



Importance of CD8 T cell-mediated immune response during intracellular parasitic infections and its implications for the development of effective vaccines

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ABSTRACT

Obligatory intracellular parasites such as *Plasmodium* sp, *Trypanosoma cruzi*, *Toxoplasma gondii* and *Leishmania* sp are responsible for the infection of hundreds of millions of individuals every year. These parasites can deliver antigens to the host cell cytoplasm that are presented through MHC class I molecules to protective CD8 T cells. The *in vivo* priming conditions of specific CD8 T cells during natural infection are largely unknown and remain as an area that has been poorly explored. The antiparasitic mechanisms mediated by CD8 T cells include both interferon- γ -dependent and -independent pathways. The fact that CD8 T cells are potent inhibitors of parasitic development prompted many investigators to explore whether induction of these T cells can be a feasible strategy for the development of effective subunit vaccines against these parasitic diseases. Studies performed on experimental models supported the hypothesis that CD8 T cells induced by recombinant viral vectors or DNA vaccines could serve as the basis for human vaccination. Regimens of immunization consisting of two different vectors (heterologous prime-boost) are much more efficient in terms of expansion of protective CD8 T lymphocytes than immunization with a single vector. The results obtained using experimental models have led to clinical vaccination trials that are currently underway.

Key words: CD8, parasites, immunity, vaccine.

INTRODUCTION

The population at risk of infection by obligatory intracellular parasites is estimated at billions of individuals living mostly in tropical and subtropical regions of the world where these parasites are endemic.

Plasmodium falciparum is responsible for the larger burden of malaria, an estimated 300 million

cases of the disease per year that result in the death of more than 1 million people, mostly African children under the age of five. Altogether, the other most important intracellular parasites (*Plasmodium vivax*, *Trypanosoma cruzi*, *Toxoplasma gondii* and *Leishmania* sp) are not responsible for as many infections or kill as many individuals as *P. falciparum*. Nevertheless, they are responsible for infecting millions of individuals yearly causing enormous social distress and economic losses.

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Although the pattern of disease transmission, infection and mortality caused by these obligatory intracellular parasites has changed in the past 50 years, worldwide they had never been a greater problem than they are today. In developing countries, these circumstances are very unlikely to improve in the next ten years. In view of these problems, the development of new strategies for prevention and control of these diseases are in great need.

Mass vaccination against these parasitic infections is not available. Research in this field can be considered important because in the future vaccines may complement other strategies such as chemotherapy, vector control etc., for the prevention and control of these parasitic diseases. Based on this conviction, studies on the mechanisms of host protective immunity and its target antigens have been pursued by immuno-parasitologists for the last 50 years.

HISTORICAL BACKGROUND

Pioneering studies performed in the sixties by Dr. Ruth S. Nussenzweig and collaborators at New York University firmly established that immunization with radiation-attenuated sporozoites could confer an exceptional degree of protection against experimental infection with viable sporozoites of *Plasmodium berghei*, a species that causes malaria in rodents. In the following decades, this work was extended to several other host species including humans. Individuals immunized with radiation-attenuated sporozoites of *P. falciparum* develop a protective immunity that is sterile, long lived and prevents the development of any symptom of the disease (Hoffman et al. 2002). Because of these exceptional attributes, this type of immunity would be ideal for an effective vaccine against malaria.

In 1987 and 1988, two groups independently reported that the immunity induced in mice by radiation-attenuated sporozoites was dependent on CD8 T lymphocytes (Schofield et al. 1987, Weiss et al. 1988). It was the first evidence that this subpopulation of lymphocytes participates in the immunity

against any form of a parasite. In the subsequent years, it was described that CD8 T cells also participate in immunity to infections caused by other intracellular parasites such as *Toxoplasma gondii* and *Trypanosoma cruzi*. In the case of *Leishmania* sp, the participation of CD8 T cells is more controversial; however, very recent studies have implicated CD8 T cells in the pathogenesis and immunity of certain types of cutaneous infection (Belkaid et al. 2002).

The definition of the importance of CD8 T lymphocytes in the immunity against these intracellular protozoan parasites has opened a new direction in the studies of the immunologic mechanisms of resistance to parasitic diseases. From a practical point of view, it may have important implications in the development of vaccines against these parasitic diseases.

CD8 T CELLS AS AN IMPORTANT MECHANISM MEDIATING PROTECTIVE IMMUNITY AGAINST INTRACELLULAR PARASITIC INFECTIONS

MALARIA LIVER STAGES

The importance of CD8 T lymphocytes in the resistance to infection against a challenge with malaria sporozoites was demonstrated in several studies performed during the last 15 years. In initial studies, BALB/c mice immunized with radiation-attenuated sporozoites were depleted of CD8 T cells before a challenge with viable sporozoites of *P. yoelii*. CD8-depleted immunized mice became completely susceptible to infection (Weiss et al. 1988). This study demonstrated that in the absence of CD8 T lymphocytes, other immunological mechanisms induced by radiation-attenuated sporozoites were not capable to promote resistance against the infection.

To further study the importance of CD8 T cells in the immunity to experimental malaria, T cell clones were obtained from spleen cells of BALB/c mice immunized with radiation-attenuated *P. yoelii* sporozoites. CD8 T cell clones were generated and characterized *in vitro* in terms of phenotype and specificity. These clones were Thy1+, $\alpha\beta$ T-Cell Receptor+, CD4- and CD8+ and they recognized

with high affinity a synthetic peptide represented by amino acids SYVPSAEQI present in the C-terminal portion of *P. yoelii* CS protein (Rodrigues et al. 1991).

In vitro these CD8 T cells presented intense lytic activity and capacity of inducing DNA degradation of H-2kd target cells in the presence of the malaria peptide. In addition, these cells secrete large amounts of IFN- γ , TNF- α and the enzyme BLT-esterase when stimulated by the antigen *in vitro* (Rodrigues et al. 1991, 1992). Most important, when adoptively transferred to naïve BALB/c mice, some of these clones were capable to provide specific immunity against the challenge with viable *P. yoelii* sporozoites (Rodrigues et al. 1991, 1992).

To demonstrate that liver stages of the malaria parasite were in fact targets for the immunity mediated by these CD8 T lymphocytes, an assay was developed to quantify the *P. yoelii* rRNA in RNA isolated from livers of mice infected with *P. yoelii*. Using this assay it was possible to demonstrate that there was a reduced amount of parasite rRNA in the liver of animals that received cells of a CD8 T cell clone 20 hours after the challenge with sporozoites. In contrast, control animals that did not receive T cells presented large amounts of parasite rRNA in their livers (Rodrigues et al. 1991).

CD8 T cells specific for epitopes present within the *P. falciparum* liver stage antigens have been commonly observed in humans immunized with radiation-attenuated sporozoites of *P. falciparum* (Doolan et al. 1997). Whether these cells are as important for human protective immunity as seen in the mouse model of infection cannot be directly addressed experimentally. Nevertheless, it is very likely that they actively participate in the protective immunity observed in human vaccinees.

During natural infection in humans, CD8 T cells specific for epitopes present within different plasmodial antigens have been detected in many individuals with previous contact with *P. falciparum* (Hill et al. 1992, Aidoo et al. 1995, Plebanski et al. 1997, Gonzalez et al. 2000). Because these individuals do not develop sterile immunity against re-

infection, these CD8 T cells do not seem to provide a degree of protective immunity similar to the one observed in individuals vaccinated with radiation-attenuated sporozoites of *P. falciparum*. Whether this is a matter of specificity or quantity of specific T cells it is unknown. Alternatively, mechanisms of parasite evasion of the immune response that are absent after immunization with radiation-attenuated sporozoites of *P. falciparum* may operate during natural human infection.

TRYPANOSOMA CRUZI

The importance of CD8 T cells in the naturally acquired immunity to *T. cruzi* infection was determined by the use of genetically modified mice that do not express either MHC class I or the CD8 molecule. These two genetically deficient mouse strains are highly susceptible to infection, being unable to control acute parasitemia (Tarleton et al. 1992, Rottenberg et al. 1993).

Subsequent studies generated CD8 T-cell lines specific for an epitope of trypomastigote surface antigen-1 (TSA). These T-cell lines were Thy1+, $\alpha\beta$ T-cell receptor+, CD4- and CD8+. *In vitro* these CD8 T cells presented intense lytic activity against *T. cruzi*-infected target cells. These cells also secreted large amounts of IFN- γ and TNF- α when stimulated with the antigen *in vitro* (Wizel et al. 1997). When adoptively transferred to naïve mice, they provided specific immunity against the challenge with infective forms of *T. cruzi* (Wizel et al. 1997).

T. cruzi is a parasite that persists for long periods of time causing a chronic inflammatory disease. Under certain circumstances T cells exacerbate the immune response causing an increase in tissue pathology or autoimmunity (Soares et al. 2001a). However, this autoimmune response has been associated with CD4 Th1 cells (dos Santos et al. 1992, Soares et al. 2001b).

As mentioned above, in the murine model, the adoptive transfer of large amounts of IFN- γ -producing CD8 T cells specific for a parasite epitope significantly reduced infection and promoted survival of mice against a lethal infection with *T.*

cruzi (Wizel et al. 1997). These results indicate that IFN- γ -producing CD8 T cells did not aggravate inflammatory reactions or autoimmunity, but rather promoted elimination of the parasites.

During natural infection in humans, CD8 T cells specific for *T. cruzi* have been detected in most individuals in the chronic phase of infection (Wizel et al. 1998b). Also, there is an association of an increase in CD8 T cells with the presence of *T. cruzi* antigens in chronic human chagasic myocarditis (Higuchi et al. 1997).

Although it is difficult to prove definitively that these cells are important in host resistance, it is plausible to believe that they exert an antiparasitic role similar to that seen in the mouse model of infection.

TOXOPLASMA GONDII

Early studies by the group of Kasper and collaborators demonstrated that mice and humans immune to *T. gondii* develop CD8 T cells that recognize parasite antigen presented by antigen-pulsed or infected target cells. These CD8 lymphocytes proliferate, secrete IFN- γ , lyse the target cells and block the multiplication of parasites *in vitro* (Khan et al. 1988, 1990).

In subsequent years, the concept that CD8 T cells are a very important effector mechanism against this parasitic infection became well established (reviewed by Denkers and Gazzinelli 1998). In mice vaccinated with an attenuated strain of *T. gondii* (ts-4), the depletion of CD8 T cells was shown to partially abrogate resistance to infection with a highly virulent strain of the parasite. Among the $\alpha\beta$ T lymphocytes, CD8 T cells seem to be the major effector mechanism because the depletion of CD4 T cells fails to produce any detectable loss of immunity. Nevertheless, both subpopulations of T lymphocytes cooperate in the immune response because the depletion of CD4 and CD8 T cells renders mice immunized with attenuated *T. gondii* ts-4 completely susceptible to infection (Gazzinelli et al. 1991).

The importance of CD8 T cells in the resistance to murine toxoplasmosis was confirmed in parallel studies in which CD4-depleted T lymphocytes were

adoptively transferred to naïve athymic recipients. These cells (mainly CD8 cells) led to a significant increase in mouse survival after infection and a significant reduction in the formation of brain cysts (Parker et al. 1991). Finally, the importance of CD8 T cells was demonstrated by the adoptive transfer of T cell clones specific for a *T. gondii* antigen. $\alpha\beta$ T-cell receptor+, CD4- and CD8+ T cells specific for the P30 (Sag1) antigen conferred a high degree of protective immunity against a lethal challenge with *T. gondii* (Khan et al. 1994).

Several lines of evidence implicate T lymphocytes in pathologic changes associated with acute infection in mice. Most evidence pointed at lymphocytes of the CD4 subset and granulocytes during this detrimental inflammatory response that can be the cause of death. Therefore, so far, CD8 T cells seem to be associated with a particularly beneficial immune response by the host, being unable to cause significant pathology (reviewed by Denkers and Gazzinelli 1998).

LEISHMANIA SP.

