A protein particle vaccine containing multiple malaria epitopes

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Ty virus-like particles consist of a single protein species that can be produced in yeast. Recombinant Ty-VLPs carrying a string of up to 15 defined cytotoxic T lymphocyte (CTL) epitopes from *Plasmodium* species prime protective CTL responses in mice following a single administration without adjuvant. Effective processing of epitopes from the string was demonstrated in vitro and in vivo and was not affected by flanking sequences.

Keywords: vaccine design, antigen presentation

Antibodies generated by vaccination against a single protein are often not sufficient to prevent infection; cellular responses, particularly cytotoxic T lymphocytes (CTL), may be required for complete protection. Unmodified soluble proteins do not prime major histocompatability complex (MHC) class I-restricted CTL responses[1] whereas particulate proteins, such as hepatitis B surface antigen particles, are extremely immunogenic and have been shown to prime CTL responses in vivo[1]. CTL epitopes and helper epitopes have been identified in proteins from many infectious pathogens. These short amino acid sequences can be produced as recombinant proteins in a 'string of beads' form[2]. The epitope string must be delivered in a form that can prime MHC class I restricted CTL responses.

Recombinant protein particles carrying one or more epitopes can be produced using the p1 protein of the retrotransposon Ty1 of *Saccharomyces cerevisiae*[3]. Sequences encoding CTL epitopes have been fused to the C-terminus of p1 (amino acids 1–381), and the resulting Ty virus-like particles (Ty-VLPs) have been shown to generate a CTL response in mice when injected via several different routes without adjuvant[4–6]. Mucosal immunization of macaques with a Ty-VLP carrying simian immunodeficiency virus p27 has been shown to elicit MHC class I restricted $CD8^+$ CTL responses[7]. In a comparison of CTL-inducing vaccines in a rodent model of malaria[5], Ty-VLPs induced the highest levels of specific lysis out of 10 vaccine/adjuvant combinations tested. In order to be used as a human vaccine, particles carrying epitopes for a wide range of HLA types would be needed. By using a 'string of beads' approach, it is possible to include epitopes from several different antigens. The addition of epitopes to stimulate human $CD4^+$ T cells, which promote the generation of antibody or CTL, may also be desirable[8].

Ty-VLPs have been used to create a candidate vaccine for *Plasmodium falciparum* malaria. There are several lines of evidence supporting a protective role for CTL against the liver-stage parasite in immunity to malaria[9]. Antigenic polymorphism in the parasite reduces the effectiveness of the host immune response. A single amino-acid change within a T-cell epitope is often sufficient to prevent recognition, either by immune escape or altered peptide ligand antagonism of the T-cell receptor[10]. It is possible to identify epitopes in conserved regions of several *P. falciparum* antigens[9]. By using these epitopes together in a vaccine it may be possible to enable the immune system of the host to mount an effective immune response against most or all strains of *P. falciparum*, not just the strain that was used to make the vaccine.

We have engineered Ty-VLPs carrying a string of 15 CTL epitopes, three T-helper epitopes, an antibody epitope, and a heparin-binding adhesion motif. We demonstrate functional expression of the three helper epitopes, the antibody epitope, and the adhesion motif. Moreover, individual CTL epitopes are effectively processed from numerous positions in the string, and they prime protective CTL responses in vivo and sensitize target cells for CTL recognition in vitro.

Results

Production of recombinant Ty-VLPs. A series of epitope cassettes were constructed from synthetic oligonucleotides, with restriction enzyme sites that allowed them to be joined together in any chosen order (Table 1). The *P. berghei* K^d-restricted epitope pb9 was included so that immunogenicity and protective efficacy against this rodent malaria could be assessed in Balb/c mice. Six different types of Ty-VLPs carrying various combinations of the cassettes A, B, C, and H fused to the C terminus of the truncated p1 coding sequence were produced in *S. cerevisiae* (ACB, ACBH, CAB, CABH, ABC, and ABCH). The order of the cassettes and the addition of H had little effect on the level of expression of the recombinant particles in yeast (Fig. 1).

