

Protection against *Plasmodium falciparum* malaria in chimpanzees by immunization with the conserved pre-erythrocytic liver-stage antigen 3

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In humans, sterile immunity against malaria can be consistently induced through exposure to the bites of thousands of irradiated infected mosquitoes. The same level of protection has yet to be achieved using subunit vaccines. Recent studies have indicated an essential function for intrahepatic parasites, the stage after the mosquito bite, and thus for antigens expressed during this stage. We report here the identification of liver-stage antigen 3, which is expressed both in the mosquito and liver-stage parasites. This *Plasmodium falciparum* 200-kilodalton protein is highly conserved, and showed promising antigenic and immunogenic properties. In chimpanzees (*Pan troglodytes*), the primates most closely related to humans and that share a similar susceptibility to *P. falciparum* liver-stage infection, immunization with LSA-3 induced protection against successive heterologous challenges with large numbers of *P. falciparum* sporozoites.

Irradiated sporozoite vaccines remain experimental because sporozoites, the parasite present in mosquito salivary glands, cannot be artificially cultured and the only effective means of administration is through 12–14 exposures to hundreds of infected mosquito bites^{1,2}. Current sporozoite candidate vaccines have not yet achieved similar protection^{3–5}, and their use is hampered by substantial polymorphism in immunologically important regions and by low immunogenicity³. The basis of the strong immunological protection induced in humans by vaccination with radiation-attenuated pre-erythrocytic malaria parasites is poorly understood. However, there is now an increasing body of evidence that the transformation of the irradiated sporozoites into live but developmentally arrested intrahepatic liver trophozoites, which occurs at low (15–20 krad) irradiation doses but not at high (23–30 krad) irradiation doses⁶, is required to induce protection^{7,8}. We therefore focused our efforts on the characterization of antigens expressed during this incompletely elucidated phase of the *Plasmodium* life cycle⁹.

Selection of liver-stage antigen 3 by human responses

The different responses of hosts immunized with irradiated sporozoites could provide a screening strategy for molecules relevant to protection. Thus, we screened 120 phage lambda clones

previously identified as expressing *P. falciparum* polypeptides that are expressed during pre-erythrocytic stage parasite development^{9,10} and that are derived from about 20 distinct genes^{9–12}. We screened a clone corresponding to each of these putative genes using eight serum samples from human volunteers (four of six protected) and from chimpanzees (one of two protected) immunized with sporozoites irradiated at low or high doses. A single clone (DG729) reacted only with sera from protected humans and chimpanzees. After confirming this differential reactivity further with a peptide derived from this fragment (Table 1), we used this clone for further investigation.

Characterization of liver-stage antigen 3

We used DG729 to probe a genomic library of *P. falciparum* (K1 strain). One clone contained the whole gene corresponding to DG729; we called it liver-stage antigen 3 (LSA-3) and determined its sequence, expression, location and conservation (Figs. 1–3 and <http://www.pasteur.fr/parmed/lsa3>). We identified a single-copy gene consisting of a 'mini-exon' 1 (198 base pairs), a 'mini-intron' (168 base pairs) and a large exon 2 (5.16 kilobases), a structure similar to that of other surface antigens of *P. falciparum*¹³. The gene for LSA-3 was located on chromosome 2 (ref. 14), where it was called ring-infected erythrocyte surface antigen

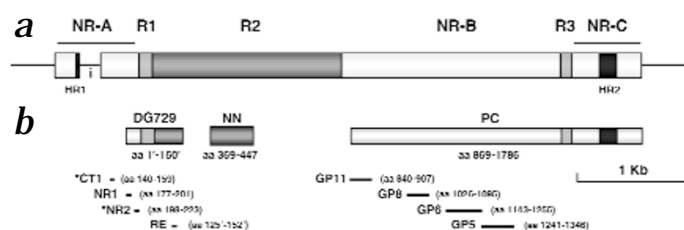


Fig. 1 Gene for LSA-3, recombinant proteins and peptides. **a**, NR-A, NR-B and NR-C, non-repeated sequences; R1–R3, three repeat blocks; HR1 and HR2, hydrophobic regions possibly corresponding to the N-terminal signal peptide and the anchor region, respectively; intron (i). **b**, Location of the sequences encoding for LSA-3 in the recombinant fusion proteins (first line) and the synthetic peptides (bars) used here (amino acid numbering, <http://www.pasteur.fr/parmed/lisa3>. aa, amino acids; *, palmitoyl-conjugated lipopeptides; ′, amino acid positions in T9/96 clone.

