Major Histocompatibility Complex and T Cell Interactions of a Universal T Cell Epitope from Plasmodium falciparum Circumsporozoite Protein*

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A 20-residue sequence from the C-terminal region of the circumsporozoite protein of the malaria parasite Plasmodium falciparum is considered a universal helper T cell epitope because it is immunogenic in individuals of many major histocompatibility complex (MHC) haplotypes. Subunit vaccines containing T* and the major B cell epitope of the circumsporozoite protein induce high antibody titers to the malaria parasite and significant T cell responses in humans. In this study we have evaluated the specificity of the T* sequence with regard to its binding to the human class II MHC protein DR4 (HLA-DRB1*0401), its interactions with antigen receptors on T cells, and the effect of natural variants of this sequence on its immunogenicity. Computational approaches identified multiple potential DR4-binding epitopes within T*, and experimental binding studies confirmed the following two tight binding epitopes: one located toward the N terminus (the T*-1 epitope) and one at the C terminus (the T*-5 epitope). Immunization of a human DR4 volunteer with a peptide-based vaccine containing the T* sequence elicited CD4+ T cells that recognize each of these epitopes. Here we present an analysis of the immunodominant N-terminal epitope T*-1. T*-1 residues important for interaction with DR4 and with antigen receptors on T*-specific T cells were mapped. MHC tetramers carrying DR4/T*-1 MHC-peptide complexes stained and efficiently stimulated these cells in vitro. T*-1 overlaps a region of the protein that has been described as highly polymorphic; however, the particular T*-1 residues required for anchoring to DR4 were highly conserved in Plasmodium sequences described to date.

To be effective in protection against malaria, subunit vaccines must include T cell epitopes capable of eliciting protective immune responses in genetically diverse populations. The T* epitope was identified during characterization of CD4+ T cell responses in human volunteers immunized with irradiated Plasmodium falciparum sporozoites, who were protected against subsequent challenge with live parasites. The T* sequence corresponding to residues 326–345 of the P. falciparum circumsporozoite (CS)3 protein (EYLNKIQNSLSTEWSPCSVT) (NF54 isolate) (1, 2) is termed a universal epitope because it elicits T cell responses restricted by many different HLA-DR allotypes in sporozoite-immunized volunteers, because it binds to multiple HLA-DR and HLA-DQ molecules in vitro and because it elicits helper T cells (Th) in mice of diverse genetic backgrounds (2, 3). Recently, in an open-label phase I trial of a tri-epitope peptide-based malaria vaccine containing T* and another T cell epitope (T1) (4) combined with a B cell epitope from the NANP repeat region of the CS protein, high levels of P. falciparum-reactive antibodies against the CS repeat epitope were elicited in most volunteers of diverse HLA types (5). After being successfully examined in different malaria vaccine trials, the T* epitope has proven to be highly immunogenic (5, 6). The strong correlation of T*-specific cellular responses with high anti-repeat antibody titers in the immunized volunteers suggests that the T* peptide functions as an effective Th (helper) epitope in vivo (5, 6). Moreover, cytokine responses from T*-specific CD4+ T cells have been detected in individuals resistant to malaria (1, 2).

In orchestrating a protective immune response to malaria parasites, the response of CD4+ T helper cells is of paramount importance for both humoral and cell-mediated immunity (4, 7–10). The characterization of the response of antigen-specific CD4+ T cells for well defined malaria peptides is required for mechanistic understanding of generation of immunity induced by CS-based malaria vaccine candidates. Since their description in 1996 (11), class I MHC/peptide tetramers have revolutionized the characterization of antigen-specific CD8+ T cells in viral infections (12–14), bacterial and other infectious diseases (15–19), and cancer (20–22). However, the low avidity of MHC class II-peptide complexes by T cell receptors and the low frequencies in which antigen-specific CD4+ T cells typically are found, compared with their CD8+ counterparts, have made difficult the use of MHC class II-peptide complexes to study the response of CD4+ T cells in vivo. Although MHC class II tetramers have been used successfully to enumerate antigen-specific CD4+ T cells in some viral and autoimmune diseases (18, 23–29), they have yet to be successfully utilized in studying parasitic infections. In malaria, besides these general problems that constrain the construction and usefulness of class II tetramers, the systematic analyses of malaria-specific human CD4+ T cells with defined
Fine Mapping of T*-1 Epitope of P. falciparum