Processing of CTL epitopes from the Ty-VLPs. Peptides that encode CTL epitopes are normally generated from whole proteins by intracellular proteases, prior to loading onto MHC class I molecules and presentation at the cell surface. When the minimal epitopes are encoded as a string, the sequence surrounding each epitope is different from that in the protein from which it was derived. We have used three different approaches to investigate whether this will affect processing and presentation.

The epitope pb9 is presented by the murine MHC molecule H2-K^d, and is the third epitope in cassette C (Table 1). Balb/c mice were immunized with different Ty-VLPs in which pb9 was present at different positions in the epitope string. Immunization of mice with Ty-VLPs carrying pb9 alone[5], or CAB, CABH, ABC, or CABDH was successful in priming CTL to pb9 (Fig. 2A and B). The remaining two combinations were also tested; 20 μg of ACB given intramuscularly resulted in 23% specific lysis at an E:T ratio

of 10:1, and 20 µg of ABCH given subcutaneously produced 18% specific lysis at an effector to target (E:T) ratio of 40:1.

J774A.1 cells, which express H2-K^d, were incubated with Ty-VLPs. The particles are taken up by the cells, and if the epitopes contained in them are processed and presented on MHC molecules at the cell surface, they can be lysed by a CTL clone that recognizes pb9. pb9 was processed out of the epitope string in all four constructs tested (Fig. 2C).

Processing of two overlapping *P. falciparum* CTL epitopes, tr 42 and tr43 (ref. 9), was assessed by using Ty-VLPs containing these epitopes to restimulate CTL in vitro from peripheral blood mononuclear cells (PBMCs) taken from a malaria-exposed donor (Fig. 2D). The Ty-VLP was able to restimulate CTL to both epitopes, indicating that the Ty-VLP had been taken up by antigen-presenting cells and that the two epitopes had been presented at the cell surface in the context of HLA-B8. Ty-VLPs were also used to stimulate primary CTL responses in vitro from PBMCs of malaria-naive donors of a known HLA type[11]. This technique involves generating numerous cell lines from PBMCs by in vitro stimulation with either peptide or Ty-VLPs. These lines are then tested individually for their ability to lyse peptide-loaded targets. Results are scored as the number of lines out of the total tested that show more than 15% specific lysis. In one experiment four lines out of 40 were positive for lysis of ls8 pulsed targets, whether the cells had been stimulated with ls8 peptide or TyCAB, indicating that ls8 is processed out of the epitope string in TyCAB. In another experiment using cp26 loaded targets, 4/40 lines stimulated with TyCAB, 9/40 lines stimulated with cp26 peptide, and 0/40 lines stimulated with ls8 peptide were positive. Similarly, 5/40 lines generated with TyCABDH particles, 4/40 generated with the tr26 peptide, and 0/40 lines with the cp36 peptide were able to lyse tr26 loaded targets. Thus all five *P. falciparum* epitopes tested, as well as the *P. berghei* epitope, can be processed out of the epitope string despite the unnatural flanking sequences.

Helper epitopes from the Ty-VLPs stimulate proliferation of human T cells in vitro. The three helper epitopes in cassette H were chosen because they all bind to many different MHC class II molecules[12–14]. A large proportion of the world population has been immunized with bacillus Calmette-Guerin (BCG) and tetanus toxoid (TT). The BCG and TT derived epitopes would thus be expected to cause proliferation of CD4+ cells in these previously primed individuals, helping to prime the CTL response to other epitopes. The CSP epitope will be recognized by CD4$^+$ cells of people who have been naturally exposed to malaria. In six cases out of seven, the proliferative response to Ty-VLPs carrying the helper epitope cassette was greater than the response to Ty-VLPs carrying the same CTL epitopes without helper epitopes (Fig. 3).

