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Long-Term Multiepitopic Cytotoxic-T-Lymphocyte Responses Induced in Chimpanzees by Combinations of *Plasmodium falciparum* Liver-Stage Peptides and Lipopeptides

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Preclinical immunogenicity studies of 12 malaria peptides, selected from four *Plasmodium falciparum* antigens (Ags), namely, LSA1, LSA3, SALSA, and STARP, that are expressed at the pre-erythrocytic (sporozoite and liver) stages of the human parasite were carried out in chimpanzees. To strengthen their immunogenicity, six of these synthetic peptides were modified by the C-terminal addition of a single palmitoyl chain (lipopeptides) and delivered without adjuvant, whereas the remaining six unmodified peptides were emulsified and delivered by using Montanide ISA51 adjuvant. We have previously reported that these peptides and lipopeptides induce high B-cell and $CD4^+$-T-helper responses in chimpanzees. In this report, we show their ability to induce multiepitopic and long-lasting antigen-specific $CD8^+$ cytotoxic-T-lymphocyte (CTL) responses. The magnitude, consistency, and memory of CTL responses generated by LSA3 peptides point to the strong immunogenicity of this liver-stage Ag. These findings support the screening strategy used to select the four *P. falciparum* pre-erythrocytic Ags and emphasize their valuable immunogenic properties. The successful immunization of nonhuman primates with combinations of corresponding peptides in a mineral oil emulsion (ISA51) and lipopeptides in saline provide a vaccine formulation that can be tested in humans.

Malaria remains one of the most devastating infectious diseases, afflicting 300 to 500 million individuals each year in many developing countries of tropical regions (7, 10, 13, 18, 33, 40, 49, 52, 53). Despite the availability of many intervention strategies, the global picture for malaria continue to deteriorate rapidly, with an eightfold increase in mortality rates predicted for the most heavily populated areas of the world (7, 10, 13, 18, 33, 41, 49, 52, 53, 55). This bleak outlook stems from the increasingly widespread development of drug-resistant forms of *Plasmodium falciparum* and insecticide-resistant *Anopheles* mosquito vectors.

The development of an effective vaccine would present an unparalleled alternative for malaria control, since it may provide the most cost-effective means to reduce the estimated annual mortality of 1.5 to 2.7 million people, caused mainly by *P. falciparum* (7, 18, 41, 52, 53). However, major challenges still face its development, including the identification of target antigens (Ags) and derived epitopes, assessment of their immunogenicity, and the need for an efficient and safe immunization strategy.

In both humans and experimental animals, repeated immunizations with radiation-attenuated sporozoites (IRRD-SPZ) induce sterile protection against a viable sporozoite challenge (20, 34, 43). For obvious practical and ethical considerations, this strategy is not appropriate for commercial vaccine production, but these findings strongly suggest the feasibility of inducing immunity to the pre-erythrocytic (sporozoite and liver) stage by vaccination. In addition, sublethally irradiated sporozoites injected intravenously into animals are able to invade the host hepatocytes but unable to undergo a complete schizogony to produce infectious merozoites (6, 43). This indicates that the Ags expressed during the liver stage (LS) of parasite development are crucial in stimulating host protective immunity (43). This early observation is supported by independent results showing induction of protective immunity in rodents after immunization with *P. berghei* LS extracts, as well as by data showing inhibition of *P. berghei* IRRD-SPZ-induced immunity when preexisting liver forms are eliminated (6, 56, 58). However, it still remains to be determined which of the numerous immune defense mechanisms described in rodent models of malaria are relevant to protection against human *P. falciparum* pre-erythrocytic stages (6, 20, 21, 34). Extrapolation from extensive rodent studies suggests that the pre-erythrocytic immunity to *P. falciparum* infection is mediated, at least in part, by major histocompatibility complex (MHC) class I-restricted cytotoxic T lymphocytes (CTLs) acting against the intrahepatic forms (20, 21). In addition, LS antigen 1 (LSA1)-specific HLA class I-restricted $CD8^+$-CTL responses correlate with resistance to severe malaria in humans (16). Based on these observations, one logical hypothesis is that an effective pre-erythrocytic malaria vaccine is likely to require the inclusion of intrahepatic T-cell epitopes capable of inducing CTL activity (20, 34, 43).

We have focused on the immunological characterization of *P. falciparum* LS Ags (LSAs), namely, LSA1, LSA3, SALSA, and STARP, that have been identified and sequenced in our laboratory (2, 15, 26, 43). Twelve synthetic peptides representing potential T-cell epitopes were selected from these four new

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TABLE 1. Peptides and lipopeptides derived from four P. *falciparum* Ags (LSA1, LSA3, SALSA, and STARP) selected for synthesis[a]

Antigen	Peptide	Residues	Sequence	No. of aa
LSA1	LSA1-REP	187–227	Ac-LAKEKLQEQQSDLEQ ERLAKEKLQEQQSDLEQERLAKEKLQ	41
	LSA1-J	1613–1636	Ac-ERRAKEKLQEQQSDLEQRKADTKK K(Pam)-NH2	24
	LSA1-NR	1633–1659	Ac-DTKKNLERKKEHGDILAEDLYGRLEIP	27
	LSA1-TER	1686–1719	Ac-NSRDSKEISIIEKTNRESITTNVEGRRDIHKGHKGHL	37
LSA3	LSA3-CT1	24–43	Ac-LLSNIEEPKENIIDNLLNNI K(Pam)-NH2	20
	LSA3-NRI	60–85	Ac-DELFNELLNSVDVNGEVKENILEESQ	26
	LSA3-NRII	81–106	Ac-LEESQVNDDIFNSLVKSVQQEQQHNV K(Pam)-NH2	26
	LSA3-RE	183–210	Ac-VESVAPSVEESVAPSVEESVAENVEESV	28
SALSA	SALSA1	23–49	Ac-SAEKKDEKEASEQGEESHKKENSQESA K(Pam)-NH2	27
	SALSA2	50–83	Ac-NGKDDVKEEKKTNEKKDDGKTDKVQEKVLEKSPK	34
STARP	STARP-R	359–378	Ac-STDNNNTKTISTDNNNTKTI K(Pam)-NH2	20
	STARP-M	512–531	Ac-STDNNNTNTISTDNNNTNTI K(Pam)-NH2 L T NTIKA S IT N D D NL D T K K K	20

[a] The amino acid sequence of each peptide (as single-letter code) and the corresponding positions and numbers of amino acids are shown. Lipopeptides were elongated with an additional lysine residue linked to a palmitic acid molecule [indicated as K(Pam)-NH2]. aa, amino acids.

LS molecules (7, 9, 11, 46, 47), and their antigenicity has been demonstrated in immunoepidemiological studies in several areas where malaria is endemic (15, 26; P. Brasseur et al., unpublished data). Our previous findings in small experimental animal models (mice and aotus) showed that covalent modification of peptides by a simple fatty acid enhances considerably their T-cell immunogenicity in the absence of adjuvant (9, 11). Therefore, as a clinically approved approach, the strategy of introducing a C-terminal palmitoylysylamide residue [K(Pam)-NH2] was extended to six of the selected peptides (lipopeptides) that were delivered in chimpanzees without adjuvant, i.e., in saline. With the perspective of using the chimpanzees as a preclinical screen, the remaining six unmodified peptides were emulsified in Montanide ISA51, an oil-in-water immunoadjuvant that can be administered safely to humans (62). We have previously reported that these LS peptides or lipopeptides induce long-lasting antibody and gamma interferon-secreting $CD4^+$–T-helper responses in nonhuman primates (7, 9, 11, 46, 47). We now show that in chimpanzees, the animal model best supporting the complete intrahepatic development of *P. falciparum* (17, 20, 34, 36, 44), combinations of LS peptides in ISA51 emulsions and of LS lipopeptides in saline also induce multiple $CD8^+$ CTLs that persist for up to 9 months postimmunization. These findings strongly suggest that *P. falciparum* LSAs display promising immunogenic properties to be included in a new vaccine against malaria.

MATERIALS AND METHODS

Animals. A total of six malaria-negative adult chimpanzees (*Pan troglodytes*) (Table 1) were enrolled in immunization experiments. The animals were randomly selected and housed in the Biomedical Primate Research Centre, Rijswijk, The Netherlands. Prior to the initiation of the study, all of the animals went through a detailed investigation, which included (i) assessment of current or past *Plasmodium* infection; (ii) determination of antibodies with specificity for sporozoites and erythrocytic stages of *P. falciparum*; (iii) determination of antibodies directed against the four pre-erythrocytic-stage Ags used for immunization; and, finally, (iv) determination of T-cell responses to LS peptides in preimmune lymphocyte cultures. Blood smears were prepared on several occasions and examined for the presence of parasites. None of these tests showed any evidence of current or past infection in any animal. Two weeks prior to the start of the study, no significant T-cell response to any LS peptides used in the present study was observed in preimmunization lymphocyte samples from the studied chimpanzees. Experimental procedures were performed under standard conditions in compliance with the institutional animal care and use committee and according to the relevant laws related to the conduct of animal experiments.