Years of studies have been unable to firmly establish that CD8 T cells are required for naturally acquired immunity against *Leishmania* sp. This is not unexpected because it is well established that MHC class II restricted CD4 T cells are extremely dominant during the development of immunity against *Leishmania* sp. The importance of CD4 T cells was illustrated in studies using mice deficient for the expression of CD8 or class II MHC molecules. After primary subcutaneous infection with *L. major*, CD8-deficient mice controlled the infection for over 1 year (Huber et al. 1998). On the other hand, mice genetically deficient for MHC class II are highly susceptible to infection (Locksley et al. 1993). These results support the notion that in the absence of CD8 T cells, other cell types (mainly class II restricted helper T cells) could provide an efficient control of a primary infection with *L. major*.

In spite of these earlier observations, very recent studies using low doses of *L. major* promastigotes inoculated into a dermal site (ear dermis) de-

monstrated that CD8 T-cell-deficient mice failed to control parasite growth. Also, these animals had a minor and delayed dermal pathology when compared to wild-type animals. In this model of infection, reconstitution of resistance was obtained when both CD4 and CD8 T cells were adoptively transferred. This study strongly suggests that CD8 T lymphocytes participate in both the pathology and immunity to primary infection with *L. major* in the skin (Belkaid et al. 2002).

These results are in agreement with a number of previous observations indicating that specific CD8 T cells are primed during natural infection or vaccination in humans. When re-stimulated *in vitro* with *Leishmania* antigens, human CD8 T cells secrete IFN- γ , an important mediator of resistance against certain types of infection with *Leishmania* sp (De Luca et al. 1999, Bottrel et al. 2001, Pompeu et al. 2001). Also, in individuals that develop the mucocutaneous form of the disease, CD8 T lymphocytes are highly cytolytic *in vitro* against *Leishmania*-infected macrophages (Brodszyn et al. 1997).

Together with the evidence obtained recently with the murine model of infection, it seems that CD8 T cells participate in both the pathogenesis and immunity to cutaneous and mucocutaneous *Leishmaniasis*. Nevertheless, the importance of these cells in relation to CD4 T cells is far from clear and remains to be further elucidated.

IDENTIFICATION OF THE EPITOPES RECOGNIZED BY PROTECTIVE CD8 T CELLS IN MICE AND HUMANS

MALARIA LIVER STAGES

Initial studies using the mouse model of malaria searched for CD8 epitopes in the only protein that had its primary structure known at that time, the CS protein. As a source of CD8 lymphocytes, spleen cells of mice vaccinated with radiation-attenuated sporozoites were used because these animals develop strong protective immunity against the infection. So far, to our knowledge, in mice vaccinated with radiation-attenuated sporozoites, CD8 lymphocytes were specific for a single epitope found in the

C-terminal region of the *P. berghei* or *P. yoelii* CS protein (Romero et al. 1989, Rodrigues et al. 1991, 1992). This epitope is recognized by CD8 T cells restricted by H-2 Kd molecules.

Figure 1 shows an outline of the *P. yoelii* CS protein with its different immunodominant epitopes recognized by antibodies (lymphocytes B) or CD4 or CD8 T lymphocytes. The epitopes of *P. yoelii* CS protein are important because the infectivity of *P. yoelii* sporozoites is comparable to that of *P. falciparum*, the most virulent human malaria parasite. Due to these characteristics, *P. yoelii* infection in mice is considered a valuable model for studies of preclinical development of subunit vaccines (see below).

Similar to the mouse studies, epitopes recognized by human CD8 T cells from individuals vaccinated with radiation-attenuated sporozoites of *P. falciparum* were initially identified within the CS protein of this human malaria parasite (Malik et al. 1991). In subsequent studies, CD8 epitopes were also found in several other distinct antigens expressed in liver stages of *P. falciparum* (Doolan et al. 1997, Wizek et al. 1995a,b, Kumar et al. 2001). Currently a total of 35 epitopes have been described in different liver stage proteins of *P. falciparum* recognized by CD8 T cells from individuals of diverse HLA haplotypes vaccinated with radiation-attenuated sporozoites (Table I).

In addition to CD8 epitopes recognized by T cells from individuals vaccinated with radiation-attenuated sporozoites, other epitopes were determined using cells collected from individuals naturally exposed to malaria (Hill et al. 1992, Aidoo et al. 1995, Plebanski et al. 1997). A list of the CD8 epitopes described in the distinct liver stage antigens of *P. falciparum* is depicted in Table II. As can be noticed, some of these epitopes are common to the ones mapped in individuals vaccinated with radiation-attenuated sporozoites. New epitopes recognized by individuals of the HLA-A2.1 haplotype have been recently described suggesting that the number of CD8 epitopes might be underestimated (Gonzalez et al. 2000).

TABLE I

Epitopes on *P. falciparum* liver stage proteins recognized by CD8 T cells from volunteers vaccinated with radiation-attenuated sporozoites of *P. falciparum*.

Protein	Residues	HLA	Sequence	Ref.
CS	368-390		KPKDEL DYENDIEKKICKMEKCS	Malik et al. 1991
CS	310-319	A1.31	EPSDKHIKE	Kumar et al. 2001
CS	394-402	A2.386	GLIMVLSFL	Doolan et al. 1997
CS	7-16	A2.7	ILSVSSFLFV	Doolan et al. 1997
CS	344-353	A3/11.336	VTCGNGIQVR	Doolan et al. 1997
CS	285-294	B7.285	MPNDPNRNV	Doolan et al. 1997
TRAP	1-15	A2	MNHLGNVKYLVIVFL	Wizel et al. 1995b
TRAP	46-60	A2	EVDLYLLMDCSGSIR	Wizel et al. 1995b
TRAP	121-135	A2	LLSTNLPYGKTNLTD	Wizel et al. 1995b
TRAP	126-140	A2	LPYGKTNLTDALLQV	Wizel et al. 1995b
TRAP	131-145	A2	TNLT DALLQVRKHLN	Wizel et al. 1995b
TRAP	136-150	A2	ALLQVRKHLNDRINR	Wizel et al. 1995b
TRAP	221-235	A2	ENVKNVIGPFMKAVC	Wizel et al. 1995b
TRAP	281-295	A2	CEEERCLPKREPLDV	Wizel et al. 1995b
TRAP	286-300	A2	CLPKREPLDVPDEPE	Wizel et al. 1995b
TRAP	521-535	A2	ALLACAGLAYKFVVP	Wizel et al. 1995b
TRAP	546-560	A2	APFDETLGEEDKDLD	Wizel et al. 1995b
TRAP	551-565	A2	TLGEEDKDLDEPEQF	Wizel et al. 1995b
TRAP	107-115	B8	ASKNKEKAL	Kumar et al. 2001
TRAP	109-117	B8	KNKEKALII	Kumar et al. 2001
TRAP	14-23	A2	FLIFFDLFLV	Doolan et al. 1997
TRAP	522-531	A3	LLACAGLAYK	Doolan et al. 1997
TRAP	523-531	A3	LACAGLAYK	Doolan et al. 1997
TRAP	539-548	B7	TPYAGEPAPF	Doolan et al. 1997
EXP1	80-88	A2	VLAGLLGNV	Doolan et al. 1997
EXP1	2-10	A2	KILSVFFLA	Doolan et al. 1997
EXP1	83-91	A2	GLLGNVSTV	Doolan et al. 1997
EXP1	91-100	A2	VLLGGVGLVL	Doolan et al. 1997
EXP1	10-18	A3	ALFFIIFNK	Doolan et al. 1997
LSA1	94-102	A3	QTNFKSLLR	Doolan et al. 1997
LSA1	105-113	A3	GVSENIFLK	Doolan et al. 1997
LSA1	59-68	A3	HVLSHNSYEK	Doolan et al. 1997
LSA1	11-20	A3	FILVNLLIFH	Doolan et al. 1997
PfS16	77-85	B7	MPLTQLAI	Doolan et al. 1997

Adapted from Kumar et al. 2001. Abbreviations: CS (circumsporozoite protein), TRAP (Thrombospondin Related Anonymous Protein), EXP1 (Excreted Protein-1), LSA1 (Liver Stage Antigen-1) and PfS16 (*P. falciparum* Surface antigen of 16 kDa).

CS Protein of *Plasmodium yoelii*

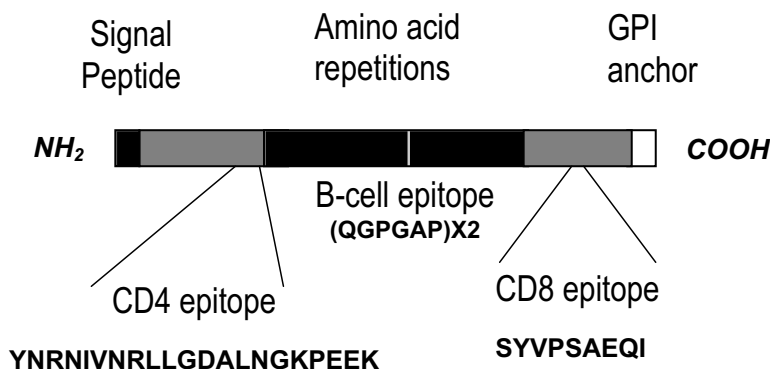


Fig. 1 – CS protein of *Plasmodium yoelii*.

TABLE II

Epitopes on *P. falciparum* liver stage proteins recognized by CD8 T cells from individuals naturally exposed to sporozoites of *P. falciparum*

Protein	Residues	HLA	Sequence	Responder / total	Ref.
CS	368-375	B35	KPKDEL DY	3/15	Hill et al. 1992
			KSKDEL DY	1/10	
CS	300-308	B7	MPNDPNRNV	1/6	Aidoo et al. 1995
CS	105-13	B8	LRKPKHKKL	2/11	Aidoo et al. 1995
TRAP	3-11	A2.1	HLGNKYLV	2/7	Aidoo et al. 1995
TRAP	500-08	A2.1	GIAGGLALL	2/7	Aidoo et al. 1995
TRAP	107-15	B8	ASKNKEKAL	1/11	Aidoo et al. 1995
TRAP	109-17	B8	KNKEKALII	2/11	Aidoo et al. 1995
LSA1	1786-94	B53	KPIVQYDNF	4/14	Hill et al. 1992
LSA1	1850-7	B35	KPNDKSLY	1/15	Hill et al. 1992
LSA1	1854-61	B17	KSLYDEHI	2/5	Aidoo et al. 1995
STARP	523-31	A2.2	MINAYLDKL	1/5	Aidoo et al. 1995

Adapted from Aidoo et al. 1995 and Plebanski et al. 1997. Abbreviations: CS (circumsporozoite protein), TRAP (Thrombospondin Related Anonymous Protein), LSA1 (Liver Stage Antigen-1) and STARP (sporozoite threonine/asparagine-rich protein).

The identification of these CD8 epitopes is the basis for the development of multi-antigen subunit vaccines aimed at the induction of CD8 T cells in humans (see below).

TRYPANOSOMA CRUZI

Spleen cells from mice chronically infected with *T. cruzi* were used to identify a number of epitopes recognized by mouse H-2Kb restricted CD8 T cells. These epitopes were located within the trypanomastig-

ote surface antigen-1 (TSA-1) and amastigote surface protein -1 or 2 (ASP-1 or ASP2, respectively, Wizel et al. 1997, Low et al. 1998). In addition, a CD8 T cell epitope was located in the *trans*-sialidase of *T. cruzi* by using spleen cells from mice immunized with plasmid DNA (Rodrigues et al. 1999). Table III contains a list of the murine CD8 epitopes that have been described so far.

Similar to mice, humans with the HLA-A2.1 haplotype chronically infected with *T. cruzi* also displayed CD8 T cells specific for epitopes within the TSA-1, ASP-1 or ASP-2 antigen (Wizel et al. 1998b, Table IV). Recently, epitopes recognized by humans with the HLA-A2.1 haplotype have been determined in the trypomastigote-secreted antigens FL-160 and cruzipain (E. Cunha-Neto, personal communication).

TOXOPLASMA GONDII AND LEISHMANIA SP.

Few publications have studied the antigens that are target of CD8 T cells specific for *T. gondii*. Khan et al. (1988), using spleen cells from infected mice, demonstrated the presence of CD8 T cells specific for the purified membrane protein denominated P30 or Surface Antigen 1 (SAG1). This result was confirmed by the immunization with native protein that elicited protective immunity dependent on the activation of this subpopulation of lymphocytes (Khan et al. 1991). CD8 T cell clones specific for the purified P30 antigen (SAG1) were generated and, when adoptively transferred to naive mice, protected them against acute infection with *T. gondii* (Khan et al. 1994).