Addition of more cassettes to the Ty-VLPs. The peptides encoded by cassettes A, B, and C encode CTL epitopes for seven

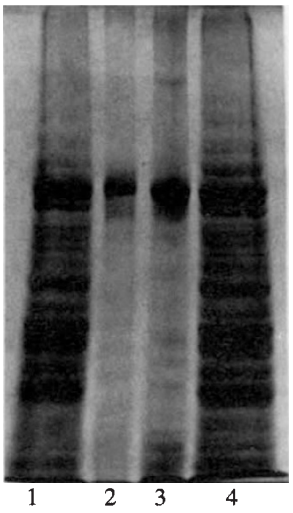


***Figure* 1. Ty-VLP production in yeast. Coomassie brilliant-blue–stained acrylamide gel. Lanes 1 and 4: Total soluble protein from two different yeast transformants producing Ty CABDHFE, after galactose induction. Lanes 2 and 3: Ty-VLPs purified from these extracts by sucrose density gradient centrifugation**

Table 1. Sequences included in the Ty-VLPs.

Cassette	Epitope	Amino acid Sequence	DNA sequence	Type	HLA restriction	Reference
A	ls8	KPNDKSLY	AAGCCGAACGACAAGTCCTTGTAT	CTL	B35	9
	cp26	KPKDELDY	AAACCTAAGGACGAATTGGACTAC	CTL	B35	9
	ls6	KPIVQYDNF	AAGCCAATCGTTCAATACGACAACTTC	CTL	B53	9
B	tr42/43	ASKNKEKALII	GCCCCAAGAACAAGGAAAAGGCTTTGATCATC	CTL	B8	9
	tr39	GIAGGLALL	GGTATCGCTGGTGGTTTGGCCTTGTTG	CTL	A2.1	9
	cp6	MNPNDPNRNV	ATGAACCCTAATGACCCAAACAGAAACGTC	CTL	B7	9
C	st8	MINAYLDKL	ATGATCAACGCCTACTTGGACAAGTTG	CTL	A2.2	9
	ls50	ISKYEDEI	ATCTCCAAGTACGAAGACGAAATC	CTL	B17	*
	pb9	SYIPSAEKI	TCCTACATCCCATCTGCCGAAAAGATC	CTL	mouse H2-K^d	26
D	tr26	HLGNVKYLV	CACTTGGGTAACGTTAAGTACTTGGTT	CTL	A2.1	9
	ls53	KSLYDEHI	AAGTCTTTGTACGATGAACACATC	CTL	B58	9
	tr29	LLMDCSGSI	TTATTGATGGACTGTTCTGGTTCTATT	CTL	A2.2	*
E	NANP	NANPNANPNANPNANP	AACGCTAATCCAAACGCAAATCCGAACGCCA ATCCTAACGCGAATCCC	B cell		12
	TRAP AM	DEWSPCSVTCGKGTRSRKRE	GACGAATGGTCTCCATGTTCTGTCACTTGTGG TAAGGGTACTCGCTCTAGAAAGAGAGAA	Heparin binding motif		27
F	cp39	YLNKIQNSL	TACTTGAACAAAATTCAAAACTCTTTG	CTL	A2.1	*
	la72	MEKLKELEK	ATGGAAAAGTTGAAAGAATTGGAAAAG	CTL	B8	*
	ex23	ATSVLAGL	GCTACTTCTGTCTTGGCTGGTTTG	CTL	B58	*
H	CSP	DPNANPNVDPNANPNV	GACCCAAACGCTAACCCAAACGTTGACCCAAA CGCCAACCCAAACGTC	T helper	Multiple	28
	BCG	QVHFQPLPPAVVKL	CAAGTTCACTTCCAACCATTGCCTCCGGCCG TTGTCAAGTTG	T helper	Universal epitope	13
	TT	QFIKANSKFIGITE	CAATTCATCAAGGCCAACTCTAAGTTCATCGGT ATCACCGAA	T helper	Universal epitope	14

*Unpublished data.