MHC class II/peptide specificities and the identification of immunodominant CD4+ T cell epitopes remain a significant challenge.

MHC class II proteins bind peptides by using a conserved network of hydrogen bonds between the peptide backbone and the conserved amino acid residues in the MHC class II binding groove, orienting peptide side chains at particular positions into defined subpockets or pockets in the overall binding site (30). In human MHC class II HLA-DR alleles, these pockets typically accommodate side chains at the P1, P4, P6, P7, and P9 pockets, with the P1 pocket playing a major role and usually occupied by a hydrophobic side chain from a residue near the N terminus of the bound peptide. The side chains of the intervening residues, at positions P3, P5, and P8 (and sometimes P1 or P11), are exposed to solvent in the MHC-peptide complex and can contact specificity loops of a T cell antigen receptor (TCR) (31). Crystallographic studies together with computational and experimental approaches have contributed to much understanding of the nature of the peptide-MHC interaction, and algorithms to predict relative binding of peptides to particular MHC class II alleles are available (32–34). However, for pathogens with very large genomes such as malaria, their usefulness has been limited, and the identification of epitopes in this parasite still largely relies on experimental work (35).

In this study, we identified the binding register of a peptide (T*-1) found within the T* sequence that has high affinity for the human class II MHC protein DR4 (HLA-DRB1*0401), and we mapped key DR4 and T cell receptor interactions for this peptide. MHC-peptide tetramers carrying this epitope proved to be useful for the detection and in vitro stimulation of several T*-1-specific T cell clones isolated from an individual vaccinated with a synthetic vaccine carrying the T* epitope (5, 36). These T cells exhibit significant cross-reactivity with synthetic peptides representing natural P. falciparum variants of the CS protein as would be required in an effective vaccine.

**EXPERIMENTAL PROCEDURES**

Peptides—Synthetic peptides were synthesized by a multiple solid-phase technique, using tert-butoxycarbonyl protection as described previously (37), and were purified by reverse-phase HPLC on a C18 LiChrospher column (Merck). The quality of the synthesized products was assessed by analytical HPLC and mass spectrometry (matrix-assisted laser desorption ionization time-of-flight). Biotinylated HA-(306–318) (PKYVKQNTLKLAT) test peptide, used for competition binding assay, was purified by analytical HPLC and mass spectrometry (matrix-assisted laser desorption ionization time-of-flight). Biotinylated HA-(306–318) (PKYVKQNTLKLAT) test peptide, used for competition binding assay, was purified by analytical HPLC and mass spectrometry (matrix-assisted laser desorption ionization time-of-flight).

**T Cell Clones and T Cell Stimulation Assays**—T cell clones were isolated from blood samples obtained at different time points during the course of the (T1BT*4-P3C vaccine trial from volunteers 09 and 10 used in this work was confirmed to be DRB1*0401 and DRB1*0403, respectively, and has been reported (36). T cell clones were expanded and maintained in culture in complete media by periodic expansion and maintained in culture in complete media by periodic addition of 5 mM dithiothreitol. Excess biotin and dithiothreitol were removed by gel filtration as above, by extensive dialysis, or by repeated cycles of concentration and dilution in a 10,000 molecular weight cut-off spiral filtration device (Millipore). DR4 and DR4Cys were purified by immunoaffinity with an LB3.1-conjugated protein A column, as described (39). The final yields of DR4 and DR4Cys were in the range of 0.2–0.5 mg/liter of cultured cells.