Peptides and lipopeptides. The sequences, localizations, and numbers of the amino acids of the 12 peptides and lipopeptides derived from four *P. falciparum* pre-erythrocytic Ags (strain T.9.96) are shown in Table 1. All peptides were synthesized by the solid-phase method on a benzhydrylamine resin (Applied Biosystems, Foster City, Calif.) by using a standard *t*-butyloxycarbonyl (Boc)-benzyl strategy and systematically acetylated at the end of the synthesis (7, 9, 11). A Boc-L-Lys (Fmoc [9-fluorenylmethoxy carbonyl]) group was introduced in the C-terminal end of the peptide and coupled to a palmitic acid moiety as previously described (7, 9–13, 19, 49). The crude peptides and lipopeptides were purified by reversed-phase high-pressure liquid chromatography. Peptides and lipopeptides were checked for homogeneity by analytical reversed-phase high-pressure liquid chromatography and for identity by amino acid analysis and molecular mass determination on a Bio Ion 20 plasma desorption mass spectrometer (Bio Ion AB, Uppsala, Sweden). Peptides and lipopeptides were 95 and 90% pure, respectively, with the exception of the STARP lipopeptide, which was insoluble in water. To prevent aggregation, the crude STARP lipopeptide (100 μg) was solubilized with trifluoroacetic acid in the presence of sodium dodecyl sulfate (600 μg), precipitated with ether, and then solubilized with water. Excess sodium dodecyl sulfate was eliminated by extensive dialysis. The synthesis of the STARP-derived mixotope was performed as described previously (45).

Immunization of chimpanzees. In view of ethical considerations and bearing in mind the requirements of a prospective vaccine in individuals with different HLA backgrounds, we designed a strategy in which reliable CTL responses could be generated in a limited number of outbred animals by vaccinating the animals with combinations of LS peptides and lipopeptides and taking into consideration previous findings that the subcutaneous route of immunization elicits strong and stable CTL responses (3, 7, 15, 48). These peptides and lipopeptides were injected subcutaneously and separately in different sites on the left or right shoulder areas. A mixture of nonlipidated peptides (100 μg of each) was injected in a single site by using Montanide ISA51 (SEPPIC, Quai d'Orsay, France). The Montanide ISA51 (100 μl) was emulsified with equal volumes of phosphate-buffered saline (pH 7.4) containing peptides. On the other hand, the mixtures of lipopeptides (100 μg of each) in phosphate-buffered saline (pH 7.4) were injected without adjuvant at a different site. The toxicity of the peptides and lipopeptides preparations was evaluated by observations of local and systemic side effect reactions after immunization.

The immunization regimen for each animal is shown in Table 2. The LSA3 in association with LSA1, SALSA, and STARP peptides and lipopeptides were injected subcutaneously in Demi, Karlien, and Iris at weeks 0, 3, and 6. The LSA1 and LSA3 peptides and lipopeptides were injected subcutaneously in Dirk at

TABLE 2. Immunization region for *P. falciparum* LS peptides or lipopeptides in chimpanzees[a]

Chimpanzee	Antigen	Lipopeptides	Peptides
Immunized animals			
Dirk	LSA3 and LSA1	LSA3-CT1-K(Pam)-NH2, LSA3-NRII-K(Pam)-NH2, and LSA1-J-K(Pam)-NH2	LSA3-NRI, LSA3-RE, LSA1-REP, LSA1-NR, and LSA1-TER
Demi	LSA3 and LSA1	LSA3-CT1-K(Pam)-NH2, LSA3-NRII-K(Pam)-NH2, and LSA1-J-K(Pam)-NH2	LSA3-NRI, LSA3-RE, LSA1-REP, LSA1-NR, and LSA1-TER
Karlien	LSA3 and SALSA	LSA3-CT1-K(Pam)-NH2, LSA3-NRII-K(Pam)-NH2, and SALSA1-K(Pam)-NH2	LSA3-NRI, LSA3-RE, and SALSA2
Iris	LSA3 and STARP	LSA3-CT1-K(Pam)-NH2, LSA3-NRII-K(Pam)-NH2, STARP-R-K(Pam)-NH2, and STARP-M-K(Pam)-NH2	LSA3-NRI and LSA3-RE
Control animals			
Bram	ISA51		
Fuad	SALINE		

[a] Animals were injected subcutaneously with a mixture of lipopeptides in saline at one site and with another mixture of different peptides emulsified in Montanide ISA51 adjuvant at another site. The chimpanzees Demi, Karlien, and Iris were immunized with LSA3 peptides and lipopeptides in combination with LSA1, SALSA, or STARP peptides and lipopeptides, respectively. Chimpanzee Dirk was injected at weeks 0, 12, and 15 with LSA1 and LSA3 peptides and lipopeptides as described in Materials and Methods. Chimpanzees Bram and Fuad served as adjuvant injected and nonimmunized controls, respectively.

weeks 0, 12, and 15. Chimpanzees Bram and Fuad served as nonimmunized and adjuvant injected controls.

Cytotoxic T-cell assays. (i) Generation of CTL lines. CTL lines were generated as previously described (9, 11, 15). Briefly, heparinized venous blood was collected from the immunized and control animals at various time points, and peripheral blood mononuclear cells (PBMC) were collected after centrifugation on a Ficoll-Hypaque density gradient (Pharmacia LKB, Uppsala, Sweden). Purified cells were then resuspended in complete medium consisting of RPMI 1640 (Gibco Laboratories, Grand Island, N.Y.) supplemented with streptomycin (100 μg/ml), penicillin (100 IU/ml), L-glutamine (50 mM), and 10% of heat-inactivated normal human AB^+ serum. The human serum was previously tested for its ability to support normal chimpanzee T-cell growth. Cells were grown at 2×10^6/ml of complete medium in 25-cm^2 Falcon flasks and restimulated with immunizing peptides (10 μg/ml of each). Cells were incubated in a humidified incubator at 37°C and 5% CO_2. Then, 10 U of human recombinant interleukin-2 (Goenzyme, San Diego, Calif.)/ml was added to the cultures after 72 h, and cytotoxic assays were performed at days 7 to 10.

(ii) Target cells. Autologous phytohemagglutinin (PHA) blasts were used as target cells. PHA blasts were generated from each animal by in vitro stimulation of 2×10^6 to 3×10^6 PBMC with 0.5 μg of L-PHA (Sigma, St. Louis, Mo.)/ml. Blasts were maintained by biweekly medium changes. Target cells were incubated overnight at 37°C in the presence or absence of 20 μg of test or control individual peptide/ml and 3 μg of human β_2-microglobulin (Sigma)/ml in RPMI medium (Gibco, San Diego, Calif.) supplemented with 20% human AB serum. Target cells were then labeled with 150 μCi of ^{51}Cr (sodium chromate; ICN Biomedical, Inc., Irvine, Calif.) for 1 h at 37°C, washed three times, and then plated in triplicate (at 5×10^3 cells per well) in a 96-well round-bottom microtiter plate (Corning, New York, N.Y.). ^{51}Cr-labeled target cells mock peptide pulsed were set up as controls. In order to minimize the nonspecific cytolytic activity caused by natural killer cells during the ^{51}Cr release assay, nonlabeled human erythroleukemia natural killer-sensitive cells (line K562) were added at 20-fold excess in each well.

(iii) Chromium release assay. CTL assays were performed as described previously (9, 11, 15). Briefly, 5×10^3 ^{51}Cr-labeled target cells, pulsed or nonpulsed with a same concentration of peptide were incubated with CTL lines in triplicate wells, either with RPMI 1640 complete medium alone (for spontaneous release) or with 5% Triton X-100 or 1 N HCl (for maximum release), in round-bottom 96-well tissue culture plates. Target and effector cells were then incubated for 5 h at 37°C in 5% CO_2-air mixture, and the ^{51}Cr activity from each well was then measured in 100 μl of supernatant by using a beta-plate scintillation counter (Wallac LKB 1205, San Jose, Calif.). Unless specified, assays were performed at a standardized effector/target ratio of 40:1. In all assays, the spontaneous ^{51}Cr release in the absence of CTLs was <20% of maximum release by 5% Triton X-100 or 1 N HCl. The classical percent lysis was calculated as follows: $100 \times$ [(experimental release − spontaneous release)/(total release − spontaneous release)]. The percent specific lysis was calculated as the percent lysis with peptide subtracted by the percent lysis without peptide. As we previously described (7, 9–11, 13), a positive result was defined as at least 10% higher specific lysis of peptide-pulsed cells than either control peptide-pulsed or nonpulsed target cells.

