematic life cycle of *Plasmodium falciparum* in relation to stage-specific expression of major candidate vaccine antigens, indicating the unique intrahepatic expression of LSA-1.



T1	aa 84-107	LTMSNVKNVSQTNFKSLLRNLGVS
LSA-J	aa 1613-1636	ERRAKEKLQEQQRDLEQRKADTKK
T2	aa 1742-1760	HTLETVNISDVNDFQISKY
ls6	aa 1786-1794	KPIVQYDNF
T3	aa 1813-1835	NENLDDLDEGIEKSSEELSEEKI
T4	aa 1836-1849	KKGKKYEKTKDNNF
T5	aa 1888-1909	DNEILQIVDELSEDITKYFMKL

Figure 3. Schematic structure of liver stage antigen-1 (LSA-1) showing distribution and sequence of T cell epitopes associated with cell-mediated protective responses.

LSA-1 AND THE BIOLOGY OF LIVER STAGE PARASITES

Sporozoite invasion occurs by a series of molecular interactions that involve sporozoite antigens such as CSP (Frevert et al. l993) and the thrombospondin-related adhesion protein (TRAP) (Müller et al. 1993, Sultan et al. l997). CSP and TRAP are carried into the hepatocyte along with the parasite but are detected only during early liver stage development (Rogers et a1. 1992). In contrast, synthesis of LSA-l begins soon after sporozoite invasion of hepatocytes and increases throughout the cycle (Hollingdale et al. 1990). Consequently, LSA-1 may be processed and presented to the immune system differently than sporozoite antigens. LSA-1 is contained in a flocculent mass that surrounds the merozoite, and may aggregate on the merozoite surface, as it does not contain a hydrophobic transmembrane sequence and appears to be secreted by the parasite into the intrahepatic parasitophorous vacuole (Hollingdale et al. l990, Fidock et al. l994). When the hepatocyte ruptures, merozoites are released within this flocculent mass (Terzakis et al. l979) containing LSA-1 into the liver sinusoid where they invade red blood cells.

LSA-1 is a 200 kDa protein (Fidock et al. 1994) that in the universally studied NF54 strain of *P. falciparum* contains a central region with 86 copies of a 17-amino acid (aa) repeat based on

EQQSDLEQERLAKEKLQ, flanked by N- and C-terminal non-repeat regions containing B and T epitopes (Zhu and Hollingdale 1991) (Fig. 3). Unlike many other vaccine candidates, the sequence of LSA-1 is highly conserved across strains (Fidock et al. 1994, Yang et al. l995), suggesting a crucial role during liver schizogony, perhaps protecting the merozoite surface.

MURINE STUDIES: EVIDENCE FOR PROTECTIVE IMMUNITY TARGETING THE INFECTED HEPATOCYTE

Mouse models have provided important insights into the nature of protective immunity directed against liver stage parasites. The environment of the liver is normally anti-inflammatory (O'Farrelly and Crispe 1999), but vaccination by irradiation-attenuated sporozoites (γ-SPZ) induces an infiltration of inflammatory cells into the mouse liver (Khan and Vanderberg 1992) and confers protection against subsequent sporozoite challenge but not against blood-borne infection. Therefore, protective immunity must be directed against pre-erythrocytic parasites, sporozoites and/or liver stages. Sera from mice immunized with γ-SPZ do not passively protect naïve mice to sporozoite challenge, whereas adoptive transfer of immune spleen cells does confer protection. These studies strongly suggest that γ-SPZ-induced protective immunity is mediated predominantly by cellular mechanisms (Hoffman et al. 1996).

The critical role of $CD8^+$ T cells was demonstrated in studies in which depletion of $CD8^+$ T cells abolished protective immunity induced by vaccination with γ-SPZ, whereas depletion of $CD4^+$ T cells had no effect (Hoffman et al. 1996). Spleen cells from γ-SPZ-immunized mice killed liver stage parasites in cultured hepatocytes in a manner that was MHC class I-specific and species-specific, suggesting that protection is mediated by $CD8^+$ cytotoxic T lymphocytes (CTL) (Hoffman et al. 1989), acting via a perforin/Fas-independent pathway (Renggli et al. 1997). $CD8^+$ T cells must recognize antigens expressed by malaria-infected hepatocytes in association with MHC class I molecules in order to confer protection in vivo (White et al. 1996).