(RESA)-H3 (Genbank accession number, AE001424). LSA-3, with a predicted molecular weight of 200 kDa (in K1), is made up of large non-repeated sequences flanking three Glu-rich repeated regions, a feature that extends the known *P. falciparum* Glu-rich antigen network¹⁵ to include a pre-erythrocytic component. We determined the location of the original fragment (DG729) and of the peptides corresponding to repeat region R2 and non-repetitive regions NR-A and NR-B (Fig. 1b). Natural or artificially induced antibodies against the non-repeated peptides and the recombinant protein glutathione S-transferase (GST)–PC (see Fig. 1) were not cross-reactive with the repeated Glu-rich regions, and we used these for further studies.

We confirmed pre-erythrocytic expression of LSA-3 (Figs. 2 and 3 and <http://www.pasteur.fr/parmed/lisa3>) by RT–PCR (primers i1 and i2) of total RNA and western blot analysis of protein extracts, isolated in both cases from sporozoites, and also by immunofluorescence antibody test (IFAT) of infected liver sections and dry or wet sporozoite preparations, using antibodies against a non-cross-reactive portion of the protein. In 5- and 6-day-old liver schizonts, LSA-3 was present in the parasitophorous vacuole and at the periphery of maturing hepatic merozoites. This location is consistent with the molecular structure of this protein, which has two hydrophobic regions (Fig. 1a). We were unable to detect mRNA of LSA-3 by northern blot analysis of RNA from erythrocytic stages. Western blot analysis and IFAT of infected red blood cells were also consistently negative with non-cross-reactive antibodies. However, we obtained reactivity using antibodies against the Glu-rich repeat region (<http://www.pasteur.fr/parmed/lisa3>), which reflects observations of a putatively homologous antigen (D260) in intra-erythrocytic parasites identified solely using such antibodies¹⁶.

Given concerns about the polymorphism of many malaria vaccine candidate molecules^{3,17,18}, we investigated naturally occurring sequence variation in LSA-3 (<http://www.pasteur.fr/parmed/lisa3>). We consistently detected the gene by PCR amplification of the NR-A region (primers S1 and S2) in a total of 111 *P. falciparum* isolates, strains or clones of various geographical origins. Using LSA-3-specific antibodies with IFAT, we also detected expression of LSA-3 in liver schizonts of two distinct strains and in all the sporozoites from 30 wild isolates that developed in mosquitoes fed *in vitro* on Thai gametocytes. The repeat regions R1 and R3 were highly conserved, but there was variation in the number and order of the repeat units of R2 among different parasite lines. However, this did not affect the predicted conserved α -helical organization, a secondary structure considered important in defining principal B-cell epitopes, as antibodies that recognize R2 did indeed produce positive results by IFAT with all the parasites tested. The non-repeated portions of exon 2, where many T-helper and cytotoxic T-lymphocyte epitopes are found^{19–21}, had a considerable degree of amino-acid sequence conservation between different

parasites (more than 95.5% homology). The sequence of the non-repetitive (NR) 2 (see Fig. 1) peptide was fully conserved among K1 and T9/96 parasites (the source of the immunizing proteins), the NF54 parasites used for sporozoite challenges and 27 *P. falciparum* samples of various geographical origins¹⁹. An HLA-B53-restricted epitope identified in the NR-B region of LSA-3 (present in GST–PC recombinant protein) was also free of variation in clone 3D7 and in 18 Gambian isolates²¹. This conservation of immunologically important epitopes contrasts with substantial polymorphism in current pre-erythrocytic vaccine candidates.

Validation of the chimpanzee for vaccine studies

The chimpanzee is the primate most closely related to humans genetically (98.4 % homology at the DNA level)²². We used many preliminary stringent tests in control chimpanzees to confirm the suitability of this model for vaccine evaluation. As cost and ethical considerations precluded the use of large numbers of animals, high reproducibility of the infection in this model system was essential. In a preliminary experiment (Table 2, group I), we confirmed that in the chimpanzee, protection by immunization with irradiated sporozoite was radiation dose-dependent, as it is in humans, and we confirmed the use of detection of the infected red blood cells as an assay of protection. The results allowed us to define many important parameters. Chimpanzees developed a powerful protective response after immunization with irradiated sporozoite, as in humans. Moreover, chimpanzees, like humans, were also susceptible to successive challenges, in contrast to lower primates or rodents, which become refractory after the first challenge²³. In two chimpanzees, five