In recent studies a putative CD8 T cell epitope recognized by spleen cells of mice immunized with a plasmid DNA containing the SAG1 gene was identified. This epitope is represented by amino acids AESKSVII and it is possibly recognized by CD8 T cells restricted by H-2Kk (Nielsen et al. 1999).

In humans with the HLA-A*0201 haplotype, one epitope recognized by CD8 T cells was also identified within the SAG1 antigen. A peptide representing amino acids 289-297 (SPEKHHCTV) was recognized by HLA-A*0201 restricted human CD8

T cells that lysed *T. gondii*-infected target cells (Yano et al. 1997).

CD8 T cells specific for a single antigen (GP46/M-2) expressed in *Leishmania amazonensis* were shown to recognize *Leishmania*-infected macrophages (Kima et al. 1997). However, no subsequent epitope mapping was performed.

CONDITIONS OF *IN VIVO* PRIMING AND EXPANSION OF PROTECTIVE CD8 T CELLS

The immune response is initiated when antigenic fragments (epitopes) of pathogen-derived proteins are presented by the molecules of the major histocompatibility complex (MHC). While MHC class I products are responsible for the presentation to classic CD8 cytotoxic T cells, MHC class II products will prime classic CD4 helper T cells.

Entry into the class I-restricted pathway of antigen presentation usually occurs through the presence of the antigenic proteins in the cytosol of antigen-presenting cells. In addition, dendritic cells can ingest proteins and have them processed and presented by the MHC class I pathway. The MHC class II pathway is mostly centered on peptides generated in the endocytic pathway. A detailed description of the MHC class I or class II pathways of antigen presentation can be found elsewhere (reviewed by Ploegh 1998).

The conditions of priming and expansion of CD8 T cells specific for any of these parasitic antigens during infection are completely obscure. It is unknown whether priming occurs through infection or antigen-pulsing of antigen-presenting cells, or by cross priming. Cross priming occurs when dendritic cells or macrophages take up cell-associated antigens and present them in the context of MHC class I molecules to classic CD8 T cells. This pathway of antigen presentation is particularly important when the antigen is not expressed in dendritic cells or macrophages. It will be very important in the future to characterize in detail the priming conditions of CD8 T cells specific for the different parasitic antigens.

The role of CD4 T cells in the priming and/or

TABLE III

Epitopes of *Trypanosoma cruzi* recognized by mouse CD8 T cells.

Peptide	Protein	Residues	H2	Sequence	Ref.
Pep77.2	TSA-1	515-522	Kb	VDYNFTIV	Wizel et al. 1997
PA14	ASP-1	509-516	Kb	VNHDFTVV	Low et al. 1998
PA5	ASP-2	285-292	Kb	KNYPFSSI	Low et al. 1998
PA6	ASP-2	274-281	Kb	NTLVFPLV	Low et al. 1998
PA7	ASP-2	545-552	Kb	DNRQYSFV	Low et al. 1998
PA8	ASP-2	552-559	Kb	VNHRFTLV	Low et al. 1998
PA10	ASP-2	399-409	Db/Kb	EKEANALYLWV	Low et al. 1998
TS359-367	TCTS	359-367	Kd	IYNVGQVSI	Rodrigues et al. 1999
P5	LYT1	190-198	Kb	ELTMKQLL	Fralish and Tarleton 2003

Adapted from Low et al. 1998. Abbreviations: TSA-1 (Trypomastigote Surface Antigen-1), ASP-1 (Amastigote Surface Protein-1), ASP-2 (Amastigote Surface Protein-2) and TCTS (*T. cruzi* trans-sialidase).

expansion of specific CD8 T cells was addressed in few studies. CD4 T cells were important for the development of CD8 T cells specific for both *T. gondii* and malaria parasites (Gazzinelli et al. 1991, Parker et al. 1991). In the case of malaria (*P. yoelii*), it was possible to elegantly demonstrate that IL-4 secreted by CD4 T cells was crucial for the maturation, but not for the initial proliferation of specific CD8 T cells (Carvalho et al. 2002).

CHARACTERIZATION OF THE *IN VIVO* EFFECTOR MECHANISMS MEDIATED BY PROTECTIVE CD8 T CELLS

MALARIA LIVER STAGES

To elucidate the role of IFN- γ as an immunological mechanism used by CD8 T cells to eliminate the liver stages of malaria parasites *in vivo*, studies were performed in mice genetically deficient for the IFN- γ receptor. After a single dose of radiation-attenuated sporozoites, these mice do not develop protective immunity against malaria liver stages. In contrast, wild type mice display remarkable immunity against these forms of malaria (Tsuji et al. 1995). This experimental evidence strongly argues for the fact that after a single immunization with radiation-attenuated sporozoites, CD8 T cells use IFN- γ -dependent mechanism(s) to elimi-

nate malaria liver stages. Because IFN- γ is a potent inducer of nitric oxide (NO) in mice, a similar approach was used to determine whether NO could mediate elimination of malaria liver stages. Unexpectedly, NO synthase 2 (NOS2)-deficient mice develop protective immunity in a manner similar to wild type animals (M. Tsuji, personal communication).

In spite of the IFN- γ dependence detected after a single immunization with radiation-attenuated sporozoites, after several doses of this immunogen, strong protective immunity can develop in mice genetically deficient for the IFN- γ receptor (Tsuji et al. 1995). The presence of IFN- γ independent mechanisms was confirmed by the observations that CD8 T cell clones, as well as CD8 T cells induced by immunization with a recombinant adenovirus, did not require a functional IFN- γ receptor to eliminate liver stages of *P. yoelii* (Rodrigues et al. 2000a). The downstream molecules activated by IFN- γ , as well as the nature of the IFN- γ -independent mechanisms remains completely unknown at present.

TRYPANOSOMA CRUZI

Although there are several lines of evidence showing that CD8 T cells participate in the protective immunity against experimental *T. cruzi* infection, the pre-

TABLE IV

Epitopes on *T. cruzi* proteins recognized by CD8 cells from individuals HLA-A2.1 chronically infected.

Peptide	Sequence	Frequency of responders	
		Positive/total	%
TSA-1(818-826)	VLLPSLFLL	3/4	75
TSA-1(818-827)	VLLPSLFLLL	18/21	86
TSA-1(89)	KLFPEVIDL	9/13	69
TSA-1(514)	FVDYNFTIV	17/21	81
ASP-1(668)	LLGLWGLTGL	14/18	78
ASP-1(666)	LLLLGLWGL	3/4	75
ASP-1(50)	VLAKDGTVEV	13/18	72
ASP-1(71)	IAGGVMAVV	4/4	100
ASP-1(508)	FVNHDFTVV	6/7	86
ASP-2(302)	WVFPESISPV	4/4	100
ASP-2(551)	FVNHRFTLV	1/4	25

Adapted from Wizel et al. 1998b. Abbreviations: TSA-1 (Trypomastigote Surface Antigen-1), ASP-1 (Amastigote Surface Protein-1), ASP-2 and (Amastigote Surface Protein-2).

cise mechanism(s) used by these cells to eliminate *T. cruzi* has(ve) not been defined.

The fact that CD8 T cells secrete IFN- γ may suggest that this mechanism may be relevant to the elimination of intracellular forms of the parasite. IFN- γ is an important mediator of naturally acquired immunity against the infection. Genetically deficient mice that do not express the IFN- γ receptor or Stat4 are unable to control *T. cruzi* infection (Holscher et al. 1998, Tarleton et al. 2000). However, a direct link between IFN- γ secretion by CD8 T cells and the *in vivo* antiparasitic activity of these cells has not been provided so far.

The IFN- γ -dependent mechanisms are at least in part mediated by the production of NO. In the absence of IFN- γ receptors, NO is not produced by macrophages, suggesting that IFN- γ is the main inducer of NOS2 activation. The evidence that NO is critical for the elimination of *T. cruzi* during acute experimental infection was obtained with genetically deficient mice that do not express NOS2. These animals are highly susceptible to infection

with different parasite strains (Holscher et al. 1998, Rodrigues et al. 2000b).

In addition to producing IFN- γ , CD8 T cells can lyse host cells infected with *T. cruzi*, or secrete other potentially active mediators such as TNF- α , granulysin or a number of different chemokines (Munoz-Fernandez et al. 1992, Aliberti et al. 1999, Stenger et al. 1999). In fact, CD8 T cells specific for amastigote or trypomastigote antigens are capable of lysing non-phagocytic cells infected with *T. cruzi in vitro* (Wizel et al. 1997, Low et al. 1998). However, it is unclear whether lysis and DNA degradation of infected target cells by CD8 T cells can be an effective mechanism to restrain *T. cruzi* infection *in vivo*. Genetically deficient mice that do not express perforin or granzyme B are not more susceptible to infection than wild type animals (Kumar and Tarleton 1998). These observations argue for the fact that in the absence of perforin- or granzyme B-mediated lysis other mechanism(s) can provide resistance against *T. cruzi* infection in mice. The elucidation of the anti-parasitic mechanisms medi-

ated by *T. cruzi*-specific CD8 T cells will require further investigation using more complex experimental models.

TOXOPLASMA GONDII

The participation of IFN- γ as an important immunological mechanism used by immune cells to eliminate tachyzoites of *T. gondii* was determined initially by the treatment of mice with specific neutralizing monoclonal antibodies. Mice injected with a monoclonal antibody to IFN- γ succumb due to acute toxoplasmosis. In contrast, animals that received control antibodies survived infection and developed chronic infection (Suzuki et al. 1988). In subsequent studies, it was shown that treatment with neutralizing monoclonal antibodies had the ability to dramatically change the resistant phenotype of mice vaccinated with an attenuated strain of *T. gondii* (Gazzinelli et al. 1991). Using genetically modified mice that do not express IFN- γ , it became clear that this cytokine is crucial for the resistance against acute toxoplasmosis in mice (Denkers et al. 1997). Because the resistance elicited by vaccination with attenuated parasites had been previously thought to be partially dependent on CD8 T cells, it was plausible to assume that the IFN- γ was secreted in part by these cells.

However, the most compelling evidence showing the importance of IFN- γ produced by CD8 T cells in mediating resistance to murine acute toxoplasmosis was obtained by the adoptive transfer of protective immunity by CD8 T cell clones specific for *T. gondii*. The treatment with specific neutralizing monoclonal antibodies to mouse IFN- γ completely reversed the immunity mediated by these T cells in naïve mice (Khan et al. 1994).

The IFN- γ secreted by CD4, CD8 and NK cells has the ability to activate *in vivo* nonhematopoietic or hematopoietic cells to eliminate intracellular *T. gondii* parasites. Using mouse bone marrow chimeras between wild-type and IFN- γ receptor-deficient mice, Yap and Sher (1999) elegantly demonstrated that both cell types have to express the IFN- γ receptor to provide efficient immunity to

acute and persistent infection.

Because IFN- γ is a major inducer of NO synthesis by phagocytic cells, the participation of NOS2 was evaluated with the aid of genetically modified mice deficient for the expression of this enzyme. NOS2-deficient mice infected with an avirulent strain of *T. gondii* die at approximately 21 to 30 days postinfection. Death was associated with large numbers of cysts and tachyzoites suggesting the importance of NO in the control of chronic infection. A similar observation was made in mice genetically deficient for IFN- γ Regulatory Factor-1, the main regulator of IFN- γ -induced NO (reviewed by Denkers and Gazzinelli 1998).

Using mouse bone marrow chimeras between wild type and NOS2-deficient mice, it was possible to determine that NO synthesis was important for the control of tachyzoite replication in infected hematopoietic but not in non-hematopoietic cells (Yap and Sher 1999).

In spite of the importance of reactive nitrogen intermediates in the control of the chronic stage of infection, several lines of evidence strongly indicate that IFN- γ induces other antiparasitic mechanisms independent of NO during murine toxoplasmosis. First, IFN- γ -deficient mice die much earlier (less than 10 days post-infection) than NOS2-deficient animals. Also, NOS2-deficient mice vaccinated with the ts-4 strain were protected against a challenge with a virulent *T. gondii* strain (Khan et al. 1998). This protective immunity had been previously shown to be entirely dependent on IFN- γ (Denkers and Gazzinelli 1998). Finally, non-hematopoietic cells deficient in the expression of NOS2 can efficiently control parasite multiplication *in vivo* (Yap and Sher 1999).