γ-SPZ invade hepatocytes and express liver stage antigens (Zhu et al. 1990), but do not progress to infect red blood cells, and become a source of persistent intrahepatic antigen (Scheller and Azad 1995). Elimination of irradiated parasites from the liver by primaquine treatment abrogates protection in mice by causing the loss of memory phenotype $CD8^+$ T cells (Krzych et al. 2000).

These home to the liver where they persist as $CD45RB^{lo}CD44^{hi}$ T cells during prolonged protection (Guebre-Xabier et al. 1999). Adoptive transfer experiments in mice demonstrated that protective T cell clones targeting liver stage parasites express CD44, a homing marker, whereas clones not expressing CD44 were not protective (Rodrigues et al. 1992).

The major mechanism by which CTL are protective is becoming increasingly apparent. The consensus view drawn from a number of elegant studies is that following γ-spz vaccination, interleukin (IL)-12 derived from macrophages, dendritic or Kupffer cells induces $CD8^+$ T cells and natural killer cells to produce the pro-inflammatory cytokine interferon (IFN)- γ. IFN- γ then induces infected hepatocytes to produce nitric oxide (NO) that kills the intrahepatic parasite (Good and Doolan 1999, Doolan and Hoffman 2000). We, like many others, are pursuing liver stage vaccine development based on this hypothesis, and specifically the central involvement of IFN-γ. Persuasive support comes from the finding of a mutation in the inducible NO synthase gene that is more frequent in children in Gabon with mild malaria than those with severe malaria (Kun et a1. 1998).

HUMAN STUDIES: EVIDENCE FOR PROTECTIVE ANTI-LSA-1 RESPONSES

Complete sterile immunity also occurs in humans after exposure to γ-spz (Clyde et al. 1973, Herrington et al. 1991), but only incomplete immunity develops after exposure to naturally transmitted parasites. A series of independent studies in humans, immunized with γ-spz or by natural infection, have all consistently correlated LSA-1-specific immune responses with protection. Proliferative cytokine and antibody responses against LSA-1 have all been associated with resistance in humans, suggesting that the molecule could play more than one role in a multi-component vaccine strategy. In studies conducted at the Walter Reed Army Institute of Research WRAIR), MD, USA, proliferative T cell responses to three LSA-1 epitopes (T1, T3, and T5) were 3-5 fold higher in protected volunteers compared to a non-protected volunteer (Krzych et al. 1995). Importantly, this provided the first link between anti-LSA-1 responses and sterile immunity in humans. Naturally acquired responses to LSA-1 were earlier implicated in human protection in studies involving Gambian children (Hill et a1. 1991). Children with mild malaria expressed the human leukocyte antigen (HLA) B53 allele more frequently than did

children with severe malaria, and adult Gambians could mount B53-restricted CTL responses to a peptide from LSA-1 (ls6) but not to peptides from three sporozoite antigens (Hill et al. 1992). The results suggested that HLA B53-restricted CTL against LSA-1 mediated resistance to severe malaria in this population.

Protection in naturally exposed populations has been directly related with anti-LSA-1 immune responses in five independent studies. Longterm studies in two geographically distinct locations have linked naturally occurring protection with IFN-γ production:

- In Papua New Guinea, IFN-γ synthesis (principally by $CD8^+$ T cells) in response to the 24 aa T1 epitope of LSA-1 (Fig. 2) was associated with an absence of parasitemia over six months (Connelly et al. 1997). This was the first direct association between anti-LSA-1 responses and resistance to *P. falciparum* in naturally exposed humans. Anti-LSA-1 proliferative responses and immunoglobulin G antibody levels were not associated with resistance. The T1 peptide variant most commonly found in the study area binds to HLA*A110l, which has a frequency of 67.5% in this population (Bucci et al. 2000), suggesting HLA class I allele selection by malaria (Hill et a1. 1992).
- In Gabon, an association was identified between protection and IFN-γ in children in an area of perennial transmission (Luty et al. 1999, May et al. 2001). Children who were admitted with mild malaria and who mounted IFN-γ responses to either LSA-J or ls6 epitopes of LSA-1 subsequently had a prolonged time to reinfection, a reduced risk of developing malaria-related anemia and a lower annual frequency of parasitemia than did children without these responses.