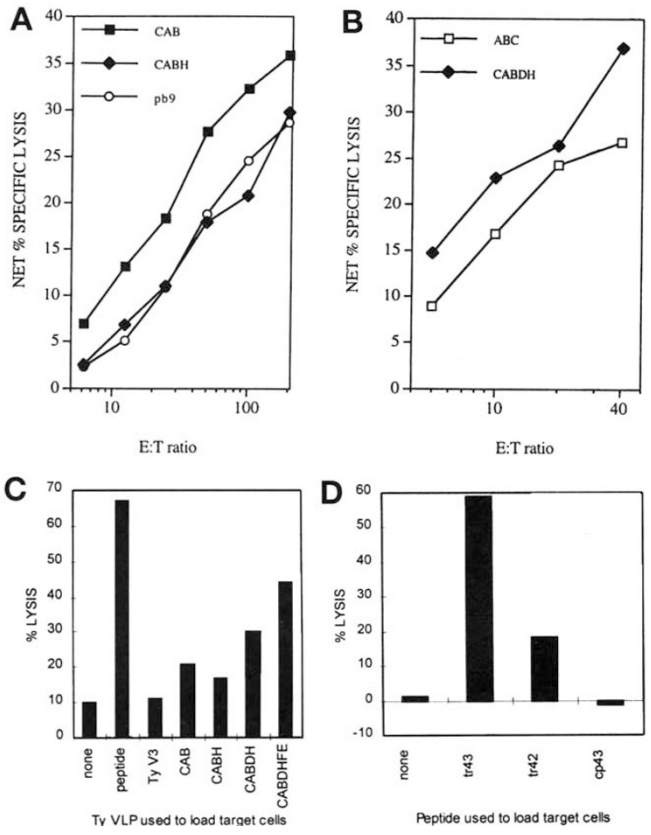
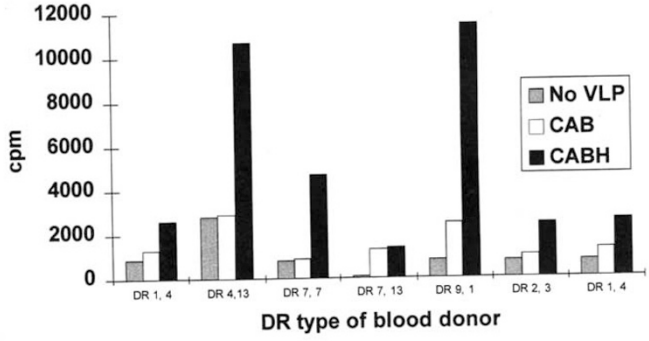


Figure 2. (A) Induction of pb9-specific CTL following a single immunization with Ty-VLPs. Mice were immunized intramuscularly with 100 μg of the particles as shown. (B) Mice were immunized intramuscularly with 50 μg Ty-VLPs. The mean of the results from three mice is shown. The maximum standard error was 1.7%. (C) Killing of J774A.1 cells by a CTL clone specific for pb9. (D) Processing of *P. falciparum* epitopes from the epitope string.



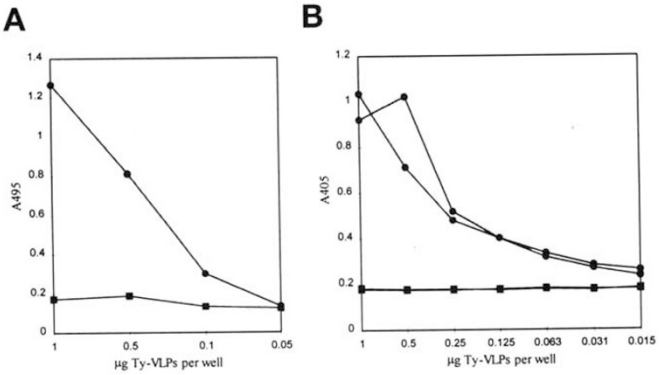


Figure 4. (A) Recognition of Ty-VLPs carrying cassette E with an anti-NANP monoclonal antibody. Results are the mean of three replicates. (B) Binding of Ty-VLPs with cassette E to dextran sulfate. Results of duplicate experiments are shown. ● CABDHFE, ■ CABH.