In some experiments, a fraction of effector cells was treated with anti-human CD4 or CD8 mouse monoclonal antibodies and thereafter with sheep anti-mouse monoclonal antibodies conjugated to magnetic beads (Dynal, Inc., Great Neck, N.Y.) to enrich CD8 or CD4 cell populations. Beads were added to T-cell lines at a bead/cell ratio of 50:1. The mixture was gently rotated at room temperature for 20 min before the beads were separated by using a magnetic particle concentrator (Dynal). Negatively selected cells were washed twice, resuspended in appropriate volume of RPMI 1640 complete medium, and used directly in CTL assays as described above. More than 95% of the cell populations were effectively depleted, as evaluated by fluorescence microscopy and flow cytometry analysis.

Statistical analysis. Figures represent data from at least two independent experiments. The data are expressed as the mean ± the standard deviation of means and analyzed by using the Student *t* test by using the Statview II statistical program (Abacus Concepts, Berkeley, Calif.).

RESULTS

Selection of potential T-cell epitopes within the LS *P. falciparum* Ags. We selected potential T-cell epitopes within the deduced primary amino acid sequence of LS protein Ags based on the fact that T-cell and B-cell epitopes have been frequently observed to cluster within limited regions of Ags (9, 38). To search for such regions, we focused on possible hinge regions between the repetitive sequences, which frequently contain B-cell epitopes, and the nonrepetitive sequences of the molecules. These hinge regions are less constrained than other parts of the molecules and therefore more readily accessible to proteolysis, an event that precedes T-cell epitope presentation in association with MHC molecules (24). We then searched for potential T-cell epitopes in the immediate vicinity of the repeat regions by using the "epitope cluster analysis" and the Garnier-Robson algorithms (29). Sequences from the repetitive areas that show putative conserved α-helical conformations, which are accessible to an acceptable mimicry by peptide synthesis, were also included. To search for potential epitopes within SALSA sequences, which in contrast to most malarial Ags does not contain repetitive regions, we selected sequences from two nonoverlapping areas that show a high tendency to adopt α-helical conformation. In contrast to the remaining Ags, the STARP structure includes a complex repeat central domain consisting of a mosaic of degenerated 10-amino-acid repeats. There is limited size variation in this domain, resulting from highly localized duplication events. The STARP-R peptide represents the nondegenerated units present as tandem copies

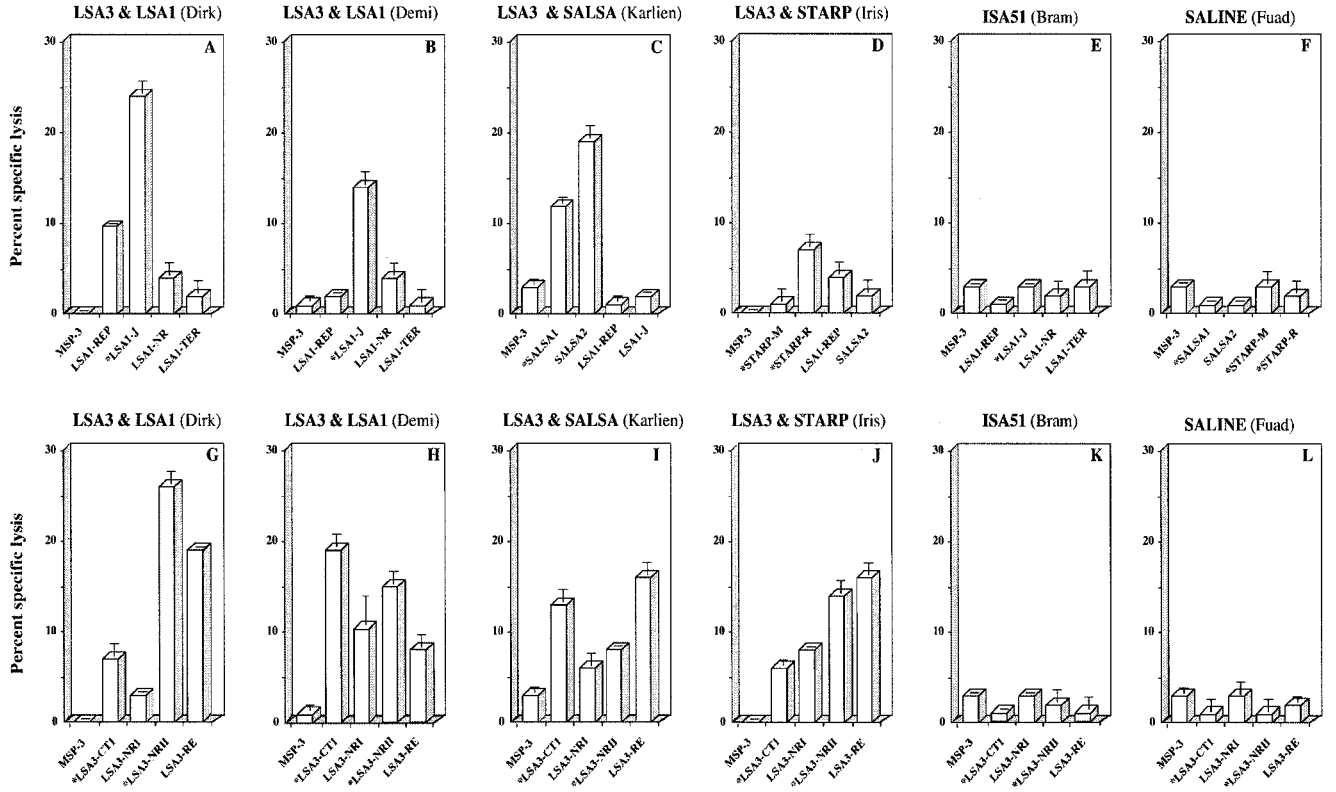

FIG. 1. CTL response induced in chimpanzees by combinations of peptides and lipopeptides derived from *P. falciparum* LSA1, LSA3, and SALSA Ags. Animals were injected subcutaneously with peptides in Montanide ISA51 and lipopeptides in saline from the indicated Ag (top of each histogram). CTL lines were derived upon in vitro stimulation of PBMC with pools of recall peptides as described in Materials and Methods. CTL activities were assayed against individual recall peptides. MSP3-C peptide (from an erythrocytic Ags) was used as the control. The CTL responses elicited by LSA1 (A and B), SALSA (C) STARP (D), and LSA3 (G to J) peptides and lipopeptide are shown at an effector/target ratio of 40:1. CTL responses recorded in control animals are shown in histograms E, F, K, and L. Assays were performed in triplicates and bars represent standard error of the mean. Asterisks in front of peptides in the *x* axis indicate that a lipopeptide form was used for in vivo immunization. The results are representative of three independent experiments.

in STARP. The STARP-M peptide is a mixotope peptide containing all of the observed degenerated sequences and, in addition, those potentially occurring but as yet nondescribed, provided they keep the same secondary structure (45). We selected 12 regions altogether within the LSA1, LSA3, SALSA, and STARP sequences, and the corresponding peptides were synthesized (Table 1). The sizes of these peptides were chosen to be between 20 and 41 residues since multiple T-cell epitopes corresponding to separate MHC class I restriction elements have been frequently described to segregate and overlap in relatively limited regions within many Ags (25, 31).