A further three studies in endemic areas have suggested that anti-LSA-1 immune responses other than IFN-γ may also contribute to naturally occurring protection:

- In a treatment-reinfection study of young males in a malaria-holoendemic area of Kenya over two transmission seasons, IL-10 responses to recombinant non-repeat regions of LSA-1, that contained the T1, T3 and T5 epitopes, predicted delayed time to reinfection, reduced frequency of parasitemia, and reduced parasite densities (Kurtis et al. 1999). Resistance to malaria was not predicted by IFN-γ or tumor necrosis factor (TNF)-α responses in either

season, and this may be due to the use of recombinant proteins containing the T1, T3 and T5 epitopes as well as others as yet uncharacterized in these flanking regions.

- An earlier study in Gabonese children reported that IL-10 responses to LSA-J and ls6, and antibodies against a 41-mer LSA-1 repeat peptide, correlated with decreased time to parasite clearance after treatment (Luty et al. 1998). However, recent investigations suggest that $CD8^+$ T cell IFN-γ responses to the T1 epitome are significantly elevated during the acute phase of infection compared to the convalescent phase, suggesting that IFN-γ responses to LSA-1 epitopes may be easily underestimated (Migot-Nabias et al. 2000, Luty et al. 2001).
- A study in another holoendemic area of Gabon reported that children with detectable anti-LSA-J antibodies had significantly longer times to reappearance of parasitemia compared to those without detectable LSA-J antibodies (Domarle et al. 1999).

Thus, exposure in endemic areas naturally induces a protective immune response to LSA-1 that variably involves $CD8^+$ T cells, IFN-γ, IL-10 and antibodies, and protection has not been exclusively associated with either type 1 or type 2 immune responsiveness. Differences between study findings may be related to the peptides or recombinant proteins used in the assays, and studies in Ghana suggest that the peptides T1, T3 and T5 may each stimulate the production of type 1 (IFN-γ) or type 2 (IL-4) cytokines, but generally not both (Migot-Nabias et al. 2000). IL-10 could mediate resistance through its ability to: (a) augment protective humoral responses by inducing B cells to secrete antibodies (Rousset et a1. 1992); (b) augment cell-mediated responses by attracting $CD8^+$ T cells (Jinquan et al. 1993) to the site of infected hepatocytes; and/or (c) enhance antibody-dependent cellular inhibition (ADCI) activity against *P. falciparum* (Kurtis et al. 1999). The putative role of anti-LSA-1 antibodies may reflect binding to LSA-1 present on the liver stage merozoite, thus reducing the initial burden of malaria-infected red blood cells. It is also possible that anti-LSA-1 antibodies cross-react with other antigens present on liver stage or red blood cell stage merozoites.

It is thus likely that while cellular immunity involving $CD8^+$ T cells, cytokines IL-12 and IFN-γ, and NO may predominate, multifactorial mechanisms act to neutralize intrahepatic parasites of *P. falciparum* (Seder and Hill 2000). These authors suggest that a pre-erythrocytic stage vaccine

should be designed to induce $CD8^+$ T cell- and IFN-γ-mediated immune responses. While there may be a need for immune cell homing to the liver to effect protection, all human studies have been performed with peripheral blood samples, indicating that protective anti-LSA-1 responses can be measured in the periphery.

LSA-1 AND VACCINE DEVELOPMENT

A number of technical hurdles still need to be overcome in order to advance basic, pre-clinical research into pre-erythrocytic malaria vaccines, including:

- Improvement of the very low efficiency of *P. falciparum* liver stage development in hepatoma cultures, facilitating dissection of protective immunity in vitro and providing a human model for pre-clinical vaccine testing.
- Development of in vitro assays that predict protective immunity acquired to LSA-1 and other hepatocyte-infected antigens, and indicate who should and who should not be prioritized for immunization, and when during life to vaccinate. Currently, the only certain way to test vaccine efficacy is to immunize volunteers and, by microscopical or PCR-based examination for blood stage parasites, determine if they are protected after exposure to *P. falciparum*-infected mosquitoes.