**Preparation of Biotinylated DR4 (DR4bio)–Peptide Complexes**—Biotin was conjugated to the unpaired cysteine on DR4bio, using the thiol-specific reagent biotinyl-3-maleimidopropionamidyl-3,6-dioxaoctanedia-mine (PEO-maleimide-biotin; Pierce). In some cases the DR4Cys was treated with 5 mM dithiothreitol for 30 min at room temperature before reaction to reduce spontaneous oxidation products, followed by gel filtration (SEC-3000; Phenomenex, Torrance, CA) in phosphate-buffered saline (PBS, 15 mM Na/KPO4, 135 mM NaCl, pH 7.0) to remove excess biotin. Biotinyl-3-maleimidopropionamidyl-3,6-dioxaoctanedia mine (50 μM) was reacted with 1–2 μM DR4bio, in PBS for 10–30 min at room temperature with the addition of 5 mM dithiothreitol. Excess biotin and dithiothreitol were removed by gel filtration as above, by extensive dialysis, or by repeated cycles of concentration and dilution in a 10,000 molecular weight cut-off spin ultrafiltration device (Centricon-10). Biotinylated DR4bio (DR4bio) was concentrated to a final concentration of 4 μM and stored at 4 °C. For peptide loading, 4–5 μM of DR4bio and 40–50 μM of T* or other peptides were incubated together for 72 h at room temperature in binding buffer (100 mM sodium phosphate, pH 5.5, 50 mM NaCl, pH 7.0) with 0.05% octyl-glucoside, 0.2% bovine serum albumin, and 1 mM phenylmethylsulfonyl fluoride) (DR4bio-peptide complexes were isolated by gel filtration and concentrated to 4–6 μM of DR4bio-peptide complexes were supplemented with 5–10 μM antibody excess of peptide for storage at 4 or −20 °C, and remained stable for up to a year. Final yield was ~0.05–0.1 mg of purified DR4bio-peptide complexes/liter of culture.

**Flow Cytometric Staining of T Cells Using MHC Tetramers**—In order to conserve reagent, T cell staining reactions were performed in the smallest practical volumes. To prepare 10 μl of fluorescent DR4-oligomer ("tetramer"), 1 μl of DR4bio-peptide complex (0.3 mg/ml stock in...
PBS was mixed with a total of 4 μl of (R)-phycocerythrin-labeled streptavidin (SA-PE, BIOSOURCE) 0.025 mg/ml prepared in PBS from 0.25 mg/ml stock, which was added stepwise in 1-μl portions over ~10 min. During the procedure the tubes were kept on ice and afterward 5 μl of PBS were added. For staining, 5 μl of fluorescent oligomer solution was added to 10⁶ T cells in 10-20 μl of culture medium in round-bottom or v-bottom 96-well plates. Cells were stained for 3 h in a 37 °C incubator, washed with PBS, and centrifuged at 1250 rpm for 10 min. At the end of the oligomer staining reaction, samples were chilled and stained with anti-CD3-FITC, anti-CD25-PE, and anti-CD69-APC for 30 min with anti-CD3-FITC, anti-CD25-PE, and anti-CD69-APC (Pharmingen), followed by washing twice with cold wash buffer (PBS, 1% fetal bovine serum, 15 mM sodium azide) and analyzed by flow cytometry (FACScan or FACS Calibur, BD Biosciences).

Stimulation of T Cells with Immobilized MHC Tetramer—Standard 96-well tissue culture plates were coated overnight at 4 °C with 50 μl of 8 μM streptavidin (Promega) in PBS, and then were blocked for 2 h at 37 °C with 200 μl of 3% bovine serum albumin in PBS. DR⁴bio-peptide complexes (serial dilutions starting from 1 μM) were bound to the streptavidin-coated plates for 6 h at 37 °C, and then the wells were washed twice with 200 μl of complete medium. For activation assay, 10⁵ T cells in 150 μl of complete medium were added to each well, and the plate was incubated for 16 h at 37 °C, 5% CO₂. Supernatants were removed, and secreted IL-2 was measured by bioassay as described above. T cells remaining in the plate were washed and stained on ice for 30 min with anti-CD3-FITC, anti-CD25-PE, and anti-CD69-APC (Pharmingen), followed by washing twice before analysis by flow cytometry.