Induction of multiepitopic CTL responses in chimpanzees by combinations of LS peptides and LS lipopeptides. To determine whether *P. falciparum* LSAs induce CTL responses in chimpanzees and to obtain maximal information from limited numbers of animals, we immunized four animals with a combination of peptides and lipopeptides derived from the LSA3 molecule, since the latter appears to be the one with greatest potential (2, 11, 12, 19), together with those derived either from LSA1 (Dirk and Demi), SALSA (Karlien), or STARP (Iris) (Table 2). After each immunization, T-cell lines were derived from each animal, and their cytolytic activities against target cells were determined upon exposure to recall individual peptides. As shown in Fig. 1, three of the six unmodified peptides (two derived from LSA3 and one from SALSA Ags) and four of the six lipopeptides (one derived from LSA1, two from LSA3, and one from SALSA Ags) elicited significant CTL responses in chimpanzees. These CTLs were perceptibly heterogeneous and multiepitopic with various intensities to a given Ag in each animal (Fig. 1). Of the four peptides and lipopeptides selected from LSA1, LSA1-J lipopeptide elicited significant CTL responses in both Dirk and Demi (Fig. 1A and B). Lower but significant levels of CTLs were generated against the LSA1-REP in Dirk, but no CTL activity was detected against the remaining LSA1 peptides (i.e., LSA1-NR and LSA1-TER) (Fig. 1A and B). T-cell lines derived from the chimpanzee Karlien showed cytolytic activity against SALSA2 peptide and low but consistent CTLs against SALSA1 peptide (Fig. 1C). None of the STARP lipopeptides elicited significant levels of CTL activities in chimpanzee Iris (Fig. 1D). The T cells lines derived from chimpanzees Dirk, Demi, Karlien, and Iris showed cytolytic activity against LSA3-NRII, LSA3-RE, and/or LSA3-CT1 peptides (Fig. 1G to J). Interestingly, all of the animals had developed responses against the various LSA3 peptides. Three animals showed good responses to LSA3-NRII (Fig. 1G, H, and J), three showed good responses to LSA3-RE (Fig. 1G, I, and J), and two showed good responses to LSA3-CT1 (Fig. 1H and I). A similar profile of LSA3-

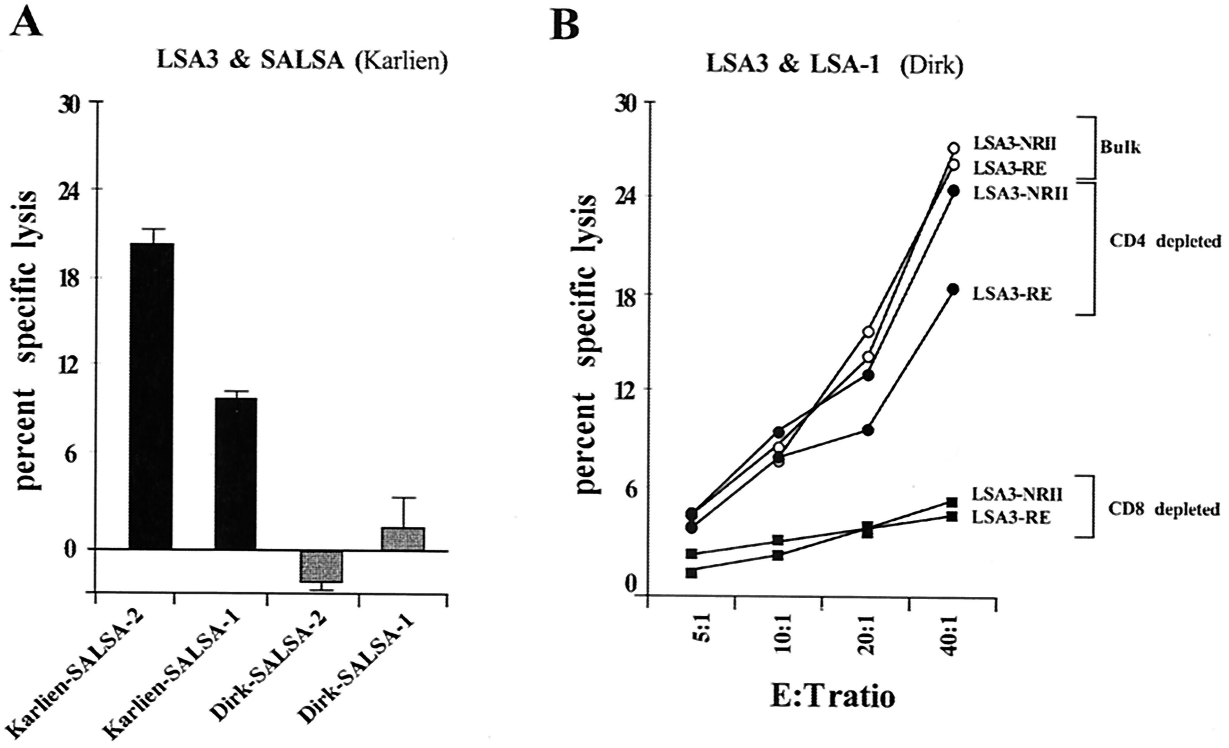

FIG. 2. Genetic restriction (A) and $CD8^+$ T-cell dependence (B) of the *P. falciparum* LSA-specific CTLs induced in chimpanzees. (A) CTLs induced in chimpanzee Karlien by SALSA peptide and lipopeptide were assayed against either autologous target cells (black histogram) or heterologous target cells (gray histogram). (B) CTL lines were derived from chimpanzee Dirk, immunized with LSA3 peptides and lipopeptides, and their cytolytic activities were assayed against LSA3-NRII or LSA3-RE peptides either as bulk CTLs (○) or after depletion of $CD4^+$ (●) or of $CD8^+$ (■) T cells. Assays were performed in triplicate, and bars represent the standard error of the mean. The results are representative of two independent experiments. E:T ratio, effector/target ratio.

specific CTL responses was recorded from Dirk immunized with LSA3, together with LSA1 peptides and lipopeptides (Fig. 1G). Collectively, although CTL activity had been detected against each of the four LSAs, the consistency and the level of CTL activity induced by LSA3 peptide epitopes points to the strong T-cell immunogenicity of LSA3.

Antigen specificity and $CD8^+$-T-cell dependence of CTL activities induced in chimpanzees by LS peptides and lipopeptides. Effector CTLs from Karlien were reactive to target cells sensitized with either SALSA1 or SALSA2 but not to target cells sensitized with heterologous control peptides from LSA1 Ag (Fig. 1C). In similar experiments with Demi, CTL lines reactive to LSA3-NRII and LSA3-CT1 did not lyse target cells sensitized with SALSA peptides (data not shown). These results indicate a lack of cross-reactive CTL epitopes within the four pre-erythrocytic Ags. The specificity of these responses was further confirmed by the lack of cytolytic activity to the control MSP3-C peptide from an erythrocytic Ags (Fig. 1) and by the lack of cytolytic activity of PBMC from both adjuvant-injected (Bram) (Fig. 1E and K) and nonimmunized control animals (Fuad) (Fig. 1F and L). The lack of cytolytic activity in PBMC sampled before immunization demonstrated that in vivo priming by LS peptides or lipopeptides was required in order to induce a CTL response (data not shown).

To address the question of MHC restriction, heterologous target cells sensitized with immunizing peptides were introduced. SALSA-specific CTL lines derived from Karlien that were able to lyse autologous target cells sensitized with either SALSA1 or SALSA2 were unable to lyse heterologous target cells from Dirk sensitized with the same peptides (i.e., SALSA1 and SALSA2) (Fig. 2A), suggesting MHC restriction of the CTL responses.

The CTL responses were $CD8^+$ T cell dependent since the cytolytic activities were abolished after in vitro depletion of $CD8^+$ effector cells, but were not affected upon depletion of $CD4^+$ cells (Fig. 2B). The cytolytic activities specific to both LSA3-NRII and LSA3-RE peptides, recorded from Dirk, were abolished by elimination of $CD8^+$ cells but were unaffected when $CD4^+$ cells were eliminated from the T-cell culture (Fig. 2B). Collectively, these results point to new regions within LSA1, LSA3, and SALSA Ags each bearing at least one $CD8^+$-CTL epitope. These epitopes are distinct from those previously identified in humans living in malaria-endemic areas (1, 2, 32, 51) and in primate models (9, 11, 12, 14, 19, 46, 47, 57). In addition, these results strongly suggest that multiepitopic and MHC-restricted $CD8^+$ CTLs can be simultaneously primed by combinations of LS peptides in Montanide ISA51 and lipopeptides in saline.

Long-lasting memory of CTL responses induced in chimpanzees by LS peptide or lipopeptide immunization. To determine whether memory CTLs had been induced, chimpanzees were monitored for up to 10 months after immunization. Nine months after Dirk's last immunization with LSA3 peptides and lipopeptides, significant CTL responses were still detectable.

The long-term CTL responses were particularly stable for two of LSA3 peptides (LSA3-RE and LSA3-NRII), which were detected even 9 months after the final immunization. Indeed, the percent specific lysis had only decreased from 22.3 to 18.7% for the LSA3-RE peptide and from 28.3 to 18.3% for the LSA3-NRII peptide. In a similar experiment with chimpanzee Iris, the LSA3-NRII-specific CTLs that were at 15.3% specific lysis 4 weeks after the third final immunization remained at a significant level 5 months postimmunization (i.e., 12.9% specific lysis). However, not all LS peptides are equally efficient at inducing a long-lasting CTL memory. For instance, although LSA3-CT1 peptide initially elicited significant CTL response in both Demi and Karlien, the response declined to a nonsignificant level within 2 months after the final immunization (i.e., from 20.7 to 5.2% in Demi and from 14.7 to 6.3% in Karlien). The specific lysis of 24.3% generated by LSA1-J peptide in chimpanzee Dirk within the first months after the final immunization declined to a nonsignificant level of 6.8% 4 months later. The CTL responses to SALSA2 peptide recorded in chimpanzee Karlien remained detectable at a significant level up to 6 months after the third immunization (i.e., from 21.9 to 18.7% specific lysis). The percentage of specific lysis of 14.3% induced by SALSA1 peptide detected in Karlien 4 weeks after the final immunization declined 6 months later to a nonsignificant level of 7.3% lysis. Finally, no CTL activity was detected in Iris, when tested 1, 2, and 4 months after STARP immunization. Together, these results showed that long-lasting CTL responses were generated in outbred chimpanzees by combinations of LS peptides or lipopeptides, particularly those derived from the LSA3 Ag.