human lymphocyte antigen (HLA) types. Approximately 77% of Africans and 70% of Europeans have at least one of these HLA types[15]. Inclusion of several epitopes for the same HLA type may prove to be important to reduce the risk of escape mutations being selected in vaccinees. We therefore sought to increase the length of the epitope string. Cassettes D, E, and F (Table 1) were constructed, and Ty-VLPs carrying CABDHFE were produced in yeast. These particles carry 14 CTL epitopes from *P. falciparum*, for eight different HLA types. The expression level of these particles and their immunogenicity in mice was not reduced by the addition of the extra sequences, which now make up an epitope string of 229 amino acids.

Cassette E contains four repeats of the B-cell epitope from circumsporozoite (CS) protein. CS is the major sporozoite coat protein, and antibodies to NANP may be of protective relevance in humans[16]. An anti-$(NANP)_n$ monoclonal antibody recognized Ty-VLPs carrying CABDHFE, but not CABH (Fig. 4). Mice were immunized intramuscularly with 50 μg CABDHFE or CABDH, without adjuvant. Serum from the mice immunized with the particles containing cassette E bound to R32LR recombinant protein, which contains multiple repeats of NANP, whereas serum from the control CABDH mice did not (data not shown).

The heparin binding adhesion motif from TRAP[17] has also been included in cassette E. This may be useful in targeting Ty-VLPs to the liver, so that the vaccine mimics the route of a natural infection. The adhesion motif mediates the binding of TRAP to sulfated glycoconjugates, such as heparin or dextran sulfate[17]. Ty-VLPs with the adhesion motif (CABDHFE) bound to dextran sulfate, but those without the motif did not (Fig. 4B). The adhesion motif is at the extreme C-terminus of the Ty-epitope fusion protein. The orientation of protein molecules forming the VLP is not known, but as large extensions at the C-terminus can be tolerated without disrupting particle formation, it is likely that the C-terminus is located on the outside of the VLP, making the C-terminus available for interaction with other molecules. Thus, unlike the CTL epitopes, which are processed out of the epitope string after entering a cell, the position of the adhesion motif in the string may be important if it is to function in targeting the VLP to liver cells. Antibodies against the adhesion motif may be generated following immunization. These antibodies could impair the ability of sporozoites to invade hepatocytes.

Ty-VLPs can prime the induction of protective CTL against *P. berghei*. Balb/c mice were challenged with 2000 infectious sporozoites intravenously. Although immunization with Ty-CABDHFE induced CTL against pb9, this immunization alone was not sufficient to protect the mice from infection following challenge (data not shown). When the immune response was boosted with a single inoculation of 10^6 recombinant attenuated vaccinia virus (Modified Virus Ankara [MVA])[18] expressing the minimal pb9 epitope as part of the CAB string, 7/7 immunized mice compared with 0/10 unimmunized mice showed sterile protection from sporozoite challenge. Out of six mice immunized twice with the recombinant MVA, none were protected. Because there are only nine amino acids of *P. berghei* sequence in these constructs the sterile protection induced must be mediated by the CD8 T-cell response to this minimal epitope.

Discussion

Ty-VLPs carrying a single *P. berghei* epitope induce CTL responses in mice[5]. When two epitopes are carried on the same Ty-VLP, CTL are generated to both[19]. We show that it is possible to produce Ty-VLPs carrying 229 amino acids encoding multiple functional epitopes. Many CTL epitopes from different antigens that are presented by a number of different HLA Class I molecules have been identified. Using minimal epitopes to produce vaccines instead of whole antigens enables the immune response to be directed towards conserved regions of antigens, thereby preventing the pathogen from escaping or antagonizing the host CTL response.

In some cases flanking sequences have been shown to be important for the efficient processing of epitopes[20], but there are now many examples of presentation of epitopes regardless of their context[21]. We find that when epitopes are linked together directly with no intervening sequences, individual epitopes can be processed by the proteolytic machinery of the cell and presented on MHC class I molecules. Even overlapping epitopes (tr42 and tr43) can both be presented by cells from the same individual. T-helper epitopes included in the particle were recognized in T-cell proliferation in vitro for all HLA-DR types tested.