Table 1 Differential reactivity of sera from protected or non-protected humans or chimpanzees with peptide NR2

Code or Name	Sporozoite irradiation dose	IFAT titer on sporozoite	Protective status	NR2 peptide (amino acids 198–223)
V4	23.6	4,096	not protected	0.5
V5	23.6	32,000	2-day delay	0.5
<i>Japie</i>	30	32,000	not protected	0.7
V6	20.8	5,120	Protected	3.8
V7	20.8	41,960	Protected	2.6
V8	20.8	40,960	Protected	4.8
WR4	15	3,200	Protected	3.4
<i>Carl</i>	18	6,400	Protected	2.3

Immunoglobulin G-specific antibodies against peptide NR2 were measured by enzyme-linked immunoassay (in arbitrary units) in sera from human volunteers (codes) and chimpanzees (names, in *italics*) immunized with sporozoites irradiated at low or high dose (in krad). Codes, immunization schemes, sporozoite IFAT titers and protective status determination for human volunteers V4–V8 and WR4 have been described^{1,2}. Similar experiments with peptides NR1 and RE (Fig. 1) yielded negative results with these sera (data not shown).

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consecutive challenges (three medium-dose and two high-dose) over 2 years provided reproducible results. Furthermore, as a result of the high dose of inoculated sporozoites, we obtained positive blood smears reproducibly in non-protected chimpanzees on days 6 or 7. This corresponded to the first invasion of red cells by merozoites released from intrahepatocytic schizonts. Finally, in the non-splenectomized chimpanzee, erythrocytic infections normally remain sub-clinical and self-limiting, which in fact occurred despite the high-dose challenges. We confirmed these results in two more chimpanzees (Table 2, Emmanuel and Fauzi).

LSA-3 induces protection in chimpanzees

Having established the suitability of the chimpanzee for our use, we next assessed the protective value of LSA-3 immunization by challenge with viable *P. falciparum* sporozoites. In preliminary experiments, we immunized two chimpanzees (Dirk and Ruud) with a mixture of LSA-3 and LSA-1 recombinant proteins. We obtained full protection against three challenges over several months only in the chimpanzee that responded to LSA-3 (Dirk), whereas both responded to LSA-1 (data not shown). In liver biopsies of Dirk on day 5, we detected only one liver schizont of 'unhealthy' appearance and infiltrated by leukocytes in the 300 liver sections screened (Fig. 3). In contrast, we found 2,500 and 750 hepatic schizonts of 'healthy' appearance in the two non-protected control chimpanzees.

Thus, we focused further immunization and challenge experiments on LSA-3 alone. We used two groups of chimpanzees to

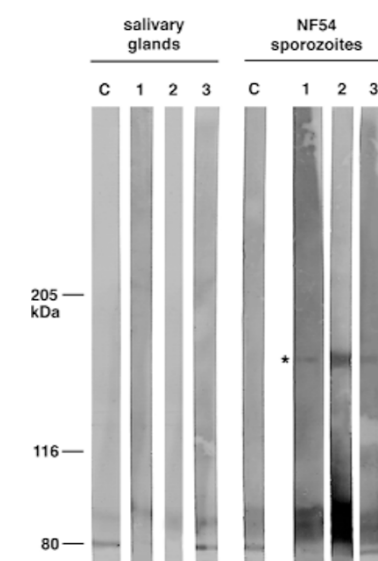


Fig. 2 LSA-3 expression in *P. falciparum* sporozoites. Western blot analysis of protein extracts from NF54 sporozoites and control uninfected mosquito salivary glands using mouse antisera against control GST (C), GST-PC (lane 1), peptides GP5, GP6, GP8 or GP11 (lane 2) or GST-729 (lane 3). *, LSA-3 (175-kDa protein, in agreement with the theoretical molecular weight of LSA-3 in this parasite strain). Left margin, molecular sizes.

evaluate lipopeptide and recombinant protein formulations (Table 2, groups II–III). In group II, one chimpanzee (Gerda) was initially immunized solely with the NR2 lipopeptide of LSA-3, and was boosted by recombinant LSA-3 molecules in Montanide ISA 51 adjuvant (SEPPIC, France). Gerda was fully protected when challenged with 1×10^7 sporozoites, whereas the control chimpanzee receiving Montanide ISA 51 alone was not (Fig. 4a).