Consistency of CTL responses induced by LS peptides or lipopeptides in outbred chimpanzees. To evaluate the consistency of the CTL induced by each LS antigen during the 10-month follow-up period for the chimpanzees, up to six PBMC samples were assayed from each animal, and the number of animals that responded persistently to a given Ag was determined. It is important to remember that CTL activity cannot be demonstrated in each assay, and thus an animal is considered a responder if CTL responses were positive at least in two of three consecutive samples. Using such criteria, both Dirk and Demi immunized with LSA1 peptides and lipopeptides developed persistent cytolytic activity to LSA1-J peptide, whereas weaker CTL responses were induced by the remaining three peptides (i.e., LSA1-REP, LSA1-NR, and LSA1-TER) (Fig. 1A and B and Table 3). Both SALSA1 and SALSA2 peptides induced persistent CTLs in Karlien (Fig. 1C); in addition to those previously recorded in two additional chimpanzees (15). In contrast, the PBMC from STARP-immunized Iris were never shown to have a CTL activity. Interestingly, the LSA3 peptides were consistently immunogenic in five of six (83% of responders) immunized animals (Fig. 1G to J). The LSA3-NRII lipopeptide generated higher and consistent CTL responses in four of six animals (Fig. 1G to J). The LSA3-RE peptide generated persistent CTLs in three of six animals (Fig. 1G to J) (A. Luty et al., unpublished data) and the LSA3-CT1 in two of six animals (Demi and Karlien). Only one in six animals immunized with LSA3 peptides and lipopeptides (Demi) developed borderline CTL response to LSA3-NRI peptide (Table 3). The magnitude and persistence of CTL responses, induced by LSA3 in five of six outbred animals strongly suggest that this Ag is immunodominant (Table 3). The $CD8^+$-CTL responses generated by the LSA3-NRII peptide showed in the present study, together with the $CD4^+$-T-cell responses previously demonstrated with the same peptide epitope (7, 9–12, 14, 19), point to LSA3-NRII sequence as bearing one or several CTL and T-helper epitopes and therefore stand as an immunologically critical region of the LSA3 Ag.

TABLE 3. Summary of the CTL and T-helper responses induced in outbred chimpanzees by LS peptide and lipopeptide[a]

Antigen	Peptide and lipopeptide	No. of animals immunized	No. of animals positive for CTL	No. of animals positive for T-helper cells
LSA1		2	2	2
	LSA1-REP	2	0	1
	LSA1-J†	2	2	1
	LSA1-NR	2	0	2
	LSA1-TER	2	0	2
SALSA*		3	3	3
	SALSA1†	3	3	3
	SALSA2	3	3	3
STARP		2	0	2
	STARP-R†	2	0	2
	STARP-M†	2	0	2
LSA3**		6	5	6
	LSA3-CT1†	6	2	6
	LSA3-NRI	6	1	6
	LSA3-NRII†	6	5	6
	LSA3-RE	6	3	6

[a] The numbers of responders developing consistent peptide-specific $CD8^+$-CTL and $CD4^+$-T-helper responses were reported as follows: *, this study, references 7, 9–15, 19, and 49, and Luty et al., unpublished; **, this study and references 7, 9–14, 19, and 49. Daggers (†) indicate that a lipopeptide form was used for in vivo immunization. LS peptides that induced both $CD8^+$- CTL and $CD4^+$- T-helper responses in chimpanzees are in boldface.

DISCUSSION

We show that immunization with combinations of *P. falciparum* LS peptides emulsified in Montanide adjuvant and lipopeptides, without any adjuvant, elicit multiepitopic and long-lasting $CD8^+$-CTL responses in the closest relative of man, the chimpanzee. The results revealed several new regions within the four LSAs that contain CTL epitopes. The data have significant implications for the development of a vaccine against malaria and bring new evidence that LSA3 is a highly immunogenic Ag and therefore an excellent candidate to be included in a *P. falciparum* pre-erythrocytic vaccine.

Thus far, only a small number of CTL epitopes have been identified within the LSAs, among which several belong to the LSA3 Ag that appears to be rather recognized frequently in humans living in malaria endemic areas (1, 2, 7, 10, 13, 32, 53). These CTL responses were obtained by sensitizing target cells with optimal short 8- to 10-amino-acid peptides selected based on HLA-allele specific amino acid motifs (2). Optimal short peptides in the range of 8 to 10 residues bind with high affinity to MHC class I molecules (65). The size of peptides used in the present study was deliberately chosen to be between 20 and 41 residues. We are aware that this strategy may lead to underestimate the level of the induced CTL responses, but we choose to use medium sized peptides mainly because (i) only a

small cohort of expensive chimpanzees was available for these experiments, (ii) T-cell epitopes have been frequently found to segregate and to overlap in relatively limited regions within many Ags (24, 31), and (iii) medium-sized peptides may contain multiple T-cell epitopes corresponding to separate MHC class I restriction elements, a factor that could be advantageous in developing a vaccine for outbred populations, such as humans. Using the long-peptide approach, it is noteworthy that CTL responses were induced in chimpanzees by 7 of the 12 peptides studied. Long peptides may very likely be processed during the in vitro and in vivo incubations into small fragments of appropriate lengths. This could be triggered either extracellularly by serum proteases (65) or at the cell surface by membrane-associated enzymes (61). It is possible that the quality of CTL responses induced may be sufficient to induce a protective immunity, since the quality of multiepitopic CTL responses, rather than their magnitude, may play a critical role against *P. falciparum-infected* hepatocytes. To our knowledge, although some of the CTL epitopes, for instance those derived from LSA1, have been reported in human living in areas where malaria is endemic (2), many epitopes, especially those derived from SALSA and LSA3 Ags, have never been described before. Together, the results of the present study revealed several new CTL epitopes on the sequence of four LSAs, expanding the panel and diversity of LS CTL epitopes and confirming the immunodominance of LSA3 (2).

Due to their limited complexity, synthetic peptides provide unique immunogens for dissecting the vaccine-induced cellular immune mechanisms against human pathogens (27, 28, 50). Peptide-based CTL vaccines are being developed against a variety of infectious pathogens, including human malaria (27, 28, 50). However, unmodified synthetic peptides usually fail to prime CTL responses in vivo unless they are delivered with a powerful immunological adjuvant (7, 8, 10–13, 19, 64). Although peptides emulsified in Freund adjuvant induce strong CTL responses in animal models this adjuvant has associated side effects and therefore is not suitable for human use (7, 8, 10, 13, 49, 64). Currently, aluminum-based mineral salts (alum), which show low toxicity, are the only adjuvants in widespread human use. However, peptides adsorbed to alum usually fail to induce Ag-specific CTLs (60). Of the large number of new adjuvants that have been tested in small laboratory animals (7, 8, 10, 13, 64), many often run into limitations due to their toxicity and/or inability to reproduce in humans the results seen in mice. In the present study, we have found that many LS peptides (i.e., LSA3-NRI, LSA3-RE, and SALSA2) emulsified in Montanide ISA51, a novel adjuvant that is acceptable for use in humans (62), were able to induce CTL responses in chimpanzees. It was confirmed that these formulations were safe and well tolerated, with no local or systemic manifestations observed.

Among the advantages of an epitope-based vaccine approach over the conventional whole protein approach is the possibility of including multiple immunodominant and subdominant epitopes within a single antigenic formulation. Several LS peptide epitopes selected in the present study induced CTL responses, indicating that each peptide defines at least one multirestricted CTL epitope or a series of adjacent or overlapping epitopes restricted by distinct class I molecules, thus underscoring the clinical potential for this set of LS peptides and lipopeptides. The present study confirms and extends recent findings of multiepitopic $CD8^+$-CTL induction by human immunodeficiency virus-derived lipopeptides (4, 5, 28). In contrast to peptides linked to the complex immunostimulating tripalmitoyl-*s*-glyceryl-cysteinyl-seryl-serine moiety, which is both mitogenic and to some extent toxic (63), the lipopeptides modified by a single palmitoyl group used in the present study proved to be well tolerated. No general or local reactions were observed in any of the immunized animals. This offers a simple answer to major questions in peptide-based vaccine development, namely, insufficient immunogenicity and the requirement for powerful vaccine adjuvants (60).