Other possible mechanisms operating *in vivo* in the control of acute toxoplasmosis can be the release of reactive oxygen intermediates or tryptophan degradation (reviewed by Denkers and Gazzinelli 1998). There is also evidence of a third still unknown mechanism of intracellular *T. gondii* elimination. Murine astrocytes can be activated by IFN- γ to eliminate intracellular forms of *T. gondii*. The

elimination of these parasites does not depend on reactive oxygen intermediates, NO or tryptophan degradation (Halonen and Weiss 2000). Whether or not these mechanisms operate *in vivo* and which one is the most relevant is still unknown.

As mentioned above, in addition to secreting IFN- γ , *T. gondii*-specific CD8 effector lymphocytes from immune mice or humans are capable of efficiently lysing parasite-infected target cells. The requirement for IFN- γ in immunity to *T. gondii* has made it difficult to evaluate the contribution of the lytic activity of CD8 T cells to the resistance to infection.

To determine the importance of perforin as a mediator of the CD8 T cell-mediated immune response, perforin-deficient mice were infected with *T. gondii*. After vaccination with the ts-4 strain, resistance to acute infection was as good in perforin-deficient mice as in wild type animals. In the chronic phase of infection there was a 3- to 4-fold increase in the number of cysts in mice infected with a non-lethal strain of *T. gondii*. This result indicates that a perforin-mediated mechanism may operate in the elimination of the brain cysts observed during the chronic stages of mouse infection (Denkers et al. 1997).

GENERATION OF SUBUNIT VACCINES CONTAINING EPITOPES RECOGNIZED BY PROTECTIVE CD8 T CELLS AND PRECLINICAL EFFICACY TRIALS IN EXPERIMENTAL MODELS

MALARIA LIVER STAGES

The fact that CD8 T cells are potent inhibitors of malaria liver stages and the characterization of epitopes recognized by these CD8 T lymphocytes prompted many investigators to explore whether induction of these T cells by subunit vaccines could be a feasible strategy for vaccination against malaria. A number of delivery systems have been used thus far to induce mouse CD8 T lymphocytes specific for the malarial epitope. They include recombinant viruses, bacteria, plasmid DNA, virus-like particles and synthetic peptides.

Recombinant viruses are the single class of im-

munogens that have been most systematically studied. Several recombinant viruses were used as vectors to elicit specific CD8 T cells and protective immunity in BALB/c mice against a challenge with sporozoites of *P. yoelii*. From these studies it became clear that, when used individually, distinct viral vectors expressing a malarial CD8 epitope provided variable degrees of protective immunity against liver stages of *P. yoelii* (reviewed by Soares and Rodrigues 1998).

When used alone in a single immunization dose, the replication-defective recombinant adenovirus containing the entire *P. yoelii* CS protein gene was shown to be the most efficient vector capable of reducing the development of liver stages of *P. yoelii* by $\sim 94\%$. Immunity was largely mediated by CD8 T lymphocytes, but CD4 T cells also participate in this process. Nevertheless, complete protection against malaria could only be observed in 40% of the mice challenged with *P. yoelii* sporozoites (Rodrigues et al. 1997). In very recent studies, the immunogenicity and protective efficacy of the recombinant adenoviral vector was improved by a simultaneous injection of the Natural Killer T cell ligand α -galactosylceramide. Under these circumstances, complete protection against infection with *P. yoelii* sporozoites was achieved in 80% of BALB/c mice (Gonzalez-Aseguinolaza et al. 2002).

Immunization with other recombinant viruses elicited CD8 T cells specific for the malarial epitope; however, the degree of elimination of malaria liver stage was lower than that observed with the adenoviral vector. Table V summarizes the results obtained after a single immunization with a variety of recombinant viral vectors.

Due to the limited degree of protective immunity observed after immunization with a single recombinant virus, studies were performed to improve protective immunity using sequential immunization with two different viral vectors expressing the same CD8 T-cell epitope for priming and boosting. This strategy has been denominated heterologous prime-boost immunization regimen.

Earlier studies performed with recombi-

TABLE V
Summary of experiments using a single recombinant virus to elicit immunological elimination of liver stages of *Plasmodium yoelii*.

Recombinant virus	Foreign gene	Liver stage inhibition	Complete protection of BALB/c mice	Immunological mechanism of protection	Reference
Adenovirus + α -galactosyl ceramide	Entire CS protein	>95%	80%	CD8 and CD4 T cells	Gonzalez-Aseguinolaza et al. 2002
Adenovirus	Entire CS protein	94%	40%	CD8 and CD4 T cells	Rodrigues et al. 1997
Sindbis	CD8 epitope	~ 75%	0%	CD8 T cells	Tsuji et al. 1998
Vaccinia	CD8 epitope	~ 75%	0%	CD8 T cells	Rodrigues et al. 1994
Vaccinia	Entire CS protein	~ 55%	0%	?	Li et al. 1993
Influenza	CD8 epitope	<10%	0%	None	Li et al. 1993

nant viruses expressing the *P. yoelii* malarial epitope showed that heterologous prime-boost immunization with recombinant influenza virus followed by recombinant vaccinia virus elicited a high degree of protective immunity against malaria infection (Li et al. 1993, Rodrigues et al. 1994). A similar protective immunity could not be achieved with two doses of only recombinant influenza or vaccinia viruses. Table VI summarizes the results obtained using different heterologous prime-boost immunization regimes to elicit immunological elimination of liver stages of *P. yoelii* and protective immunity against malaria.

This unexpected observation was explained by the fact that mice immunized with the combination of these two different carrier viruses had a frequency of epitope-specific CD8 T-cells more than 20 times higher than animals immunized twice with one of these recombinant viruses (Murata et al. 1996).

In figure 2 there is an example of the synergistic effect observed after the heterologous prime-boost immunization with recombinant influenza virus followed by recombinant vaccinia virus expressing the malarial epitope. As it can be noticed there is an increase of more than 40 folds in the number of malaria peptide specific CD8 T cells.

The expansion of epitope-specific CD8 T-cells

depends on the sequence of immunization with these two viruses. In mice primed with recombinant vaccinia virus followed by a booster injection with recombinant influenza virus, the frequency of CD8 T-cells was 10 times lower than after priming with influenza virus followed by a vaccinia virus booster (Figure 2 and Murata et al. 1996). No protective immunity could be observed in this case (Li et al. 1993). These findings were recently extended to other malaria CD8 epitopes expressed in the CS protein of *P. falciparum*, a human malaria parasite (Miyahira et al. 1998).

Based on these observations, several authors have explored the heterologous prime-boost immunization to increase the CD8 immune response. Independently performed studies using two distinct rodent malaria parasites, *P. yoelii* and *P. berghei*, observed that the heterologous prime-boost immunization regimes with a plasmid containing the CS gene followed by injection with recombinant vaccinia virus greatly enhanced the specific CD8 T cell response. This protocol of immunization did not only lead to a significant increase in the CD8 immune response, but also provided a significant degree of protection against a challenge with *P. berghei* or *P. yoelii* sporozoites (Table VI and Schneider et al. 1998, Sedegah et al. 1998).

TABLE VI

Summary of experiments using heterologous prime/boost regimes to elicit immunological elimination of liver stages and complete protection against challenge with *Plasmodium yoelii* sporozoites.

Recombinant viruses, particles of DNA		Liver stage inhibition	Complete protection of BALB/c mice	Immunological mechanism of protection	Ref.
Prime	Boost				
Adenovirus Entire CS protein	Vaccinia Entire CS protein	100%	100%	CD8 and CD4 T cells	Bruna-Romero et al. 2001
Plasmid Entire CS protein + GMCSF	Vaccinia Entire CS protein	?	80-87.5%	CD8 T cells	Sedegah et al. 2000
Plasmid Entire CS protein	Vaccinia Entire CS protein	?	50-69%	CD8 T cells	Sedegah et al. 1998
TyVLPs particles CD8 epitope	Vaccinia Entire CS protein	>95%	62%	CD8 T cells	Oliveira-Ferreira et al. 2000
Influenza CD8 epitope	Vaccinia Entire CS protein	>95%	60%	CD8 T cells	Li et al. 1993
Influenza CD8 epitope	Vaccinia Entire CS protein	>95%	?	CD8 T cells	Rodrigues et al. 1994
Vaccinia Entire CS protein	Influenza CD8 epitope	<10%	0%	None	Li et al. 1993
Vaccinia Entire CS protein	Plasmid Entire CS protein	?	13%	None	Sedegah et al. 1998

The increase in frequency of specific CD8 cells was dependent on the order of immunization. Priming with recombinant vaccinia virus followed by a booster injection of DNA did not evoke a significant increase in the frequency of specific CD8 T-cells or protection against malaria infection (Table VI and Schneider et al. 1998, Sedegah et al. 1998).

The immunogenicity of DNA vaccines can be further enhanced by co-immunization with plasmids containing genes of cytokines or co-stimulatory molecules of the immune system. Using *P. yoelii* infection, it was possible to demonstrate that co-administration of a plasmid containing the gene of murine GM-CSF enhanced both the CD8 response and the protective immunity to malaria in mice immunized with the CS gene and subsequently boosted with a recombinant vaccinia virus (Table VI and Sedegah et al. 2000).

Also using murine malaria as a vaccination model, it has been shown that priming with recom-

binant virus-like particles derived from a yeast retro-transposon (TyVLPs) containing the *P. berghei* or *P. yoelii* CS CD8 epitope could only induce strong CD8 T cell responses and protective immunity to malaria when followed by a booster injection with the recombinant vaccinia virus expressing the *P. berghei* or *P. yoelii* CS protein (Table VI and Plebanski et al. 1998, Oliveira-Ferreira et al. 2000).

Very recently, total inhibition of liver stage development and 100% protection against malaria was obtained in mice primed with the recombinant adenovirus expressing the *P. yoelii* CS protein, followed by a booster injection with a recombinant vaccinia virus expressing this same antigen. This protocol of immunization led to increased numbers specific CD8 and CD4 T cells, high anti-sporozoite antibody titers, and 100% protection of BALB/c mice when the time between priming and boosting with these two vectors was extended from 2 to 8 weeks. Most importantly, this immunization proto-

Heterologous prime-boost regimen of immunization

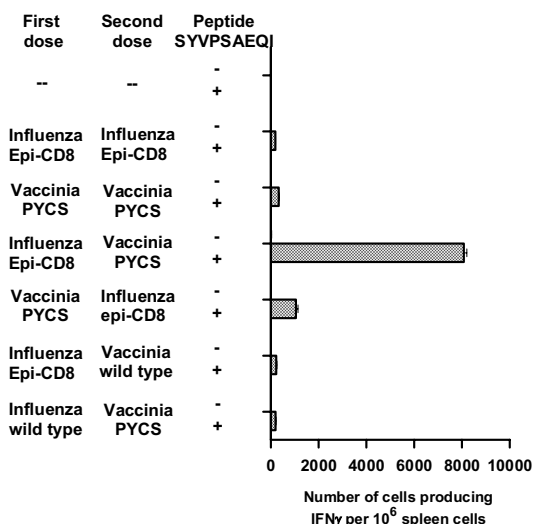


Fig. 2 – **Influenza Epi-CD8** – Recombinant Influenza virus containing the CD8 epitope of *P. yoelii* CS protein (SYVPSAEQI). **Vaccinia PYCS** – Recombinant Vaccinia virus containing the entire gene of *P. yoelii* CS protein. Adapted from Murata et al. 1996.

col elicited long-lasting protective immunity against experimental infection (Table VI and Bruna-Romero et al. 2001).

In addition to malaria, similar observations have been made in several experimental models, thereby confirming the potential of the heterologous prime-boost immunization regimen to enhance the specific immune response against multiple pathogens (reviewed by Zavala et al. 2001). It is also important to point out that this increase in the frequency of specific CD8 T cells described initially in mice was recently confirmed in several types of non-human primates (Kent et al. 1998, Robinson et al. 1999, Hanke et al. 1999, Allen et al. 2000).