Recombinant Ty-VLPs are a flexible epitope delivery system. These particles are prepared from a generally recognized as safe microorganism by a simple purification protocol, which can easily be scaled up. They can be administered without adjuvant by various routes leading to strong CTL responses against the included epitopes. In mice these responses are long lasting and can be boosted[5]. Trials of recombinant Ty-VLPs in humans have shown them to be safe, and to elicit cellular and proliferative responses to the vaccine[22]. The use of alum as an adjuvant impairs CTL responses, but in a phase I trial where no adjuvant was used, CTL responses were induced[23]. These particles therefore represent a promising candidate both for travellers and individuals living in malaria-endemic areas, and should also be considered as an antigen delivery system for vaccination against other diseases where CTLs are required for protection.

Experimental protocol

Construction of plasmids and preparation of Ty-VLPs. All of the CTL epitopes are from conserved regions of *P. falciparum* antigens with the exceptions of cp26 and cp6. Variation in st8 and the previously unpublished epitopes has not yet been studied. To make each epitope cassette, four synthetic oligonucleotides encoding the required sequences (Table 1) using yeast codon bias with a BglII site at the 5′ end and a BamHI site at the 3′ end were annealed. For example, to make cassette A the following oligonucleotides were synthesized:
A1, GATCTAAGCCGAACGACAAGTCCTTGTATAAACCTAA;
A2, TCGTCCTTAGGTTTATACAAGGACTTGTCGTTCGGCTTA;
A3, GGACGAATTGGACTACAAGCCAATCGTTCAATACGACAACTTCG;
A4, GATCCGAAGTTGTCGTATTGAACGATTGGCTTGTAGTCCAAT, where A1 and A3 form the top strand, and A2 and A4 form the bottom strand. The annealed oligonucleotides were ligated into the pUC-based cloning vector pIC20R between the BglII and BamHI sites. After sequencing, cassettes were ligated together as required. The junction formed by ligating the BamHI site at the 3′ end of one cassette to the BglII site at the 5′ end of the next destroys both restriction sites, facilitating the addition of further cassettes to either end of the string. When the required sequence had been established, it was then inserted at the BamHI site of the TyA expression vector pOGS40, forming a C-terminal fusion to the Ty p1 protein, under the control of the strong, galactose-inducible PAL promoter. Recombinant Ty-VLPs were prepared from *S. cerevisiae* strain MC2 following galactose induction[4], and resuspended in PBS for injection. The following combinations of cassettes were fused to p1 and formed particles in *S. cerevisiae:* ACB, ACBH, CAB, CABH, ABC, ABCH, CABDH, and CABDHFE.

Immunological methods. Female Balb/c mice 6–8 weeks of age were immunized with doses of Ty-VLPs ranging from 20 to 100 μg, given subcutaneously or intramuscularly as described in the figure legends and text. Pb9 alone is a Ty-VLP carrying only the pb9 sequence. Cytotoxicity assays were performed as described[5]. Briefly, spleen cells were cultured with the pb9 peptide (SYIPSAEKI) for 5 days. Interleukin 2 was added on day 3. Targets were ^{51}Cr loaded P815 H-2K^d cells used alone (background) or prepulsed for 1 h with 10 μg/ml pb9 peptide. Supernatants were harvested after 4 h. For challenge studies, mice were injected intravenously with Ty-CABDHFE particles at 100 μg/mouse. Mice were challenged after 3 weeks or boosted with recombinant MVA-CAB at 10^6 pfu/mouse and challenged 3 weeks later. Each animal was injected intravenously with 2000 freshly isolated viable *P. berghei* sporozoites dissected out from the salivary glands of ANKA strain *Anopheles stephensi* mosquitoes. Thin smears of venous blood were collected 8–12 days after challenge and stained for the presence of blood stage parasites with Giemsa stain.