An effective pre-erythrocytic vaccine may have to include multiple immunodominant epitopes from different proteins because of the genetic restriction of T-cell responses (39, 66). By combining multiple T-cell epitopes, identified through in vivo immunogenicity studies in nonhuman primates, into a single vaccine, one can target efficiently protective CTLs to the parasite in diverse MHC genetic backgrounds (2, 37). In the present study, we conceived a strategy in which in vivo multiepitopic CTL responses could be generated in outbred animals by immunization with combinations of peptides and lipopeptides from several LSAs. In the case of LSA3, which was combined with each one of the remaining three LSAs, most of the CTLs targeted LSA3-NRII and LSA3-RE peptides. These results are consistent with those obtained in additional chimpanzees, immunized with the same set of LSA3 peptides, which led to optimal expansion of $CD8^+$ CTLs (7, 9–14, 19; Luty et al., unpublished). Despite their distinct MHC backgrounds (as determined by sequence analysis [R. Bontrop et al., unpublished data]), all of the LSA3-immunized animals showed CTL responses to components of LSA3, suggesting that an outbred human population could behave similarly. These results may reflect either promiscuous LSA3 epitopes for different MHC molecules, as recently reported for other pre-erythrocytic Ags (22, 54), or may be attributed to a high circulating precursor frequency. The latter is supported by a recent study showing high frequencies of LSA3-specific CTLs in humans living in areas where malaria is endemic (2). After immunization, a loss in CTL activity in the periphery could be anticipated, due to the homing of memory CTLs within the lymphoid organs (e.g., spleen), as documented in other systems (35). In this context, the CTL responses generated here proved to be long-lasting, with some responses being maintained in peripheral blood for up to 9 months after cessation of immunization.

Among the immune effectors against LS malaria stages, CTLs have been shown to play an important role in murine models, and there have been indirect indications for their role in the immunity to *P. falciparum* LS infection in humans (6, 7, 10, 13, 18, 20, 30, 34, 41, 52, 53, 55). In addition, several studies have stressed the requirement for a concomitant activation of $CD4^+$ T cells in the generation and maintenance of efficient CTL responses (7, 9–11, 13, 14, 22, 42). In this case a natural boosting of immunity by a malaria vaccine would require T-helper epitopes of parasite origin. Each of the 12 peptides used in the present study, with seven proved to be efficient in inducing CTL responses, was previously found to contain at least one $CD4^+$–T-helper epitope. (Table 3) (7, 10–13, 19). Therefore, the induction and maintenance of CTLs by this set of LS peptides or lipopeptides could rely upon the coexistence in the

sequences used of multiepitopes capable of inducing both $CD8^+$ CTLs and $CD4^+$–T-helper cells. In addition to CD8 CTL responses, several of the LS peptides and lipopeptides used in the present study have also been found previously able to induce CD4 and antibody responses (2, 9, 11, 12, 14, 19). This is important since developing a vaccine against *P. falciparum* will likely require generating a breadth of responses that include both humoral and $CD8^+$ $CD4^+$ cellular immunity. It is also very likely that these 20 to 41 amino acids large peptides contain multiple overlapping epitopes. However, their fine mapping is beyond the scope of the present study.

Today, with the genome of *P. falciparum* at hand, optimism abounds that new therapies and vaccines will emerge based on this information (23). However, in the case of LS antigens, malarial immunologists are still facing the considerable challenge of deciding which screen could be used to first assign a given gene to the LS and, second, to decide which (if any) could identify among the ca. 1,000 to 2,000 expressed molecules in these stages those that induce protection. We have previously developed and described a strategy based on the use of human immune responses from human living in areas where malaria is endemic to identify antigens expressed during the intrahepatic stage of the malaria life cycle (43, 59). We thereafter completed this by another strategy in which protective and nonprotective immune responses from volunteers immunized by irradiated sporozoites and/or chimpanzees were used to designate among the huge array of pre-erythrocytic molecules, the ones with valuable vaccine potential (2, 19). Up to now, a decade of molecular and immunological research has supported our strategy and confirmed the interest of the four pre-erythrocytic antigens mentioned in the present study. The present results, which define the CTL immunogenicity of these four LS antigens in nonhuman primates, bring additional support to the strategy we used in selecting the pre-erythrocytic antigens and demonstrate that they have valuable immunological characteristics.