TRYPANOSOMA CRUZI

The recent identification of epitopes recognized by *T. cruzi*-specific CD8 T lymphocytes has led to studies aiming at the development of subunit vaccines against experimental Chagas' disease. So far, to our

knowledge, the only delivery system used to induce *T. cruzi*-specific CD8 T lymphocytes was plasmid DNA.

DNA vaccination studies were performed with 3 distinct genes of *T. cruzi* encoding for major surface antigens of trypomastigote forms of this parasite. In studies conducted in our laboratory we used a gene encoding for the catalytic domain of the enzyme *trans*-sialidase (TS). Immunization of BALB/c mice with the TS gene elicited specific antibodies and promoted T-cell activation (Costa et al. 1998). The T-cell immune response was later characterized as being mediated mainly by CD4 Th1 and CD8 TC1 cells, which secreted a large amount of IFN- γ , but not IL-4 or IL-10 (Rodrigues et al. 1999). Upon challenge with infective *T. cruzi* trypomastigotes, immunized mice showed a significant reduction in parasitemia and survived acute lethal infection (Costa et al. 1998).

Parallel studies were performed on BALB/c and C57BL/6 mice immunized with plasmids containing the TSA-1 gene. DNA vaccination was shown to generate antibodies, as well as CD8 T cells specific for parasitic epitopes. After challenge with *T. cruzi* trypomastigotes, a significant reduction in parasitemia was observed in C57BL/6 mice, but not in BALB/c mice. Nevertheless, in both cases the DNA-vaccinated mice were more likely to survive acute infection than mice injected with the plasmid vector alone (Wizel et al. 1998a).

DNA-based immunization was also performed with a plasmid containing the gene of *T. cruzi* complement regulatory protein. This study demonstrated that both recombinant protein and DNA immunization could elicit activation of specific CD4 T cells. However, only DNA immunization could confer protective immunity on BALB/c mice. Based on that comparison, the authors proposed that CD8 T cell activation occurring during DNA immunization could be important for the protective immunity observed (Sepulveda et al. 2000).

Most recently, studies were carried out with genes encoding antigens expressed by the intracellular replicative forms of *T. cruzi*. They demonstrated

that the genes encoding ASP-1, ASP-2 and LYT1 could provide a high degree of protective immunity against experimental infection (Garg and Tarleton 2002 and Fralish and Tarleton 2003).

From mice immunized with the TS gene, we isolated CD4 Th1 and CD8 Tc1 clones which displayed remarkable antiparasitic activities *in vitro* (Rodrigues et al. 1999, 2000b). Because DNA immunization with the TS gene could elicit distinct immunological mechanisms, we considered that a detailed comparison of the immunogenicity of plasmids containing either the entire TS gene or DNA sequences encoding its immunogenic portions would be important. For that purpose, the levels of T cell response and protective immunity against experimental infection were evaluated in mice immunized with plasmids containing DNA sequences encoding either the TS CD4 or CD8 or both T-cell epitopes.

Distinct plasmids containing DNA sequences encoding both TS CD4 and CD8 T-cell epitopes could provide a degree of immunity sufficient to reduce the parasitemia and mortality of DNA-immunized animals caused by a lethal challenge with *T. cruzi*. On the other hand, plasmids expressing either CD4 or CD8 T-cell epitopes of TS were unable to provide a similar degree of protective immunity against infection (Table VII). These observations suggest that activation of CD4 Th1 and CD8Tc1 T cells are important for host immunoprotection induced by immunization with a DNA subunit vaccine (Fujimura et al. 2001).

TOXOPLASMA GONDII AND LEISHMANIA SP.

The fact that no clear epitope recognized by CD8 T cells has been characterized in *T. gondii* or *Leishmania* sp. precludes testing of subunit vaccines in experimental models. Several reports using plasmid immunization have been able to demonstrate heterogeneous degrees of protective immunity against *T. gondii* infection in immunized mice. These studies used genes encoding the antigens SAG1, GRA1, GRA7 and ROP2.

Although DNA immunization can elicit some degree of protective immunity, the activation of spe-

cific CD8 T cells has been poorly or not at all characterized (Nielsen et al. 1999, Angus et al. 2000, Vercammen et al. 2000, Desolme et al. 2000). Whether subunit vaccines may induce protective CD8 T cells specific for these antigen remains to be further characterized in the future.

In *Leishmania major*, s.c. immunization with a plasmid containing the gene coding *L. major* LACK elicited protective immunity against infection in a highly susceptible mouse strain (BALB/c). The protective immunity detected was mostly mediated by specific CD4 Th1 cells, although protective immunity was also partly dependent on CD8 T lymphocytes (Gurunathan et al. 1997). A further evaluation of the role of CD8 T cells found that they may function as helpers for the development of effector CD4 T cells. No evidence so far has indicated that the participation of CD8 T cells in protective immunity against *L. major* is related to their effector function (Gurunathan et al. 2000). Also, a CD8 T-cell epitope is yet to be identified within the LACK antigen.

HUMAN CLINICAL TRIALS USING SUBUNIT VACCINES

The successful results of preclinical vaccination studies performed on rodent malarial have encouraged human phase I/IIa vaccination trials aimed at inducing CD8 T cells specific for malaria liver stage antigens. Phase I/IIa trials tested the safety, tolerability and immunogenicity of plasmid DNA, recombinant viruses and synthetic peptides. Table VIII presents a summary of the different human trials.

The human vaccination trials performed so far have provided evidence that DNA vaccines can be successfully used to elicit a specific CD8 T cells response (Doolan and Hoffman 2001). In the first study, groups of 5 healthy individuals were immunized with different doses of a plasmid containing the *P. falciparum* CS protein gene produced under Good Manufacturing Practice conditions. Individuals received 3 i.m. doses of plasmid, each consisting of 20 to 2,500 µg of DNA. Specific cytotoxic T lymphocytes (CTL) responses were detected in 2 of 5 individuals immunized with 3 doses of ei-

TABLE VII
Requirement of DNA coding sequences for CD4 Th1 and CD8 Tc1 epitopes during
DNA immunization against experimental *T. cruzi* infection.

Plasmid	Base pairs (aminoacids)	TS region(s)	CD4 Th1 immune response	CD8 Tc1 immune response	Protective immunity
pcDNA3-TS	1-3180(1-1060)	Entire protein	Yes	Yes	Yes
p154/13	1-2034(1-678)	Catalytic domain	Yes	Yes	Yes
pΔ154/13	1-825(1-275) catalytic domain (CD4 epitopes)	Part of the	Yes	No	No
pCD8-epitope	1077-1101(359-367)	CD8 epitope	No	No	No
pΔ154/13-CD8	1-825+1077-1101 (1-275+359-367)	Part of the catalytic domain (CD4 epitopes) and CD8 epitopes	Yes	Yes	Yes
pcDNA3	None	None	No	No	No

Adapted from Fujimura et al. 2001.

TABLE VIII
Summary of the Phase I/IIa human vaccination trials carried out to elicit CD8 T-cell-mediated immunity against malaria.

Immunogen	Antigen	Safety and tolerability	Responders /Total no. of individuals tested (CD8 or CTL)	Reference
Plasmid	<i>P. falciparum</i> CS protein	Good	13/14	Wang et al. 2001
Vaccinia virus	<i>P. falciparum</i> CS protein, TRAP, LSA1, MSP1, SERA, AMA1 and Pfs25	Good	7/15	Ockenhouse et al. 1998
Synthetic peptide	<i>P. falciparum</i> CS protein (amino acids 282-383)	Good	6/8	Lopez et al. 2001

ther 20 or 100µg of DNA. The higher immunizing doses (500 and 2, 500µg of DNA) generated a CTL response in 3 of 5 and 4 of 5 individuals, respectively. MHC-restricted CD8 T-cells mediated CTL activity. CTL responses were significantly higher in individuals who received either 500 or 2, 500µg of DNA. However, no difference was observed in the magnitude of the CTL response after 3 doses in individuals immunized with these two doses of DNA. In

general, the CTL responses were stronger in DNA-immunized vaccinees compared to individuals naturally exposed to *P. falciparum* infection (Wang et al. 1998).

A second human trial was designed to test immune responses induced by different routes of immunization. Individuals received three doses of 2.5 mg of DNA i.m. by needle injection or i.m. by needle-less Biojector™ 2000, or i.m. and i.d.

by Biojector™ 2000 (70% of the total dose i.m. and 30% i.d.). Thirteen of the 14 volunteers had antigen-specific CD8 T cells that produced IFN- γ responses against 20-100% of peptides containing defined class I binding epitopes. The data also suggested that Biojector injection i.m. was the most effective route of immunization (Wang et al. 2001).

Current studies are being performed to improve cell-mediated immune responses by the co-administration of a plasmid containing the human GM-CSF gene. Also, individuals are being immunized with several plasmids containing different malarial genes. Results of these new trials are expected to be published soon (reviewed by Doolan and Hoffman 2001).

Phase I/IIa trials have also been performed with NYVAC-Pf7, a highly attenuated vaccinia virus containing 7 *P. falciparum* genes inserted into its genome. The vaccine was considered to be safe and well tolerated. Seven of the 15 individuals that received three doses of 10E8 virus developed a detectable CTL response to at least one of the three liver stage antigens (CS or LSA-1 or TRAP antigen, Ockenhouse et al. 1998). Nevertheless, it is important to notice that the individuals immunized were not HLA matched. Therefore, it is difficult to determine whether the absence of the immune response reflected variable immunogenicity of the vector or lack of CD8 T-cell epitopes.

In a third Phase I/IIa vaccination trial, individuals were immunized with a synthetic peptide representing the C-terminal region of the *P. falciparum* CS protein (amino acids 282-383). Individuals were immunized i.m. with the peptide in the presence of the adjuvants Montanide 720 or Alum. The CD8 T cell immune response was evaluated in HLA-A*0201 individuals. Upon vaccination, 6 of 8 individuals developed a CD8 T lymphocyte response, as measured by IFN- γ secretion. CD8 T cells were mainly directed toward epitopes 327-335 (5 of 8 volunteers) and 299-308 (2 of 8 volunteers, Lopez et al. 2001).

In a very recent publication, a heterologous prime-boost vaccination regime of DNA either in-

tramuscularly or epidermally, followed by intradermal recombinant modified vaccinia virus Ankara (MVA), induced high frequencies of interferon IFN- γ -secreting, antigen-specific T-cell responses in humans to a pre-erythrocytic malaria antigen, thrombospondin-related adhesion protein (TRAP, McConkey et al. 2003). T-cell responses induced by the DNA vaccine priming-recombinant MVA boosting produced partial protection manifested as delayed parasitemia after sporozoite challenge with *Plasmodium falciparum*.

Taken together, these human vaccination trials open new avenues for new vaccination strategies aimed at inducing malaria-specific CD8 T cells.

EVASION OF CD8 T-CELL IMMUNE RESPONSE

Viruses are the best known class of pathogens that use a variety of mechanisms to evade the immune recognition by MHC class I restricted CD8 T cells. Some of these strategies may function at the level of the antigen presentation by infected cells such as: i) down regulation of MHC class I expression, ii) interference with cytosolic proteolysis, iii) inhibition of peptide transport to the endoplasmic reticulum (ER), iv) retention and destruction of MHC class I molecules in the ER, v) presence of mutant epitopes that do not bind to MHC I class molecules, etc. Under these circumstances, infected cells have a limited amount of peptide/MHC-class I complexes on their surface that makes them extremely difficult to be recognized by specific CD8 T cells. A detailed review and examples of these evasion mechanisms can be found in Ploegh 1998.

Alternatively, escape mechanisms can interfere with the recognition by MHC I class restricted CD8 T cells. For example, mutant viruses can be selected with amino acid variations that retain binding to MHC class I molecules but result in reduced recognition by T cells or generate antagonistic peptides that inhibit activation of specific T cells by the MHC-peptide complex (reviewed by Ploegh 1998).

Most recently, viral proteins have been described that interfere with the effector mechanisms

TABLE IX
Polymorphism of a human CD8 T cell epitope restricted by HLA-A*0201.