Our strategy for LSA-1 vaccine development is derived from the aforementioned mouse immunobiology and human field studies. This concurs with the view held by Doo1an and Hoffman (2000) that an LSA-1 vaccine should elicit memory $CD8^+$ T cells that home to the liver and secrete IFN-γ in order to confer protection. Other mediators targeting LSA-1, like IL-10 and antibodies, could enhance vaccine efficacy. Since LSA-1 is likely to be one element of a mu1ti-componentc vaccine, responses specific to LSA-1 should be additive or synergistic with those elicited by other antigens. This may be particularly relevant if LSA-1 is combined with the RTS,S vaccine (Stoute et al. 1997). An attenuated vaccinia virus expressing seven malaria genes, including the N- and C-terminal non-repeat regions of LSA-1, has already undergone human clinical trials in the USA, and successfully induced CTL and proliferative responses (Ockenhouse et al. 1998). Several epitopes on LSA-1 and other pre-erythrocytic antigens restricted by different

HLA class I alleles have been identified (Aidoo et al. 1995), and a vaccine construct containing some of these epitopes is undergoing phase I trials at the University of Oxford, UK.

In work at the University of Leeds particulate MS2 bacteriophage that expresses multiple copies of the T1 epitope (which contains overlapping HLA A3/11 and MHC $H2^{-d}$ binding epitopes) has been shown to induce a strongly polarized type 1 response in $H\text{-}2^{d}$ mice (Heal et a1. 1999). The $CD8^{+}$ T cell population was the major source of the pronounced specific IFN-γ synthesis (Brown et al. 2002) (Fig. 4). This merits further evaluation of MS2 as a vector for malaria T cell epitopes and validates RNA phage capsid display of immunogenic peptides as a basis to develop novel synthetic vaccines against other infectious diseases. Leeds scientists are now examining a fusion protein comprising LSA-1 and hepatitis B core antigen as another particulate delivery system (Rowlands and Taylor-Robinson, unpublished data). In collaboration with WRAIR, we are evaluating LSA-1 as a soluble, *Escherichia coli*-expressed protein (comprising N- and C-terminal regions separated by two 17-aa repeats) formulated with type 1 adjuvants, and incorporation of LSA-1 into attenuated Venezuelan equine encephalitis virus, a live vector delivery system.

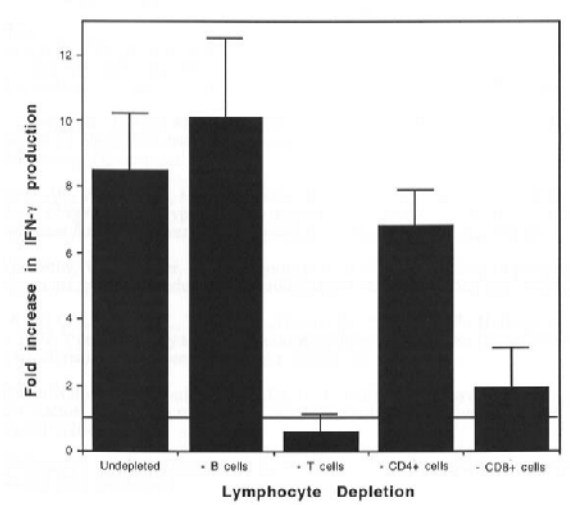


Figure 4. Depletion of $CD8^{+}$ T cells from immunologically primed splenocytes ablates the IFN-γ response to stimulation with LSA-1 T1 peptide.

Lymphocyte subset depletion was performed by immunomagnetic cell sorting to > 98% purity on splenocytes from mice immunized with MS2-Tl following ex vivo restimulation with LSA-1 T1 peptide (25 μg/ml), as previously described (Heal et al. 1999, Brown et a1. 2002). Data are fold increases in cytokine secretion over that of splenocytes from identically immunized mice not

restimulated ex vivo (horizontal line, value 1.0). Data are representative of four similar experiments.

CONCLUSIONS

Clinical infection with falciparum malaria may be completely averted by a vaccine against the liver stage that gives rise to the pathogenic erythrocytic forms of the parasite. Sterilizing immunity can be conferred by inoculating humans with γ-spz, and a recombinant pre-erythrocytic vaccine partially protects humans from infection. In the search for an efficacious vaccine, attention is increasingly turning to LSA-1, one of only a few proteins known to be expressed by *P. falciparum*-infected hepatocytes, and which holds particular promise as a vaccine candidate antigen. We are evaluating LSA-1 as part of a multi-component malaria vaccine using different vehicle technologies. Studies of malaria-exposed individuals consistently relate anti-LSA- 1 responses with protection, and upcoming clinical trials of the various LSA-1 constructs should identify the optimal approach that exploits this antigen in a vaccine against malaria. Although deployment of an effective malaria vaccine will take several years longer, in the present decade significant or even dramatic research advances can be anticipated towards this important goal.

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