In Gerda, boosting with the recombinant LSA-3 formulation did not induce any detectable increase in the strong B-cell, T helper-cell and cytotoxic T-lymphocyte responses already evoked by the initial lipopeptide-peptide injections^{19,20}. Therefore, we sought to determine whether the simple and well-tolerated peptide formulation alone could induce protection. We immunized two chimpanzees, Mopia and Mgbado, with LSA-3 lipopeptide-peptides alone (Table 2, group III). We obtained protection against a first challenge with 2×10^4 sporozoites in both. Experiments with the same group included an investigation of the effects of microbead presentation of recombinant proteins without adjuvant in one chimpanzee (Judy), which resulted in a 1-day delay to 'patency' (time of blood infection after sporozoite challenge) (Fig. 4b). After a subsequent high-dose sporozoite challenge (5×10^6 sporozoites), both Mopia and Mgbado demonstrated a distinct 2-day delay to patency

Table 2: Results of challenge experiments in chimpanzees

Animal Groups		Immunization dates (weeks)	Challenge dates (weeks)	Protection	
Chimpanzee	Immunization protocols			low-dose 2×10^4	high-dose 10^7
Group I ^a		0,8,24,44,65	93,123		
Carl	18 krad-irradiated sporozoites			+	+
Japie	30-krad-irradiated sporozoites			-	-
Marcel	unimmunised control			-	-
Theo	unimmunised control			-	-
Group II		0,3,13,31	60		
Gerda	(lipopep. NR2) then GST-rec in ISA51	then		nd	+
Lianne	control ISA 51	40,45,48,50		nd	-
Group III		0,3,6	21,29 ^b		
Mopia	(lipo)pep.			+	d2
Mgbado	(lipo)pep.			+	d2
Judy	GST-rec/microbeads			d1	-
Ondele	control GST/microbeads			-	-
Makata	unimmunized control			-	-
Group IV		0,4,8	13		
Patty	(lipo)pep. ^c			+	nd
Wendy	GST-rec in SBAS2			+	nd
Willy	GST-rec in SBAS2			-	nd
Helen	control SBAS2			-	nd
Group V			33,46		
Cindy	GST-rec in SBAS2	0,4,8,26 ^d		+	+
Marty	GST-rec in SBAS2			d1	-
Emmanuel	18 krad-irradiated sporozoites	5,8,11,26 ^e		+	+
Fauzi	30 krad-irradiated sporozoites			-	-

Challenges used either 2×10^4 (low-dose) or 1×10^7 (high-dose) NF54 *P. falciparum* sporozoites (Protection). ^aGroup I chimpanzees received three additional challenges (two low-dose and one high-dose), which led each time to similar results; that is, a reproducible protection only in Carl (data not shown). ^bHigh-dose challenge with 5×10^6 sporozoites. ^cSame mixture as in group III but without peptide CT1. ^dCindy and Marty. ^eFauzi. +, complete protection; d1 and d2: 1-day (d1) and 2-day (d2) delay to patency compared with that of controls; nd, not done; lipopep., lipopeptide; (lipo)pep., mixture of NR1, NR2 and CT1 lipopeptides and RE peptide; GST-rec, mixture of recombinants GST-DG729, GST-NN and GST-PC.

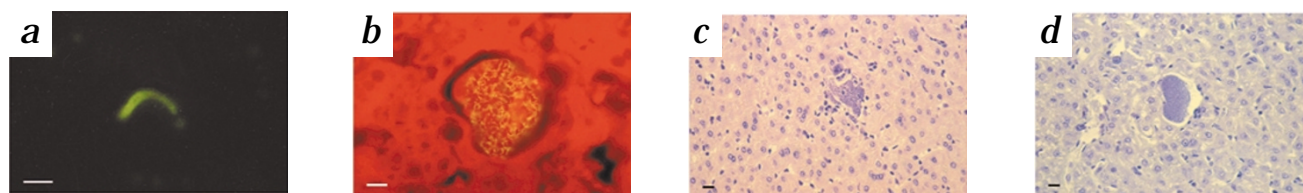


Fig. 3 Immunostaining of *P. falciparum* pre-erythrocytic stages with antibodies against LSA-3. **a**, Sporozoites stained by IFAT with human antibodies affinity-purified on recombinant bgal-DG729. **b**, Staining by IFAT of *P. falciparum* liver stages on day 6 after challenge³² from a chimpanzee, using the antibodies induced by lipopeptide NR2 injection¹⁹ in chimpanzee Gerda (additional pictures, <http://www.pasteur.fr/parmed/lsa3>). **c** and **d**, The sin-