In vitro processing The macrophage H-2K^d cell line J774A.1 (ECCC) was ^{51}Cr loaded for 1 h, washed and then cultured for 4 h with 100 μg/ml of the corresponding Ty particle construct. After washing it was used as a target for the pb9 epitope-specific effector CTL clone CS.C3 at an E:T ratio of 20:1 in a standard 4 h ^{51}Cr release assay. Cells that have been incubated for the same period with no additions, or with Ty-VLPs lacking the pb9 sequence (Ty-V3 [ref. 24]) were used as controls.

Human T-cell responses. Proliferation assays were carried out as described[25]. Briefly, PBMC were isolated from blood donors of known HLA type. 2.5×10^6 cells were prepulsed for 1 h with 50 μg/ml of Ty particles with or without the helper epitope cassette, irradiated, washed and then cultured with 2.5×10^6 untreated cells in a total volume of 2 ml. From days 5–10 triplicate 50 μl aliquots were taken and ^{3}H-thymidine incorporation was measured after an overnight incubation. Standard deviation was <10%. Peak proliferation is shown. Donors are adult Oxford residents who were immunized in childhood with BCG and TT. Secondary and primary CTL responses were induced as described[11]. Briefly, PBMC from blood donors of known HLA type were prepulsed for 1 h with peptide or Ty particles (50 μg/ml) and then diluted 1/20 by the addition of culture medium plus 25 ng/ml interleukin 7 (for primaries and secondaries) and 5 μg/ml KLH (for primaries). Cells were cultured in 2 ml and interleukin 2 (IL2) (10 U/ml) was added on day 4. For primary responses, on day 7 cells were "semi-cloned" at 5000 cells/well into 96 U bottom well plates with 100,000 autologous irradiated peptide prepulsed (10 μg/ml) PBMC/well and 10 U/ml of IL2. A week later 40 wells were tested for specific cytotoxic activity in the chromium release assay. Positive wells were scored as those having >15% SL[11]. Secondary responses were tested for specific cytotoxic activity on day 9 of culture at an E:T ratio of 40:1. PBMCs from a malaria-exposed HLA-B8 donor were restimulated in vitro using Ty-VLPs containing the overlapping B8 epitopes tr42 and tr43. Targets were HLA-matched Epstein-Barr virus transformed B cell lines alone (background) or prepulsed with 10 μg/ml of peptide tr42, tr43, or the control cp43, another malaria B8 epitope that is not included in the Ty-VLP used to restimulate

NANP ELISA. To detect the $(NANP)_n$ B cell epitope on Ty-VLPs, particles with either CABH or CABDHFE were bound to microtiter plates in 50 mM carbonate buffer pH 9.6 at either 1, 0.5, 0.1, or 0.05 μg per well by overnight incubation at 4°C. The wells were washed 5 times with PBS containing 0.1% Tween 20 (PBST), then the peroxidase-conjugated anti-NANP monoclonal antibody PF2A10 was diluted 1/1000 in PBST containing 1% bovine serum albumin (PBST/BSA; Sigma, St. Louis, MO) and 50 μl added to each well. After incubation at 37°C 2 h, the wells were washed 5 times with PBST, and 200 μl peroxidase substrate (OPD; Sigma) was added. The color was allowed to develop in the dark for 30 min, then the reaction was stopped with 50 μl 3 M HCL and the A_{495} read using an ELISA plate reader. The recombinant protein R32LR, which contains repeats of NANP, was used as a positive control, resulting in an A_{495} of 2.6 when 0.1 μg was used to coat wells.

Dextran sulfate binding assay. Dextran sulfate 500 (Sigma) was used to coat microtiter plates at 1 μg per well. The wells were washed 5 times with PBST, and Ty-VLPs were added in PBST/BSA, using a serial twofold dilution starting at 1 μg/well. After 2 h at 37°C, the plates were washed, and bound Ty-VLPs were detected using a mix of 5 anti-Ty-VLP antibodies, then alkaline phosphatase conjugated antimouse antibody. Cleavage of NPP (Sigma) was then detected by reading A_{450}.

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