Sequence	Isolates of <i>P. falciparum</i>	No. of substitutions	HLA-A*0201 binding relative activity ^a	Recognition (% lysis ^b)
YLNKIQNSL	NF54, 427	0	0.12	100
YL K KIKNSI	7G8	3	<0.03	9
YL K KIQNSL	WEL, It2G1, T9-101, T4R	1	0.25	83
YL K TIQNSL	LE5	2	0.03	0
YL K T IKNSL	T9-98, 366a, 399, 406, 419	3	0.10	10
YL Q KIQNSL	366b	1	0.20	91
YL Q KIKNSL	406, 419	2	0.30	22
YLN T IQNSL	427	1	0.20	4
YL Q KIRNSL	366c	2	0.28	14

Adapted from Bonelo et al. 2000. a = Relative activity in relation to the binding of MA influenza virus peptide. b = Percent maximal lysis induced by a human CD8 T cell clone specific for peptide YLNKIQNSL.

mediated by T cells. For example, poxviruses encode several soluble cytokine receptors that bind to and block the activity of IFN- α , β and γ TNF, IL-1 etc. Also, these viruses express a protein termed crmA that functionally inhibits Asp-specific cysteine proteases (caspases), blocking apoptosis induced by CTL, TNF, or Fas (reviewed by Ploegh 1998).

It is unknown whether protozoan parasites have evolved in such a way as to use all these mechanisms of immune evasion. So far very few examples of mechanisms of evasion have been confirmed in the case of the CD8 T cell-mediated immune response to parasites. This lack of information most likely reflects the fact that the CD8 T cell-mediated immune response to parasites only recently has attracted the attention of most investigators.

Amino acid variations in CD8 epitopes within the CS protein of *P. falciparum* malaria parasites have been described. These amino acid substitutions affected binding to HLA I class molecules, generated peptides that failed to stimulate specific CD8 T cells or behaved as antagonistic peptides (Udhayakumar et al. 1997, Gilbert et al. 1998, Bonelo et al. 2000).

The best described example that a variation in a

malaria parasite epitope may interfere with binding to HLA I class molecules or fail to stimulate specific naturally induced human CD8 T cells was described by Bonelo et al. (2000). As shown in Table IX, nine variant peptides based on the sequences of different strains of *P. falciparum* were tested for the ability to bind to HLA-A*0201 and to be recognized by a human CD8 T cell clone obtained from an individual naturally exposed to the parasite.

Variant peptides from strains 7G8 and LE5 of *P. falciparum* displayed much lower binding affinities for HLA-A*0201 and were not recognized by the human CD8 T cell clone. Four other variant peptides still retain the ability to bind to HLA-A*0201. However, because of their amino acid substitutions, they also failed to be recognized by the human CD8 T cell clone. Finally, it is noteworthy that this T cell clone could still recognize fairly well two other variant epitopes represented in several strains of *P. falciparum*. A total of 7 of the 18 different strains could be recognized by a single T cell clone.

This experiment illustrates that there might be naturally occurring variant CD8 epitopes that interfere with recognition of MHC class I restricted T cells. The identification of these variant epitopes may increase our understanding of the importance

of certain effector immune mechanisms, and ways in which vaccines can be best designed.

PERSPECTIVES

The knowledge that CD8 T cells are essential for efficient immunity to intracellular parasites has raised important questions with basic and applied implications. Several gaps in basic knowledge are expected to be filled in the years to come. Some of the most relevant questions are: i) How are specific CD8 T cells induced and maintained *in vivo*? ii) What are the main effector mechanisms operating *in vivo* against the different parasites? Are there specific or general mechanisms of immune evasion used by each of these parasites? These questions in humans are even more relevant and still more complex to study due to the obvious limitations.

From the applied point of view, it is now clear that the heterologous prime-boost regimes of immunization can be highly efficient in terms of the expansion of protective CD8 T lymphocytes. Nevertheless, the reason why this type of immunization is more efficient than the others is still largely unknown and should be addressed in the future. To our knowledge, few hypotheses have been formulated thus far to explain this event and none was tested experimentally with success.

Finally, human vaccination trials aimed at inducing CD8 T cell-mediated immunity have been initiated and should be further intensified to provide important information about the feasibility of this strategy to develop a new generation of vaccines against parasitic infections.

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RESUMO

Parasitas intracelulares obrigatórios como *Plasmodium* sp, *Trypanosoma cruzi*, *Toxoplasma gondii* e *Leishmania* sp são responsáveis pela infecção de milhões de indivíduos a cada ano. Estes parasitas são capazes de liberar antígenos no citoplasma de células infectadas do hospedeiro que são apresentados por moléculas de MHC classe I para células T CD8 protetoras. As condições de estímulo *in vivo* destas células T CD8 específicas durante a infecção natural são pouco conhecidas e constituem uma área pouco explorada. Os mecanismos anti-parasitários mediados por células T CD8 incluem vias dependentes e independentes do interferon- γ . O fato que células T CD8 são potentes inibidores do desenvolvimento parasitário levou diversos investigadores a explorarem se a indução destes linfócitos T poderia constituir uma estratégia factível para o desenvolvimento de vacinas efetivas contra estas doenças parasitárias. Estudos feitos em modelos experimentais suportam a hipótese que células T CD8 induzidas por vetores recombinantes virais ou vacinas de DNA podem servir de base para a vacinação humana. Regimes de imunização consistindo de dois vetores distintos (prime-boost heterólogo) são muito mais eficientes em termos da expansão de linfócitos T CD8 protetores do que a imunização com um único vetor. Os resultados obtidos usando modelos experimentais levaram a vacinações clínicas que estão atualmente em curso.

Palavras-chave: CD8, parasitas, imunidade, vacinas.

REFERENCES

- AIDOO M, LALVANI A, ALLSOPP CE, PLEBANSKI M, MEISNER SJ, KRAUSA P, BROWNING M, MORRIS-JONES S, GOTCH F, FIDOCK DA AND AL E. 1995. Identification of conserved antigenic components for a cytotoxic T lymphocyte-inducing vaccine against malaria. *Lancet* 345: 1003-1007.
- ALIBERTI JC, MACHADO FS, SOUTO JT, CAMPANELLI AP, TEIXEIRA MM, GAZZINELLI RT AND SILVA JS. 1999. beta-Chemokines enhance parasite uptake and promote nitric oxide-dependent microbiostatic activity in murine inflammatory macrophages infected with *Trypanosoma cruzi*. *Infect Immun* 67: 4819-4826.
- ALLEN TM, VOGEL TU, FULLER DH, MOTHE BR, STEFFEN S, BOYSON JE, SHIPLEY T, FULLER J, HANKE T,

- SETTE A, ALTMAN JD, MOSS B, McMICHAEL AJ AND WATKINS DI. 2000. Induction of AIDS virus-specific CTL activity in fresh, unstimulated peripheral blood lymphocytes from rhesus macaques vaccinated with a DNA prime/modified vaccinia virus Ankara boost regimen. *J Immunol* 164: 4968-4978.
- ANGUS CW, KLIVINGTON-EVANS D, DUBEY JP AND KOVACS JA. 2000. Immunization with a DNA plasmid encoding the SAG1 (P30) protein of *Toxoplasma gondii* is immunogenic and protective in rodents. *J Infect Dis* 181: 317-324.
- BELKAID Y, VON STEBUT E, MENDEZ S, LIRA R, CALER E, BERTHOLET S, UDEY MC AND SACKS D. 2002. CD8+ T cells are required for primary immunity in C57BL/6 mice following low-dose, intradermal challenge with *Leishmania major*. *J Immunol* 168: 3992-4000.
- BONELO A, VALMORI D, TRIPONEZ F, TIERCY JM, MENTHA G, OBERHOLZER J, CHAMPAGNE P, ROMERO JF, ESPOSITO F, NEBIE I, BARBEY C, ROMERO P, HERRERA S, CORRADIN G AND LOPEZ JA. 2000. Generation and characterization of malaria-specific human CD8(+) lymphocyte clones: effect of natural polymorphism on T cell recognition and endogenous cognate antigen presentation by liver cells. *Eur J Immunol* 30: 3079-3088.
- BOTTREL RL, DUTRA WO, MARTINS FA, GONTIJO B, CARVALHO E, BARRAL-NETTO M, BARRAL A, ALMEIDA RP, MAYRINK W, LOCKSLEY R AND GOLLOB KJ. 2001. Flow cytometric determination of cellular sources and frequencies of key cytokine-producing lymphocytes directed against recombinant LACK and soluble *Leishmania* antigen in human cutaneous *Leishmaniasis*. *Infect Immun* 69: 3232-3239.
- BRODSKYN CI, BARRAL A, BOAVENTURA V, CARVALHO E AND BARRAL-NETTO M. 1997. Parasite-driven *in vitro* human lymphocyte cytotoxicity against autologous infected macrophages from mucosal *Leishmaniasis*. *J Immunol* 159: 4467-4473.
- BRUNA-ROMERO O, GONZALEZ-ASEGUINOLAZA G, HAFALLA JC, TSUJI M AND NUSSENZWEIG RS. 2001. Complete, long-lasting protection against malaria of mice primed and boosted with two distinct viral vectors expressing the same plasmodial antigen. *Proc Natl Acad Sci USA* 98: 11491-11496.
- CARVALHO LH, SANO G, HAFALLA JC, MORROT A, CUROTTO DE LAFAILLE MA, ZAVALA F. 2002. IL-4-secreting CD4+ T cells are crucial to the development of CD8+ T-cell responses against malaria liver stages. *Nat Med* 8: 166-170.
- COSTA F, FRANCHIN G, PEREIRA-CHIOCCOLA VL, RIBEIRAO M, SCHENKMAN S AND RODRIGUES MM. 1998. Immunization with a plasmid DNA containing the gene of trans-sialidase reduces *Trypanosoma cruzi* infection in mice. *Vaccine* 16: 768-774.
- DE LUCA PM, MAYRINK W, LVES CR, COUTINHO SG, OLIVEIRA MP, BERTHO AL, TOLEDO VP, COSTA CA, GENARO O AND MENDONCA SC. 1999. Evaluation of the stability and immunogenicity of autoclaved and nonautoclaved preparations of a vaccine against American tegumentary *Leishmaniasis*. *Vaccine* 17: 1179-1185.
- DENKERS EY AND GAZZINELLI RT. 1998. Regulation and function of T-cell-mediated immunity during *Toxoplasma gondii* infection. *Clin Microbiol Rev* 11: 569-588.
- DENKERS EY, YAP G, SCHARTON-KERSTEN T, CHAREST H, BUTCHER BA, CASPAR P, HEINY S AND SHER A. 1997. Perforin-mediated cytolysis plays a limited role in host resistance to *Toxoplasma gondii*. *J Immunol* 159: 1903-1908.
- DESOLME B, MEVELEC MN, BUZONI-GATEL D AND BOUT D. 2000. Induction of protective immunity against toxoplasmosis in mice by DNA immunization with a plasmid encoding *Toxoplasma gondii* GRA4 gene. *Vaccine* 18: 2512-2521.
- DOOLAN DL AND HOFFMAN SL. 2001. DNA-based vaccines against malaria: status and promise of the Multi-Stage Malaria DNA Vaccine Operation. *Int J Parasitol* 31: 753-762.
- DOOLAN DL, HOFFMAN SL, SOUTHWOOD S, WENTWORTH PA, SIDNEY J, CHESNUT RW, KEOGH E, APPELLA E, NUTMAN TB, LAL AA, GORDON DM, OLOO A AND SETTE A. 1997. Degenerate cytotoxic T cell epitopes from *P. falciparum* restricted by multiple HLA-A and HLA-B supertype alleles. *Immunity* 7: 97-112.
- DOS SANTOS RR, ROSSI MA, LAUS JL, SILVA JS, SAVINO W AND MENGEL J. 1992. Anti-CD4 abrogates rejection and reestablishes long-term tolerance to syngeneic newborn hearts grafted in mice chronically infected with *Trypanosoma cruzi*. *J Exp Med* 175: 29-39.