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REFERENCES

1. **Aidoo, M., A. Lalvani, C. E. Allsopp, M. Plebanski, S. J. Meisner, P. Krausa, M. Browning, S. Morris-Jones, F. Gotch, D. A. Fidock, et al.** 1995. Identification of conserved antigenic components for a cytotoxic T lymphocyte-inducing vaccine against malaria. Lancet **345:**1003–1007.
2. **Aidoo, M., A. Lalvani, S. C. Gilbert, J. T. Hu, P. Daubersies, N. Hurt, H. C. Whittle, P. Druihle, and A. V. Hill.** 2000. Cytotoxic T-lymphocyte epitopes for HLA-B53 and other HLA types in the malaria vaccine candidate liver-stage antigen 3. Infect. Immun. **68:**227–232.
3. **Alexander, J., C. Oseroff, C. Dahlberg, M. Qin, G. Ishioka, M. Beebe, J. Fikes, M. Newman, R. W. Chesnut, P. A. Morton, K. Fok, E. Appella, and A. Sette.** 2002. A decaepitope polypeptide primes for multiple $CD8^+$ IFN-γ and Th lymphocyte responses: evaluation of multiepitope polypeptides as a mode for vaccine delivery. J. Immunol. **168:**6189–6198.
4. **Andrieu, M., J. F. Desoutter, E. Loing, J. Gaston, D. Hanau, J. G. Guillet, and A. Hosmalin.** 2003. Two human immunodeficiency virus vaccinal lipopeptides follow different cross-presentation pathways in human dendritic cells. J. Virol. **77:**1564–1570.
5. **Andrieu, M., E. Loing, J. F. Desoutter, F. Connan, J. Choppin, H. Gras-Masse, D. Hanau, A. Dautry-Varsat, J. G. Guillet, and A. Hosmalin.** 2000. Endocytosis of an HIV-derived lipopeptide into human dendritic cells followed by class I-restricted $CD8^+$ T lymphocyte activation. Eur. J. Immunol. **30:**3256–3265.
6. **Belnoue, E., F. T. Costa, T. Frankenberg, A. M. Vigario, T. Voza, N. Leroy, M. M. Rodrigues, I. Landau, G. Snounou, and L. Renia.** 2004. Protective T-cell immunity against malaria liver stage after vaccination with live sporozoites under chloroquine treatment. J. Immunol. **172:**2487–2495.
7. **BenMohamed, L., Y. Belkaid, E. Loing, K. Brahimi, H. Gras-Masse, and P. Druilhe.** 2002. Systemic immune responses induced by mucosal administration of lipopeptides without adjuvant. Eur. J. Immunol. **32:**2274–2281.
8. **BenMohamed, L., G. Bertrand, C. D. McNamara, H. Gras-Masse, J. Hammer, S. L. Wechsler, and A. B. Nesburn.** 2003. Identification of novel immunodominant $CD4^+$ Th1-type T-cell peptide epitopes from herpes simplex virus glycoprotein D that confer protective immunity. J. Virol. **77:**9463–9473.
9. **BenMohamed, L., H. Gras-Masse, A. Tartar, P. Daubersies, K. Brahimi, M. Bossus, A. Thomas, and P. Druilhe.** 1997. Lipopeptide immunization without adjuvant induces potent and long-lasting B, T helper, and cytotoxic T lymphocyte responses against a malaria liver stage antigen in mice and chimpanzees. Eur. J. Immunol. **27:**1242–1253.
10. **BenMohamed, L., R. Krishnan, C. Auge, J. F. Primus, and D. J. Diamond.** 2002. Intranasal administration of a synthetic lipopeptide without adjuvant induces systemic immune responses. Immunology **106:**113–121.
11. **BenMohamed, L., R. Krishnan, J. Longmate, C. Auge, L. Low, J. Primus, and D. J. Diamond.** 2000. Induction of CTL response by a minimal epitope vaccine in HLA A*0201/DR1 transgenic mice: dependence on HLA class II restricted T_H response. Hum. Immunol. **61:**764–779.
12. **BenMohamed, L., A. Thomas, M. Bossus, K. Brahimi, J. Wubben, H. Gras-Masse, and P. Druilhe.** 2000. High immunogenicity in chimpanzees of peptides and lipopeptides derived from four new *Plasmodium falciparum* pre-erythrocytic molecules. Vaccine **18:**2843–2855.
13. **BenMohamed, L., S. L. Wechsler, and A. B. Nesburn.** 2002. Lipopeptide vaccines: yesterday, today, and tomorrow. Lancet Infect. Dis. **2:**425–431.
14. **Bossus, M., L. BeMohamed, A. Londono, B. Barbier, A. Tartar, P. Druilhe, and H. Gras-Masse.** 1997. Improved detection of human antibodies to a *Plasmodium* antigen using a peptide modified with Aib residues. J. Peptide Sci. **3:**47–53.
15. **Bottius, E., L. BenMohamed, K. Brahimi, H. Gras, J. P. Lepers, L. Raharimalala, M. Aikawa, J. Meis, B. Slierendregt, A. Tartar, A. Thomas, and P. Druilhe.** 1996. A novel *Plasmodium falciparum* sporozoite and liver stage antigen (SALSA) defines major B, T helper, and CTL epitopes. J. Immunol. **156:**2874–2884.
16. **Bowen, J. C., H. O. Alpar, R. Phillpotts, and M. R. Brown.** 1992. Mucosal delivery of herpes simplex virus vaccine. Res. Virol. **143:**269–278.
17. **Bray, R. S.** 1960. Studies on malaria in chimpanzees. VIII. The experimental transmission and pre-erythrocytic phase of *Plasmodium malariae*, with a note on the host-range of the parasite. Am. J. Trop. Med. Hyg. **9:**455–465.
18. **Charlotte-Gruner, A., G. Snounou, K. Brahimi, F. Letourneur, L. Renia, and P. Druilhe.** 2003. Pre-erythrocytic antigens of *Plasmodium falciparum*: from rags to riches? Trends Parasitol. **19:**74–78.
19. **Daubersies, P., A. W. Thomas, P. Millet, K. Brahimi, J. A. Langermans, B. Ollomo, L. BenMohamed, B. Slierendregt, W. Eling, A. Van Belkum, G. Dubreuil, J. F. Meis, C. Guerin-Marchand, S. Cayphas, J. Cohen, H. Gras-Masse, and P. Druilhe.** 2000. Protection against *Plasmodium falciparum* malaria in chimpanzees by immunization with the conserved pre-erythrocytic liver-stage antigen 3. Nat. Med. **6:**1258–1263.
20. **Doolan, D. L., R. C. Hedstrom, W. O. Rogers, Y. Charoenvit, M. Rogers, P. de la Vega, and S. L. Hoffman.** 1996. Identification and characterization of the protective hepatocyte erythrocyte protein 17-kDa gene of *Plasmodium yoelii*, homolog of *Plasmodium falciparum* exported protein 1. J. Biol. Chem. **271:**17861–17868.
21. **Doolan, D. L., and S. L. Hoffman.** 2000. The complexity of protective immunity against liver-stage malaria. J. Immunol. **165:**1453–1462.
22. **Doolan, D. L., S. Southwood, R. Chesnut, E. Appella, E. Gomez, A. Richards, Y. I. Higashimoto, A. Maewal, J. Sidney, R. A. Gramzinski, C. Mason, D. Koech, S. L. Hoffman, and A. Sette.** 2000. HLA-DR-promiscuous T-cell epitopes from *Plasmodium falciparum* pre-erythrocytic-stage antigens restricted by multiple HLA class II alleles. J. Immunol. **165:**1123–1137.
23. **Doolan, D. L., S. Southwood, D. A. Freilich, J. Sidney, N. L. Graber, L. Shatney, L. Bebris, L. Florens, C. Dobano, A. A. Witney, E. Appella, S. L. Hoffman, J. R. Yates III, D. J. Carucci, and A. Sette.** 2003. Identification of *Plasmodium falciparum* antigens by antigenic analysis of genomic and proteomic data. Proc. Natl. Acad. Sci. USA **100:**9952–9957.
24. **Elliott, T. J., V. Cerundolo, C. Ohlen, H. G. Ljunggren, K. Karre, and A. Townsend.** 1991. Antigen presentation and the association of class-I molecules. Acta Biol. Hung. **42:**213–229.
25. **Elvin, J., C. Potter, T. Elliott, V. Cerundolo, and A. Townsend.** 1993. A method to quantify binding of unlabeled peptides to class I MHC molecules and detect their allele specificity. J. Immunol. Methods **158:**161–171.
26. **Fidock, D. A., E. Bottius, K. Brahimi, I. I. Moelans, M. Aikawa, R. N. Konings, U. Certa, P. Olafsson, T. Kaidoh, A. Asavanich, et al.** 1994. Cloning and characterization of a novel *Plasmodium falciparum* sporozoite surface antigen, STARP. Mol. Biochem. Parasitol. **64:**219–232.
27. **Gahery-Segard, H., G. Pialoux, B. Charmeteau, S. Sermet, H. Poncelet, M. Raux, A. Tartar, J. P. Levy, H. Gras-Masse, and J. G. Guillet.** 2000. Mul-