gle residual liver schizont detected in chimpanzee Dirk (day 5 after challenge) seems infiltrated by lymphomononuclear cells (c, compared with one of the many healthy schizonts in control chimpanzee Peer (d) (total of about 2,500 schizonts per 300 liver sections). Scale bars represent 5 mm (a) and 20 mm (b–d).

and a low transient parasitemia, whereas no protection was obtained for Judy (Fig. 4c). The delay to patency indicates that the immune responses had caused a reduction exceeding 90% of the intrahepatic schizont load²⁴ (Fig. 4).

In chimpanzees from groups IV and V, we investigated the efficacy of a less-complex lipopeptide mixture alone, or of recombinants emulsified in the SmithKline Beecham adjuvant system (SBAS) 2, a new adjuvant whose efficacy has been established recently in humans^{4,5}. As immunogenicity studies^{19,20} and analysis of previous chimpanzee data had indicated that peptide CT1 (see Fig. 1) was poorly immunogenic and thus might not be essential, we immunized chimpanzee Patty using a mixture of three instead of four peptides. This chimpanzee showed protection after challenge. Among four chimpanzees receiving SBAS2-emulsified LSA-3 proteins, two showed full, sterile protection against a medium-dose challenge. One showed a delay in patency, which may be indicative of partial protection, whereas neither the fourth chimpanzee nor the control chimpanzee receiving SBAS2 alone were protected. One of the two fully protected chimpanzees further challenged with a high dose 3 months later still showed full protection.

Discussion

We have presented here evidence that LSA-3 of *P. falciparum* is a valuable candidate for effective vaccination against pre-erythrocytic stages. We obtained data by challenge of immunized chimpanzees, a new model for human malaria vaccine development. The chimpanzee is the only nonhuman primate fully susceptible to complete intrahepatic development of *P. falciparum*, with a rate of sporozoite transformation to liver forms similar to that seen in humans⁸. Furthermore, detailed investigations of chim-

panzee immune responses can be made and legitimately compared with those of humans^{19,20}. The fact that parasitological and immunological events can be directly examined in liver biopsies, a possibility excluded for infected humans, is of considerable relevance.

We immunized nine chimpanzees using lipopeptides in saline or polypeptides in either Montanide or SBAS2. Full sterile protection was induced in six of these nine chimpanzees on first challenge. After taking into consideration the substantial delay compared with that of controls, which corresponds to a considerable decrease in liver parasite load²⁴, we were able to extend the protective effect induced by LSA-3 to eight of the nine chimpanzees. We obtained protection in 11 of the 14 challenges, which were done in chimpanzees immunized with LSA-3. This protection was complete for 7 of the 11 challenges. All the control chimpanzees here showed a consistent pattern in the early appearance and the course of the blood-stage parasitemia after each of the 12 challenges with viable parasites. Demonstration of this reproducibility in controls is essential in the interpretation of our data. After taking into consideration other groups of chimpanzees used in preliminary experiments in which complex schemes of immunization or other pre-erythrocytic antigens were tested (data not shown), we were able to confirm the reproducibility of the model with an additional 26 successful challenges.

A chief concern of vaccine candidates at present is genetic polymorphism^{17,18}. It is therefore encouraging that protection was induced against a heterologous challenge (NF54 strain) in outbred chimpanzees immunized with LSA-3 molecules whose sequences were derived from K1 and T9/96 parasites. An advantage of LSA-3 may reside in its limited sequence polymorphism.