- FRALISH BH AND TARLETON RL. 2003. Genetic immunization with LYT1 or a pool of trans-sialidase genes protects mice from lethal *Trypanosoma cruzi* infection. *Vaccine* 21: 3070-3080.
- FUJIMURA AE, KINOSHITA SS, PEREIRA-CHIOCCOLA VL AND RODRIGUES MM. 2001. DNA sequences encoding CD4+ and CD8+ T-cell epitopes are important for efficient protective immunity induced by DNA vaccination with a *Trypanosoma cruzi* gene. *Infect Immun* 69: 5477-5486.
- GARG N AND TARLETON RL. 2002. Genetic immunization elicits antigen-specific protective immune responses and decreases disease severity in *Trypanosoma cruzi* infection. *Infect Immun* 70: 5547-5555.
- GAZZINELLI RT, HAKIM FT, HIENY S, SHEARER GM AND SHER A. 1991. Synergistic role of CD4+ and CD8+ T lymphocytes in IFN-gamma production and protective immunity induced by an attenuated *Toxoplasma gondii* vaccine. *J Immunol* 146: 286-292.
- GILBERT SC, PLEBANSKI M, GUPTA S, MORRIS J, COX M, AIDOO M, KWIATKOWSKI D, GREENWOOD BM, WHITTLE HC AND HILL AV. 1998. Association of malaria parasite population structure, HLA, and immunological antagonism. *Science* 279: 1173-1177.
- GONZALEZ JM, PETER K, ESPOSITO F, NEBIE I, TIERCY JM, BONELO A, AREVALO-HERRERA M, VALMORI D, ROMERO P, HERRERA S, CORRADIN G AND LOPEZ JA. 2000. HLA-A*0201 restricted CD8+ T-lymphocyte responses to malaria: identification of new *Plasmodium falciparum* epitopes by IFN-gamma ELISPOT. *Parasite Immunol* 22: 501-514.
- GONZALEZ-ASEGUINOLAZA G, VAN KAER L, BERGMANN CC, WILSON JM, SCHMIEG J, KRONENBERG M, NAKAYAMA T, TANIGUCHI M, KOEZUKA Y AND TSUJI M. 2002. Natural killer T cell ligand alpha-galactosylceramide enhances protective immunity induced by malaria vaccines. *J Exp Med* 195: 617-624.
- GURUNATHAN S, SACKS DL, BROWN DR, REINER SL, CHAREST H, GLAICHENHAUS N AND SEDER RA. 1997. Vaccination with DNA encoding the immunodominant LACK parasite antigen confers protective immunity to mice infected with *Leishmania major*. *J Exp Med* 186: 1137-1147.
- GURUNATHAN S, STOBIE L, PRUSSIN C, SACKS DL, GLAICHENHAUS N, IWASAKI A, FOWELL DJ, LOCKSLEY RM, CHANG JT, WU CY AND SEDER RA. 2000. Requirements for the maintenance of Th1 immunity *in vivo* following DNA vaccination: a potential immunoregulatory role for CD8+ T cells. *J Immunol* 165: 915-924.
- HALONEN SK AND WEISS LM. 2000. Investigation into the mechanism of gamma interferon-mediated inhibition of *Toxoplasma gondii* in murine astrocytes. *Infect Immun* 68: 3426-3430.
- HANKE T, SAMUEL RV, BLANCHARD TJ, NEUMANN VC, ALLEN TM, BOYSON JE, SHARPE SA, COOK N, SMITH GL, WATKINS DI, CRANAGE MP AND MCMICHAEL AJ. 1999. Effective induction of simian immunodeficiency virus-specific cytotoxic T lymphocytes in macaques by using a multiepitope gene and DNA prime-modified vaccinia virus Ankara boost vaccination regimen. *J Virol* 73: 7524-7532.
- HIGUCHI MD, RIES MM, AIELLO VD, BENVENUTI LA, GUTIERREZ PS, BELLOTTI G AND PILEGGI F. 1997. Association of an increase in CD8+ T cells with the presence of *Trypanosoma cruzi* antigens in chronic, human, chagasic myocarditis. *Am J Trop Med Hyg* 56: 485-489.
- HILL AV, ELVIN J, WILLIS AC, AIDOO M, ALLSOPP CE, GOTCH FM, GAO XM, TAKIGUCHI M, GREENWOOD BM, TOWNSEND AR AND AL E. 1992. Molecular analysis of the association of HLA-B53 and resistance to severe malaria. *Nature* 360: 434-439.
- HOFFMAN SL, GOH LM, LUKE TC, SCHNEIDER I, LE TP, DOOLAN DL, SACCI J, DE LA VEGA P, DOWLER M, PAUL C, GORDON DM, STOUTE JA, CHURCH LW, SEDEGAH M, HEPPNER DG, BALLOU WR AND RICHIE TL. 2002. Protection of humans against malaria by immunization with radiation-attenuated *Plasmodium falciparum* sporozoites. *J Infect Dis* 185: 1155-1164.
- HOLSCHER C, KOHLER G, MULLER U, MOSSMANN H, SCHAUB GA AND BROMBACHER F. 1998. Defective nitric oxide effector functions lead to extreme susceptibility of *Trypanosoma cruzi*-infected mice deficient in gamma interferon receptor or inducible nitric oxide synthase. *Infect Immun* 66: 1208-1215.
- HUBER M, TIMMS E, MAK TW, ROLLINGHOFF M AND LOHOFF M. 1998. Effective and long-lasting immunity against the parasite *Leishmania major* in CD8-deficient mice. *Infect Immun* 66: 3968-3970.
- KENT SJ, ZHAO A, BEST SJ, CHANDLER JD, BOYLE DB AND RAMSHAW IA. 1998. Enhanced T-cell immuno-

- genicity and protective efficacy of a human immunodeficiency virus type 1 vaccine regimen consisting of consecutive priming with DNA and boosting with recombinant fowlpox virus. *J Virol* 72: 10180-10188.
- KHAN IA, SMITH KA AND KASPER LH. 1988. Induction of antigen-specific parasiticidal cytotoxic T cell splenocytes by a major membrane protein (P30) of *Toxoplasma gondii*. *J Immunol* 141: 3600-3605.
- KHAN IA, SMITH KA AND KASPER LH. 1990. Induction of antigen-specific human cytotoxic T cells by *Toxoplasma gondii*. *J Clin Invest* 85: 1879-1886.
- KHAN IA, ELY KH AND KASPER LH. 1991. A purified parasite antigen (p30) mediates CD8+ T cell immunity against fatal *Toxoplasma gondii* infection in mice. *J Immunol* 147: 3501-3506.
- KHAN IA, ELY KH AND KASPER LH. 1994. Antigen-specific CD8+ T cell clone protects against acute *Toxoplasma gondii* infection in mice. *J Immunol* 152: 1856-1860.
- KHAN IA, MATSUURA T AND KASPER LH. 1998. Inducible nitric oxide synthase is not required for long-term vaccine-based immunity against *Toxoplasma gondii*. *J Immunol* 161: 2994-3000.
- KIMA PE, RUDDLE NH AND McMAHON-PRATT D. 1997. Presentation via the class I pathway by *Leishmania amazonensis*-infected macrophages of an endogenous *Leishmania* antigen to CD8+ T cells. *J Immunol* 159: 1828-1834.
- KUMAR A, KUMAR S, LE TP, SOUTHWOOD S, SIDNEY J, COHEN J, SETTE A AND HOFFMAN SL. 2001. HLA-A*01-restricted cytotoxic T-lymphocyte epitope from the *Plasmodium falciparum* circumsporozoite protein. *Infect Immun* 69: 2766-2771.
- KUMAR S AND TARLETON RL. 1998. The relative contribution of antibody production and CD8+ T cell function to immune control of *Trypanosoma cruzi*. *Parasite Immunol* 20: 207-216.
- LI S, RODRIGUES M, RODRIGUEZ D, RODRIGUEZ JR, ESTEBAN M, PALESE P, NUSSENZWEIG RS AND 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.
- LOCKSLEY RM, REINER SL, HATAM F, LITTMAN DR AND KILLEEN N. 1993. Helper T cells without CD4: control of *Leishmaniasis* in CD4-deficient mice. *Science* 261: 1448-1451.
- LOPEZ JA, WEILENMAN C, AUDRAN R, ROGGERO MA, BONELO A, TIERCY JM, SPERTINI F AND CORRADIN G. 2001. A synthetic malaria vaccine elicits a potent CD8(+) and CD4(+) T lymphocyte immune response in humans. Implications for vaccination strategies. *Eur J Immunol* 31: 1989-1998.
- LOW HP, SANTOS MA, WIZEL B AND TARLETON RL. 1998. Amastigote surface proteins of *Trypanosoma cruzi* are targets for CD8+ CTL. *J Immunol* 160: 1817-1823.
- MALIK A, EGAN JE, HOUGHTEN RA, SADOFF JC AND HOFFMAN SL. 1991. Human cytotoxic T lymphocytes against the *Plasmodium falciparum* circumsporozoite protein. *Proc Natl Acad Sci USA* 88: 3300-3304.
- McCONKEY SJ, REECE WH, MOORTHY VS, WEBSTER D, DUNACHIE S, BUTCHER G, VUOLA JM, BLANCHARD TJ, GOTHARD P, WATKINS K, HANNAN CM, EVERAERE S, BROWN K, KESTER KE, CUMMINGS J, WILLIAMS J, HEPPNER DG, PATHAN A, FLANAGAN K, ARULANANTHAM N, ROBERTS MT, ROY M, SMITH GL, SCHNEIDER J, PETO T, SINDEN RE, GILBERT SC AND HILL AV. 2003. Enhanced T-cell immunogenicity of plasmid DNA vaccines boosted by recombinant modified vaccinia virus Ankara in humans. *Nat Med* 9: 729-735.
- MIIYAHIRA Y, GARCIA-SASTRE A, RODRIGUEZ D, RODRIGUEZ JR, MURATA K, TSUJI M, PALESE P, ESTEBAN M, ZAVALA F AND NUSSENZWEIG RS. 1998. Recombinant viruses expressing a human malaria antigen can elicit potentially protective immune CD8+ responses in mice. *Proc Natl Acad Sci USA* 95: 3954-3959.
- MUNOZ-FERNANDEZ MA, FERNANDEZ MA AND FRESNO M. 1992. Synergism between tumor necrosis factor-alpha and interferon-gamma on macrophage activation for the killing of intracellular *Trypanosoma cruzi* through a nitric oxide-dependent mechanism. *Eur J Immunol* 22: 301-307.
- MURATA K, GARCIA-SASTRE A, TSUJI M, RODRIGUES M, RODRIGUEZ D, RODRIGUEZ JR, NUSSENZWEIG RS, PALESE P, ESTEBAN M AND ZAVALA F. 1996. Char-