Peptide Binding Assays—Peptide binding to DR4 was analyzed using an enzyme-linked immunosorbent assay-based competition assay as described previously (3, 32). Purified HLA-DR4 molecules (3.8 pmol) were diluted into freshly prepared binding buffer containing bovine serum albumin in PBS. DR⁴bio-peptide complexes (serial dilutions starting from 1 μM) were bound to the streptavidin-coated plates for 6 h at 37 °C, and then the wells were washed twice with 200 μl of complete medium. For activation assay, 10⁵ T cells in 150 μl of complete medium were added to each well, and the plate was incubated for 16 h at 37 °C, 5% CO₂. Supernatants were removed, and secreted IL-2 was measured by bioassay as described above. T cells remaining in the plate were washed and stained on ice for 30 min with anti-CD3-FITC, anti-CD25-PE, and anti-CD69-APC (Pharmingen), followed by washing twice before analysis by flow cytometry.

Native Gel Electrophoresis—Peptide binding was also followed qualitatively by a native gel electrophoresis assay. DR4 (100 pmol) and T* or any other T* peptide derivative (1000 pmol) was mixed in binding buffer in a total volume of 25 μl. After 72 h of incubation at room temperature, the samples were analyzed by 10% PAGE in the absence of SDS. The electrophoresis was run under conditions in which gel and running buffer were kept at 4 °C to avoid heating the sample during electrophoresis. After the gels were run, they were stained with Coomassie Blue R-250 and destained with a mixture of methanol (30%) and acetic acid (10%) in water.

Prediction of MHC Peptide Binding Activity—Potential T cell epitopes were evaluated using a local modification of the matrix-based approach of Hammer et al. (32). Matrix-based methods for predicting class II MHC peptide binding affinity assign a value to each amino acid residue at each position in a sliding nine-residue binding frame. In the original implementation, only peptides with Tyr, Trp, and Phe at the P1 position were considered (32). In our local modification, values are assigned for all possible amino acid residues at P1, with high values for Tyr, Trp, and Phe, intermediate values for Leu, Ile, Val, and Met, and low values for the other amino acids, essentially as described (34). Peptide binding prediction using other algorithms generally provided similar results (33, 41).

Molecular Modeling—The coordinates of DR4 in complex with collagen II peptide (42) were obtained from a crystal structure in the Protein Data Bank (code 2SEB) and were used as a template for modeling. After manual replacement of the lateral chain of the collagen peptide with the amino acids of T*-1 in the binding frame, as suggested by the alanine-scanning experiments using Insight II (2000) Biopolymer Group software (Accelrys Software, San Diego), the complex was minimized, and the energetics of the nonbonded DR4-peptide interaction were determined using the Docking Program (Accelrys).