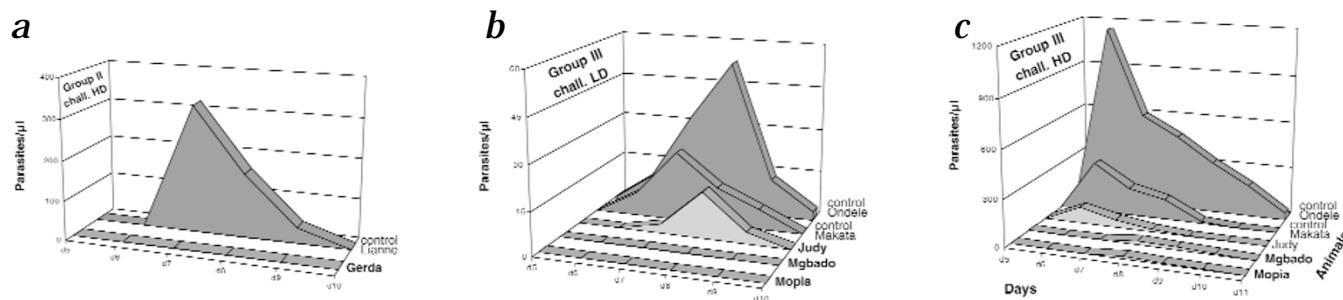


Fig. 4 Blood parasitemia courses in groups II and III. Chimpanzees from group II (a) and group III (b and c) after high-dose (HD) or low-dose (LD) challenge (chall.) with NF54 sporozoites. Bold names, totally or partially protected chimpanzees. Parasitemia scales are different for each challenge,

as expected from challenges with different numbers of sporozoites. The day of patency in control and non-protected chimpanzees is the same for a given challenge inoculum within each group (here and in other groups not shown here).

We investigated a variety of immunization strategies in the course of this work. Our data confirm the utility of SBAS2. The results we obtained with simple peptide and lipopeptide formulations are also particularly encouraging, as these vaccines do not require adjuvant and are relatively easy to produce with Good Manufacturing Practice conditions²⁵. We detected no local or general reactions in our chimpanzees after lipopeptide injections, an observation consistent with previous experience with similar formulations derived from simian immunodeficiency virus in macaques²⁶ and hepatitis B surface antigen²⁷ or human immunodeficiency virus²⁵ in humans. This bodes well for future clinical trials.

Methods

Selection of clone DG729. Dot-blot analysis of the β -galactosidase-fused recombinant proteins encoded by the pre-erythrocytic clones was done on nitrocellulose as described⁹, using human and chimpanzee sera diluted 1:100. Enzyme-linked immunoassays were done in duplicate as described²⁸ on sera diluted 1:100, using coating solutions of 0.3, 3 and 10 μ g/ml NR1, NR2 and (RE) (see Fig. 1) peptides, respectively, in PBS. Enzyme-linked immunoassay titers are expressed in arbitrary units representing the ratio of the mean absorbance values from test sera to the mean absorbance value plus three standard deviations from 10 control samples assayed in parallel in the same plate. Results were considered positive for ratios above one (ref. 19).

LSA-3 cloning and characterization. Detailed description of molecular methods, gene cloning, sequence data, protein characteristics and description of the recombinant proteins and of the peptides are available at <http://www.pasteur.fr/parmed/lsa3>. The primers used for PCR were S1 (nucleotides 161–184) and S2 (nucleotides 454–432), and those for RT-PCR were i1 (nucleotides 695–722) and i2 (nucleotides 824–799); numbering refers to the sequence of the gene for LSA-3 sequence of K1 (Genbank accession number, AJ007010). All mouse sera used for the western blot (at a dilution of 1:100) in Fig. 2 were obtained after three subcutaneous injections of 100 mg immunogen emulsified in SBAS2 (ref. 4). Long synthetic peptides GP5, GP6, GP8 and GP11 (see Fig. 1) were synthesized as described²⁹ (Fig. 1, position).

Immunogens injected in chimpanzees. Sequences of the immunogens consisted of clone DG729 and inserts NN (see Fig. 1) and PC, as well as peptides NR1, NR2, RE and CT1 (Fig. 1, location; details available at <http://www.pasteur.fr/parmed/lsa3>). Clone DG729, as well as inserts NN and PC were expressed as GST-fused recombinants and were purified according to the manufacturer's recommendations (Invitrogen, Carlsbad, California). Recombinant proteins GST-DG729, GST-NN and GST-PC were designed to cover 95% of the LSA-3 antigen and were used as a mixture (called LSA-3 GST-rec). Peptides NR1, NR2 and CT1 were also synthesized as palmitoyl-conjugated lipopeptides (* in Fig. 1), as described¹⁹. The combination of synthetic compounds consisted of a mixture of NR1, NR2 and CT1 lipopeptides and of RE peptide. All peptides and lipopeptides were purified to a purity of more than 90% by reverse-phase chromatography, and the impurities consisted essentially of related peptides of shorter sequences¹⁹.