- acterization of *in vivo* primary and secondary CD8+ T cell responses induced by recombinant influenza and vaccinia viruses. *Cell Immunol* 173: 96-107.
- NIELSEN HV, LAUEMOLLER SL, CHRISTIANSEN L, BUUS S, FOMSGAARD A AND PETERSEN E. 1999. Complete protection against lethal *Toxoplasma gondii* infection in mice immunized with a plasmid encoding the SAG1 gene. *Infect Immun* 67: 6358-6363.
- OCKENHOUSE CF, SUN PF, LANAR DE, WELDE 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 AND AL E. 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, MIYAHIRA Y, LAYTON GT, SAVAGE N, ESTEBAN M, RODRIGUEZ D, RODRIGUEZ JR, NUSSENZWEIG RS AND 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.
- PARKER SJ, ROBERTS CW AND ALEXANDER J. 1991. CD8+ T cells are the major lymphocyte subpopulation involved in the protective immune response to *Toxoplasma gondii* in mice. *Clin Exp Immunol* 84: 207-212.
- PLEBANSKI M, AIDOO M, WHITTLE HC AND HILL AV. 1997. Precursor frequency analysis of cytotoxic T lymphocytes to pre-erythrocytic antigens of *Plasmodium falciparum* in West Africa. *J Immunol* 158: 2849-2855.
- PLEBANSKI M, GILBERT SC, SCHNEIDER J, HANNAN CM, LAYTON G, BLANCHARD T, BECKER M, SMITH G, BUTCHER G, SINDEN RE AND HILL AV. 1998. Protection from *Plasmodium berghoi* 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.
- PLOEGH HL. 1998. Viral strategies of immune evasion. *Science* 280: 248-253.
- POMPEU MM, BRODSKYN C, TEIXEIRA MJ, CLARENCO J, VAN WEYENBERG J, COELHO IC, CARDOSO SA, BARRAL A AND BARRAL-NETTO M. 2001. Differences in gamma interferon production *in vitro* predict the pace of the *in vivo* response to *Leishmania amazonensis* in healthy volunteers. *Infect Immun* 69: 7453-7460.
- ROBINSON HL, MONTEFIORI DC, JOHNSON RP, MANSON KH, KALISH ML, LIFSON JD, RIZVI TA, LU S, HU SL, MAZZARA GP, PANICALI DL, HERNDON JG, GLICKMAN R, CANDIDO MA, LYDY SL, WYAND MS AND McCLURE HM. 1999. Neutralizing antibody-independent containment of immunodeficiency virus challenges by DNA priming and recombinant pox virus booster immunizations. *Nat Med* 5: 526-534.
- RODRIGUES EG, ZAVALA F, EICHINGER D, WILSON JM AND TSUJI M. 1997. Single immunizing dose of recombinant adenovirus efficiently induces CD8+ T cell-mediated protective immunity against malaria. *J Immunol* 158: 1268-1274.
- RODRIGUES EG, CLAASSEN J, LEE S, WILSON JM, NUSSENZWEIG RS AND TSUJI M. 2000a. Interferon-gamma-independent CD8+ T cell-mediated protective anti-malaria immunity elicited by recombinant adenovirus. *Parasite Immunol* 22: 157-160.
- RODRIGUES M, NUSSENZWEIG RS, ROMERO P AND ZAVALA F. 1992. The *in vivo* cytotoxic activity of CD8+ T cell clones correlates with their levels of expression of adhesion molecules. *J Exp Med* 175: 895-905.
- RODRIGUES M, LI S, MURATA K, RODRIGUEZ D, RODRIGUEZ JR, BACIK I, BENNINK JR, YEWDELL JW, GARCIA-SASTRE A, NUSSENZWEIG RS, ESTEBAN M, PALESE P AND ZAVALA F. 1994. Influenza and vaccinia viruses expressing malaria CD8+ T and B cell epitopes. Comparison of their immunogenicity and capacity to induce protective immunity. *J Immunol* 153: 4636-4648.
- RODRIGUES MM, CORDEY AS, ARREAZA G, CORRADIN G, ROMERO P, MARYANSKI JL, NUSSENZWEIG RS AND ZAVALA F. 1991. CD8+ cytolytic T cell clones derived against the *Plasmodium yoelii* circumsporozoite protein protect against malaria. *Int Immunol* 3: 579-585.
- RODRIGUES MM, RIBEIRAO M, PEREIRA-CHIOCCOLA V, RENIA L AND COSTA F. 1999. Predominance of CD4 Th1 and CD8 Tc1 cells revealed by characterization

- of the cellular immune response generated by immunization with a DNA vaccine containing a *Trypanosoma cruzi* gene. *Infect Immun* 67: 3855-3863.
- RODRIGUES MM, RIBEIRAO M AND BOSCARDIN SB. 2000b. CD4 Th1 but not Th2 clones efficiently activate macrophages to eliminate *Trypanosoma cruzi* through a nitric oxide dependent mechanism. *Immunol Lett* 73: 43-50.
- ROMERO P, MARYANSKI JL, CORRADIN G, NUSSENZWEIG RS, NUSSENZWEIG V AND ZAVALA F. 1989. Cloned cytotoxic T cells recognize an epitope in the circumsporozoite protein and protect against malaria. *Nature* 341: 323-326.
- ROTTENBERG ME, BAKHIET M, OLSSON T, KRISTENSSON K, MAK T, WIGZELL H AND ORN A. 1993. Differential susceptibilities of mice genomically deleted of CD4 and CD8 to infections with *Trypanosoma cruzi* or *Trypanosoma brucei*. *Infect Immun* 61: 5129-5133.
- SCHNEIDER J, GILBERT SC, BLANCHARD TJ, HANKE T, ROBSON KJ, HANNAN CM, BECKER M, SINDEN R, SMITH GL AND HILL AV. 1998. Enhanced immunogenicity for CD8+ T cell induction and complete protective efficacy of malaria DNA vaccination by boosting with modified vaccinia virus Ankara. *Nat Med* 4: 397-402.
- SCHOFIELD L, VILLAQUIRAN J, FERREIRA A, SCHELLEKENS H, NUSSENZWEIG R AND NUSSENZWEIG V. 1987. Gamma interferon, CD8+ T cells and antibodies required for immunity to malaria sporozoites. *Nature* 330: 664-666.
- SEDEGAH M, JONES TR, KAUR M, HEDSTROM R, HOBART P, TINE JA AND HOFFMAN SL. 1998. Boosting with recombinant vaccinia increases immunogenicity and protective efficacy of malaria DNA vaccine. *Proc Natl Acad Sci USA* 95: 7648-7653.
- SEDEGAH M, WEISS W, SACCI JBJ, CHAROENVIT Y, HEDSTROM R, GOWDA K, MAJAM VF, TINE J, KUMAR S AND HOBART P HS. 2000. Improving protective immunity induced by DNA-based immunization: priming with antigen and GM-CSF-encoding plasmid DNA and boosting with antigen-expressing recombinant poxvirus. *J Immunol* 164: 5905-5912.
- SEPULVEDA P, HONTEBEYRIE M, LIEGEARD P, MASCILLI A AND NORRIS KA. 2000. DNA-Based immunization with *Trypanosoma cruzi* complement regulatory protein elicits complement lytic antibodies and confers protection against *Trypanosoma cruzi* infection. *Infect Immun* 68: 4986-4991.
- SOARES IS AND RODRIGUES MM. 1998. Malaria vaccine: roadblocks and possible solutions. *Braz J Med Biol Res* 31: 317-332.
- SOARES MB, PONTES-DE-CARVALHO L AND RIBEIRO-DOS-SANTOS R. 2001a. The pathogenesis of Chagas' disease: when autoimmune and parasite-specific immune responses meet. *An Acad Bras Cienc* 73: 547-559.
- SOARES MB, SILVA-MOTA KN, LIMA RS, BELLINTANI MC, PONTES-DE-CARVALHO L AND RIBEIRO-DOS-SANTOS R. 2001b. Modulation of chagasic cardiomyopathy by interleukin-4: dissociation between inflammation and tissue parasitism. *Am J Pathol* 159: 703-709.
- STENGER S, ROSAT JP, BLOOM BR, KRENSKY AM AND MODLIN RL. 1999. Granulysin: a lethal weapon of cytolytic T cells. *Immunol Today* 20: 390-394.
- SUZUKI Y, ORELLANA MA, SCHREIBER RD AND REMINGTON JS. 1988. Interferon-gamma: the major mediator of resistance against *Toxoplasma gondii*. *Science* 240: 516-518.
- TARLETON RL, KOLLER BH, LATOUR A AND POSTAN M. 1992. Susceptibility of beta 2-microglobulin-deficient mice to *Trypanosoma cruzi* infection. *Nature* 356: 338-340.
- TARLETON RL, GRUSBY MJ AND ZHANG L. 2000. Increased susceptibility of Stat4-deficient and enhanced resistance in Stat6-deficient mice to infection with *Trypanosoma cruzi*. *J Immunol* 165: 1520-1525.
- TSUJI M, MIYAHIRA Y, NUSSENZWEIG RS, AGUET M, REICHEL M AND ZAVALA F. 1995. Development of antimalaria immunity in mice lacking IFN-gamma receptor. *J Immunol* 154: 5338-5344.
- TSUJI M, BERGMANN CC, TAKITA-SONODA Y, MURATA K, RODRIGUES EG, NUSSENZWEIG RS AND ZAVALA F. 1998. Recombinant Sindbis viruses expressing a cytotoxic T-lymphocyte epitope of a malaria parasite or of influenza virus elicit protection against the corresponding pathogen in mice. *J Virol* 72: 6907-6910.
- UDHAYAKUMAR V, ONGECHA JM, SHI YP, AIDOO M, ORAGO AS, OLOO AJ, HAWLEY WA, NAHLEN BL, HOFFMAN SL, WEISS WR AND LAL AA. 1997. Cy-

- toxic T cell reactivity and HLA-B35 binding of the variant *Plasmodium falciparum* circumsporozoite protein CD8+ CTL epitope in naturally exposed Kenyan adults. *Eur J Immunol* 27: 1952-1957.
- VERCAMMEN M, SCORZA T, HUYGEN K, DE BRAEKELEER J, DIET R, JACOBS D, SAMAN E AND VERSCHUEREN H. 2000. DNA vaccination with genes encoding *Toxoplasma gondii* antigens GRA1, GRA7, and ROP2 induces partially protective immunity against lethal challenge in mice. *Infect Immun* 68: 38-45.
- WANG R, DOOLAN DL, LE TP, HEDSTROM RC, COONAN KM, CHAROENVIT Y, JONES TR, HOBART P, MARGALITH M, NG J, WEISS WR, SEDEGAH M, DE TAISNE C, NORMAN JA AND HOFFMAN SL. 1998. Induction of antigen-specific cytotoxic T lymphocytes in humans by a malaria DNA vaccine. *Science* 282: 476-480.
- WANG R, EPSTEIN J, BARACEROS FM, GORAK EJ, CHAROENVIT Y, CARUCCI DJ, HEDSTROM RC, RAHARDJO N, GAY T, HOBART P, STOUT R, JONES TR, RICHIE TL, PARKER SE, DOOLAN DL, NORMAN J AND HOFFMAN SL. 2001. Induction of CD4(+) T cell-dependent CD8(+) type 1 responses in humans by a malaria DNA vaccine. *Proc Natl Acad Sci USA* 98: 10817-10822.
- WEISS WR, SEDEGAH M, BEAUDOIN RL, MILLER LH AND GOOD MF. 1988. CD8+ T cells (cytotoxic/suppressors) are required for protection in mice immunized with malaria sporozoites. *Proc Natl Acad Sci USA* 85: 573-576.
- WIZEL B, HOUGHTEN RA, PARKER KC, COLIGAN JE, CHURCH P, GORDON DM, BALLOU WR AND HOFFMAN SL. 1995a. Irradiated sporozoite vaccine induces HLA-B8-restricted cytotoxic T lymphocyte responses against two overlapping epitopes of the *Plasmodium falciparum* sporozoite surface protein 2. *J Exp Med* 182: 1435-1445.
- WIZEL B, HOUGHTEN R, CHURCH P, TINE JA, LANAR DE, GORDON DM, BALLOU WR, SETTE A AND HOFFMAN SL. 1995b. HLA-A2-restricted cytotoxic T lymphocyte responses to multiple *Plasmodium falciparum* sporozoite surface protein 2 epitopes in sporozoite-immunized volunteers. *J Immunol* 155: 766-775.
- WIZEL B, NUNES M AND TARLETON RL. 1997. Identification of *Trypanosoma cruzi* trans-sialidase family members as targets of protective CD8+ TC1 responses. *J Immunol* 159: 6120-6130.
- WIZEL B, GARG N AND TARLETON RL. 1998a. Vaccination with trypomastigote surface antigen 1-encoding plasmid DNA confers protection against lethal *Trypanosoma cruzi* infection. *Infect Immun* 66: 5073-5081.
- WIZEL B, PALMIERI M, MENDOZA C, ARANA B, SIDNEY J, SETTE A AND TARLETON R. 1998b. Human infection with *Trypanosoma cruzi* induces parasite antigen-specific cytotoxic T lymphocyte responses. *J Clin Invest* 102: 1062-1071.
- YANO A, AOSAI F, YANG TH, HE N, MUN HS, LIU H, INOKO H AND NOROSE K. 1997. Correlation between direct binding ability of synthetic *T. gondii* SAG1 peptides to HLA-A2 measured by a sensor for surface plasmon resonance and antigenicity of the peptides for *T. gondii*-infected cell-specific CTL. *Biochem Biophys Res Commun* 236: 257-261.
- YAP GS AND SHER A. 1999. Effector cells of both non-hemopoietic and hemopoietic origin are required for interferon (IFN)-gamma- and tumor necrosis factor (TNF)-alpha-dependent host resistance to the intracellular pathogen, *Toxoplasma gondii*. *J Exp Med* 189: 1083-1092.
- ZAVALA F, RODRIGUES M, RODRIGUEZ D, RODRIGUEZ JR, NUSSENZWEIG RS AND ESTEBAN M. 2001. A striking property of recombinant poxviruses: efficient inducers of *in vivo* expansion of primed CD8(+) T cells. *Virology* 280: 155-159.