RESULTS

Defining the Minimal DR4-binding Sequence in T*—The T* epitope has been defined previously as a 20-amino acid long sequence (EYLNIKIQNFLSTPWCSETVFT) from the C-terminal region of the malaria CS protein (Fig. 1A) that binds to DRB1*0401 and other human class II MHC variants and when included in a subunit vaccine induced immunodominant CD4⁺ T cell responses (2, 3, 5, 36). Class II MHC proteins bind peptides in a canonical polyproline-like extended conformation, interacting with approximately nine residues of the bound peptide (30). The ends of the peptide binding groove are open, and a bound peptide can extend out of the site at either end. Thus, a long peptide like T* can have multiple potential binding registers. We combined computational and experimental approaches to determine which of the potential binding registers correspond to the immunogenic epitope(s). First, we analyzed the sequence using a matrix-based binding prediction algorithm for HLA-DRB1*0401 (32). In this approach to predicting MHC-peptide binding affinities, an independent site assumption is used; the matrix contains values representing the effect of every possible side chain at each position in a sliding nine-residue binding frame. Such matrices are available for a wide variety of class II MHC allotypes (32–34). Typically strong allele-specific preferences are seen at positions P1, P4, P6, and P9, consistent with the location of significant MHC-peptide contact as revealed in the crystal structures (30). We used this approach to evaluate each of the 12 possible 9-mer registers in the T* peptide. Positive values indicative of potential tight binding interactions were obtained for the four registers Leu328, Asn329, Ile331, and Leu335, labeled according to the residue predicted to occupy the major hydrophobic pocket P1 (Table 1).

In order to evaluate experimentally which, if any, of these predicted registers bind to DR4 with reasonable affinity, the set of peptides T*-1 through T*-6 (Table 2) was designed, synthesized, and tested for DR4 binding in a competition binding assay as shown in Fig. 1B, with IC₅₀ values given in Table 2. These peptides were designed to allow formation of MHC-peptide main-chain interactions that flank the 9-residue binding registers (30), and thus contain 11 or 12 residues. The almost

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4 L. J. Stern, personal communication.
TABLE 1
Predicted binding of epitopes in T* to HLA-DR4

| Register | Sequence (amino acid position) | Register | IC50 μM |
|----------|--------------------------------|----------|---------|
| T*       | EYLNIKQNSLSTEWSPCSVT         | All      | ≤0.1    |
| T*-1     | EYLNIKQNSLSTWPEYS            | L328 + N329 | 0.2     |
| T*-2     | oNKLQNSLSTRT                 | L328     | >30     |
| T*-3     | YLNKQNLSTd                   | L328     | >30     |
| T*-4     | KQNLSTEWWS                   | L331     | NB*     |
| T*-5     | SLSTEWSPCSV                  | L335     | 0.9     |
| T*-6     | SLSTEWSPCST                  | L335     | 3.0     |

* NB indicates no binding.

Completely overlapping registers Leu328 and Asn329 were difficult to distinguish. We attempted to evaluate their relative roles by using the peptides T*-1, T*-2, and T*-3 (Table 2). T*-1 contains both registers and one flanking amino acid on each end; T*-2 carries a L328G substitution designed to disrupt the Leu328 register; and T*-3 carries a T337D substitution designed to disrupt the Asn329 register. The partially overlapping registers Ile331 and Leu335 were addressed in a more straightforward way, using the 11-mer peptides T*-4 and T*-5 that carry the desired binding register with one flanking amino acid on each end. Peptide T*-6 additionally carries a C342S substitution to alleviate potential problems with cysteine oxidation and/or modification during synthesis and assay. In vitro peptide binding assays were used to examine the binding capacity for DR4 of T* and T* derivative peptides carrying the predicted DR4 binding registers. Binding was evaluated by using a competition assay with a biotinylated high affinity DR4-binding peptide from influenza HA-(306–318) (Fig. 1A). IC50 values for these responses are given in Table 2. The T*-1 peptide, carrying the Leu328 and Asn329 registers, binds with high affinity, only slightly worse than the parent peptide. The substitution scheme employed did not distinguish the relative binding affinity of the two likely registers, as both mutated peptides (T*-2 and T*-3) bound very weakly. Peptide T*-4, carrying the Ile331 register, did not exhibit detectable binding by this assay. Peptide T*-5, carrying the Leu335 register, bound less tightly, almost 10 times weaker than the parent T* peptide. The T*-6 peptide, which also carries the Leu335 register but with a C342S substitution, bound similarly, although the cysteine substitution results in a 3-fold reduction in half-maximal inhibitory concentration when compared with T*-5.