Chimpanzee immunizations and challenges. None of the chimpanzees here had been exposed to malaria infections or malarial antigens previously. Recombinant and synthetic compounds were injected subcutaneously, at a dose of 100 μ g for each peptide and/or lipopeptides, and/or 50 μ g for each protein. Lipopeptides were always injected in PBS and, except where described otherwise, peptides and recombinants were emulsified in Montanide ISA51. Chimpanzees in group I (Carl and Japie) were immunized by five intravenous injections of 5×10^6 gamma-irradiated sporozoites at day 0 and weeks 8, 24, 44 and 65, and received three challenges at weeks 97 and 123 (Table 2, challenge doses). One year after the three challenges reported here, these chimpanzees were re-immunized once, and received one low-dose challenge and one high-dose challenge,

which showed the same pattern of protection (data not shown; J.A.M.L. *et al.*, manuscript in preparation). In group II, Gerda received NR2 lipopeptide at day 0 and weeks 3, 13 and 31 as described¹⁹. She was then boosted with the mixture of LSA-3 GST-rec at weeks 40, 45, 48 and 50. Control chimpanzee Lianne received Montanide ISA51 alone. Challenges were done at week 60. Chimpanzees in group III were immunized at day 0 and weeks 3 and 6. Mopia and Mgbado received LSA-3 as a mixture of NR1, NR2 and CT1 lipopeptides and of RE peptide, whereas Judy was injected with LSA-3 GST-rec adsorbed to latex microbeads. Low-dose and high-dose challenges were done at weeks 21 and 29, respectively. In group IV, Patty received LSA-3 (lipo)peptides, but without lipopeptide CT1, whereas Wendy and Willy were injected with LSA-3 GST-rec emulsified in SBAS2 (SmithKline Beecham Biologicals, Rixensart, Belgium), whose efficacy has been recently established in humans^{4,5}. Control chimpanzee Helen received SBAS2 only. All chimpanzees were immunized at weeks 0, 4 and 8 and were challenged with 2×10^4 sporozoites at week 13. In group V, Cindy and Marty were both immunized at weeks 0, 4, 8 and 26 with LSA-3 GST-rec in SBAS2 (as in group IV). Emmanuel and Fauzi were immunized at weeks 5, 8, 11 and 26 with 18 krad- and 30 krad-irradiated sporozoites, respectively, as were Carl and Japie (group I). Fauzi was considered the negative control and Emmanuel, the positive control for this group. Low-dose and high-dose challenges were done at weeks 33 and 46, respectively, in all four chimpanzees.

NF54 sporozoites were obtained from dissected salivary glands of infected *Anopheles gambiae* as described³⁰. Sporozoites were pooled, resuspended in PBS and injected intravenously. All chimpanzees in each group were challenged with the same pool of sporozoites. Because of the cost, extensive evaluation of the minimal infective dose has not been undertaken; however, challenge with 5×10^3 sporozoites, the lowest dose used so far, has proven infective in four other chimpanzees (A.W.T., unpublished data).

Determination of the protective status. For groups I, II, IV and V, chimpanzee blood was obtained on days 5–10, evaluated by thick- and thin-film Giemsa-stained preparations, and confirmed in all cases by *in vitro* culture (data not shown), as described²⁴. For chimpanzees in group III, blood obtained every day on days 5–18, then every other day up to day 30, was used to prepare thin and thick smears, which were Giemsa-stained and examined by two separate microscopists. A chimpanzee was considered totally protected when no parasites could be detected in the circulation blood, by direct microscopy and by long-term culture, or partially protected when time to patency was delayed by 1 or more days compared with that in control chimpanzees. In mice, these delays correspond to a protection of 80% (24 h) or 96% (48 h) against sporozoite challenges. In humans, a 12-hour delay was calculated to correspond to a 92% reduction of liver forms after sporozoite challenges²⁴. In a limited number of chimpanzees, a liver biopsy was done by a veterinary doctor on anesthetized chimpanzees on day 5 after a high-dose challenge. Material was fixed, and sections 4 μ m in thickness were made and stained by Giemsa-collophonium³¹ before the liver forms in 300 sections (average area, 0.8 cm²) were counted microscopically. All chimpanzees were treated with chloroquine immediately after the period of observation regardless of their protective status.

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