tiepitopic B- and T-cell responses induced in humans by a human immunodeficiency virus type 1 lipopeptide vaccine. J. Virol. **74:**1694–1703.
28. **Gahery-Segard, H., G. Pialoux, S. Figueiredo, C. Igea, M. Surenaud, J. Gaston, H. Gras-Masse, J. P. Levy, and J. G. Guillet.** 2003. Long-term specific immune responses induced in humans by a human immunodeficiency virus type 1 lipopeptide vaccine: characterization of $CD8^+$-T-cell epitopes recognized. J. Virol. **77:**11220–11231.
29. **Garnier, A., and L. Tosi.** 1978. Circular dichroism investigation of the (L lysine)n-Cu(II) (1:1) systems with n = 4 and n = 25. Bioinorg. Chem. **8:** 493–501.
30. **Good, M. F., and D. L. Doolan.** 1999. Immune effector mechanisms in malaria. Curr. Opin. Immunol. **11:**412–419.
31. **Higgins, J. A., C. J. Thorpe, J. D. Hayball, R. E. O'Hehir, and J. R. Lamb.** 1994. Overlapping T-cell epitopes in the group I allergen of Dermatophagoides species restricted by HLA-DP and HLA-DR class II molecules. J. Allergy Clin. Immunol. **93:**891–899.
32. **Hill, A. V., J. Elvin, A. C. Willis, M. Aidoo, C. E. Allsopp, F. M. Gotch, X. M. Gao, M. Takiguchi, B. M. Greenwood, A. R. Townsend, et al.** 1992. Molecular analysis of the association of HLA-B53 and resistance to severe malaria. Nature **360:**434–439.
33. **Hill, A. V., W. Reece, P. Gothard, V. Moorthy, M. Roberts, K. Flanagan, M. Plebanski, C. Hannan, J. T. Hu, R. Anderson, P. Degano, J. Schneider, E. Prieur, E. Sheu, and S. C. Gilbert.** 2000. DNA-based vaccines for malaria: a heterologous prime-boost immunization strategy. Dev. Biol. **104:**171–179.
34. **Hoffman, S. L., and E. D. Franke.** 1994. Inducing protective immune responses against the sporozoite and liver stages of *Plasmodium*. Immunol. Lett. **41:**89–94.
35. **Hoffman, S. L., D. Isenbarger, G. W. Long, M. Sedegah, A. Szarfman, S. Mellouk, and W. R. Ballou.** 1990. T lymphocytes from mice immunized with irradiated sporozoites eliminate malaria from hepatocytes. Bull. W. H. O. **68**(Suppl.)**:**132–137.
36. **Hollingdale, M. R., M. Aikawa, G. X. Chen, J. F. Meis, K. Sakhuja, B. Sina, and J. D. Zhu.** 1990. Pre-erythrocytic stage malaria parasites: non-circumsporozoite protein antigens. Bull. W. H. O. **68**(Suppl.)**:**178–180.
37. **Joshi, S. K., P. R. Suresh, and V. S. Chauhan.** 2001. Flexibility in MHC and TCR recognition: degenerate specificity at the T-cell level in the recognition of promiscuous Th epitopes exhibiting no primary sequence homology. J. Immunol. **166:**6693–6703.
38. **Kabilan, L., M. Troye-Blomberg, H. Perlmann, G. Andersson, B. Hogh, E. Petersen, A. Bjorkman, and P. Perlmann.** 1988. T-cell epitopes in Pf155/RESA, a major candidate for a *Plasmodium falciparum* malaria vaccine. Proc. Natl. Acad. Sci. USA **85:**5659–5663.
39. **Khusmith, S., M. Sedegah, and S. L. Hoffman.** 1994. Complete protection against *Plasmodium yoelii* by adoptive transfer of a $CD8^+$ cytotoxic T-cell clone recognizing sporozoite surface protein 2. Infect. Immun. **62:**2979–2983.
40. **Lalvani, A., and A. V. Hill.** 1998. Cytotoxic T lymphocytes against malaria and tuberculosis: from natural immunity to vaccine design. Clin. Sci. **95:**531–538.
41. **Lalvani, A., P. Moris, G. Voss, A. A. Pathan, K. E. Kester, R. Brookes, E. Lee, M. Koutsoukos, M. Plebanski, M. Delchambre, K. L. Flanagan, C. Carton, M. Slaoui, C. Van Hoecke, W. R. Ballou, A. V. Hill, and J. Cohen.** 1999. Potent induction of focused Th1-type cellular and humoral immune responses by RTS,S/SBAS2, a recombinant *Plasmodium falciparum* malaria vaccine. J. Infect. Dis. **180:**1656–1664.
42. **Leclerc, C., R. Lo-Man, C. Fayolle, A. Charbit, J. M. Clement, P. Martineau, D. O'Callaghan, and M. Hofnung.** 1994. Molecular and cellular parameters controlling the immunogenicity of foreign B- or T-cell epitopes expressed by recombinant vectors. Dev. Biol. Stand. **82:**193–199.
43. **Lunel, F., and P. Druilhe.** 1989. Effector cells involved in nonspecific and antibody-dependent mechanisms directed against *Plasmodium falciparum* blood stages in vitro. Infect. Immun. **57:**2043–2049.
44. **Mizukoshi, E., M. Nascimbeni, J. B. Blaustein, K. Mihalik, C. M. Rice, T. J. Liang, S. M. Feinstone, and B. Rehermann.** 2002. Molecular and immunological significance of chimpanzee major histocompatibility complex haplotypes for hepatitis C virus immune response and vaccination studies. J. Virol. **76:**6093–6103.
45. **Pasquetto, V., D. A. Fidock, H. Gras, E. Badell, W. Eling, W. R. Ballou, J. Belghiti, A. Tartar, and P. Druilhe.** 1997. *Plasmodium falciparum* sporozoite invasion is inhibited by naturally acquired or experimentally induced polyclonal antibodies to the STARP antigen. Eur. J. Immunol. **27:**2502–2513.
46. **Perlaza, B. L., M. Arevalo-Herrera, K. Brahimi, G. Quintero, J. C. Palomino, H. Gras-Masse, A. Tartar, P. Druilhe, and S. Herrera.** 1998. Immunogenicity of four Plasmodium falciparum preerythrocytic antigens in *Aotus lemurinus* monkeys. Infect. Immun. **66:**3423–3428.
47. **Perlaza, B. L., J. P. Sauzet, A. T. Balde, K. Brahimi, A. Tall, G. Corradin, and P. Druilhe.** 2001. Long synthetic peptides encompassing the *Plasmodium falciparum* LSA3 are the target of human B and T cells and are potent inducers of B helper, T helper and cytolytic T-cell responses in mice. Eur. J. Immunol. **31:**2200–2209.
48. **Perlaza, B. L., C. Zapata, A. Z. Valencia, S. Hurtado, G. Quintero, J. P. Sauzet, K. Brahimi, C. Blanc, M. Arevalo-Herrera, P. Druilhe, and S. Herrera.** 2003. Immunogenicity and protective efficacy of *Plasmodium falciparum* liver-stage Ag-3 in *Aotus lemurinus* griseimembra monkeys. Eur. J. Immunol. **33:**1321–1327.
49. **Perng, G. C., B. Maguen, L. Jin, K. R. Mott, J. Kurylo, L. BenMohamed, A. Yukht, N. Osorio, A. B. Nesburn, G. Henderson, M. Inman, C. Jones, and S. L. Wechsler.** 2002. A novel herpes simplex virus type 1 transcript (AL-RNA) antisense to the 5′ end of the latency-associated transcript produces a protein in infected rabbits. J. Virol. **76:**8003–8010.
50. **Pialoux, G., H. Gahery-Segard, S. Sermet, H. Poncelet, S. Fournier, L. Gerard, A. Tartar, H. Gras-Masse, J. P. Levy, and J. G. Guillet.** 2001. Lipopeptides induce cell-mediated anti-HIV immune responses in seronegative volunteers. AIDS **15:**1239–1249.
51. **Plebanski, M., M. Aidoo, H. C. Whittle, and A. V. Hill.** 1997. Precursor frequency analysis of cytotoxic T lymphocytes to pre-erythrocytic antigens of *Plasmodium falciparum* in West Africa. J. Immunol. **158:**2849–2855.
52. **Plebanski, M., and A. V. Hill.** 2000. The immunology of malaria infection. Curr. Opin. Immunol. **12:**437–441.
53. **Plebanski, M., O. Proudfoot, D. Pouniotis, R. L. Coppel, V. Apostolopoulos, and G. Flannery.** 2002. Immunogenetics and the design of *Plasmodium falciparum* vaccines for use in malaria-endemic populations. J. Clin. Investig. **110:**295–301.
54. **Ravichandran, M., D. L. Doolan, J. Cox-Singh, S. L. Hoffman, and B. Singh.** 2000. Research note: HLA degenerate T-cell epitopes from *Plasmodium falciparum* liver stage-specific antigen 1 (LSA-1) are highly conserved in isolates from geographically distinct areas. Parasite Immunol. **22:**469–473.
55. **Reece, W. H., M. Plebanski, P. Akinwunmi, P. Gothard, K. L. Flanagan, E. A. Lee, M. Cortina-Borja, A. V. Hill, and M. Pinder.** 2002. Naturally exposed populations differ in their T1 and T2 responses to the circumsporozoite protein of *Plasmodium falciparum*. Infect. Immun. **70:**1468–1474.
56. **Renia, L., M. M. Rodrigues, and V. Nussenzweig.** 1994. Intrasplenic immunization with infected hepatocytes: a mouse model for studying protective immunity against malaria pre-erythrocytic stage. Immunology **82:**164–168.
57. **Sauzet, J. P., B. L. Perlaza, K. Brahimi, P. Daubersies, and P. Druilhe.** 2001. DNA immunization by *Plasmodium falciparum* liver-stage antigen 3 induces protection against *Plasmodium yoelii* sporozoite challenge. Infect. Immun. **69:**1202–1206.
58. **Scheller, L. F., and A. F. Azad.** 1995. Maintenance of protective immunity against malaria by persistent hepatic parasites derived from irradiated sporozoites. Proc. Natl. Acad. Sci. USA **92:**4066–4068.
59. **Sherman, I. W., J. R. Greenan, and P. de la Vega.** 1988. Immunofluorescent and immunoelectron microscopic localization of protein antigens in red cells infected with the human malaria *Plasmodium falciparum*. Ann. Trop. Med. Parasitol. **82:**531–545.
60. **Singh, M., and D. T. O'Hagan.** 2002. Recent advances in vaccine adjuvants. Pharm. Res. **19:**715–728.
61. **Theobald, M., T. Ruppert, U. Kuckelkorn, J. Hernandez, A. Haussler, E. A. Ferreira, U. Liewer, J. Biggs, A. J. Levine, C. Huber, U. H. Koszinowski, P. M. Kloetzel, and L. A. Sherman.** 1998. The sequence alteration associated with a mutational hotspot in p53 protects cells from lysis by cytotoxic T lymphocytes specific for a flanking peptide epitope. J. Exp. Med. **188:**1017–1028.
62. **Turner, J. L., R. J. Trauger, A. E. Daigle, and D. J. Carlo.** 1994. HIV-1 immunogen induction of HIV-1-specific delayed-type hypersensitivity: results of a double-blind, adjuvant-controlled, dose-ranging trial. AIDS **8:**1429–1435.
63. **Uhl, B., V. Speth, B. Wolf, G. Jung, W. G. Bessler, and S. Hauschildt.** 1992. Rapid alterations in the plasma membrane structure of macrophages stimulated with bacterial lipopeptides. Eur. J. Cell Biol. **58:**90–98.
64. **Vichier-Guerre, S., R. Lo-Man, L. BenMohamed, E. Deriaud, S. Kovats, C. Leclerc, and S. Bay.** 2003. Induction of carbohydrate-specific antibodies in HLA-DR transgenic mice by a synthetic glycopeptide: a potential anti-cancer vaccine for human use. J. Pept. Res. **62:**117–124.
65. **Widmann, C., J. L. Maryanski, P. Romero, and G. Corradin.** 1991. Differential stability of antigenic MHC class I-restricted synthetic peptides. J. Immunol. **147:**3745–3751.
66. **Wizel, B., R. Houghten, P. Church, J. A. Tine, D. E. Lanar, D. M. Gordon, W. R. Ballou, A. Sette, and S. L. Hoffman.** 1995. 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.

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