These results were confirmed using a different assay. Using a gel shift assay to monitor the direct binding of unlabeled peptide to DR4 in solution, the same pattern of reactivity was observed (Fig. 1C), with stable MHC-peptide complexes formed by T*, T*-1, and T*-6 peptides. Thus, of the potential binding registers predicted by the matrix analysis, the Leu328/Asn329 register (T*-1 peptide) and also the Leu335 register (T*-6 peptide) allow for tight binding to DR4, but the Ile331 register (T*-4 peptide) does not bind to DR4 with sufficient affinity to allow detection in our assays. Furthermore, neither Asn329 nor Leu335 registers (T*-2 and T*-3 peptides respectively) demonstrated binding in this assay.

**Immunogenicity of T* Peptides**—To assess the immunogenicity of the T*-derived peptides, a T*-specific CD4+ T cell clone (92E10) isolated from a DR4 volunteer who participated in the phase I (T1BT*4)-P3C polyoxime vaccine trial (volunteer 09 (5)) was used. Recognition of T* peptide sequences by this clone was measured by using DR81*0401-transfected fibroblasts as APCs (data not shown). Table 3 summarizes the results of T cell proliferation and IL-2 secretion for clone 92E10 tested for activation using T* and the T* derivative peptides. Although peptides T*, T*-1, and T*-3 induced cellular proliferation and IL-2 production by the 92E10 clone, peptides T*-4, T*-5, or T*-6 induced neither cellular proliferation nor IL-2 production. Peptide T*-2 induced a weak response, significantly above background but clearly weaker than for peptides T*, T*-1, and T*-3. These data strongly suggest that clone
92E10 requires an immunostimulatory determinant present in T*-1 and T*-3 that is partially compromised in the T*-2 peptide. Presumably, the high sensitivity of the T cell assays allows detection of the response to peptide T*-3 despite its low binding affinity, although it is possible that kinetic effects not directly measured in our competition assay are important in determining T cell recognition (43).

Identifying MHC and TCR Contact Residues in T*-1—Key residues within the T* epitope were defined by using alanine-scanning mutagenesis in order to map the predominant MHC and TCR contacts of the immunogenic T*1 epitope, and to gain insight into whether the dominant binding register was Leu328 or Asn333 (Table 4). Single alanine substitutions of each residue in T*-1 were tested in DR4-binding assays and T cell activation assays as described above for clone 92E10, also for clones 5 and 6 that also recognize an epitope in the N-terminal region of T* (Table 5), and for control clone 19 that recognizes a T*-derived epitope not present in T*-1. Alanine substitutions at Leu328 and Asn333 positions blocked binding to DR4, as evaluated in the competition binding assay (Table 4). Substitutions at other positions had no significant effect. These results clearly establish the binding register as Leu328 occupies the P1 pocket and Asn333 the P6 pocket in the T*-1 peptide. Alanine substitutions at these positions and also at Lys330, Ile331, Gin332, and Ser334 blocked activation of T cell clone 92E10. The impaired T cell response to Ala substitution of residues Lys330, Ile331, Gin332, and Ser334 in the absence of appreciable effects on MHC-peptide binding suggests that these four residues make critical peptide-TCR contacts. Similar but not identical patterns of reactivity were observed for clones 5 and 6, isolated from the same individual as clone 92E10 and also recognizing the T*-1 epitope (Table 5). The two residues Tyr327 and Ser334 that flank the central MHC binding determinants (Leu328 and Asn333) differentially affect recognition by the T*-specific clones; Tyr327 substitution affects clones 5 and 6 but not 92E10, and Ser334 substitution affects 92E10 but not the others. Clone 19, a C-terminal-T*-specific T cell clone, is stimulated by T* but not by any of the T*-1 analogues and is included as a negative control to show the specificity of the responses by the T*-1-specific clones.

Additional DR4-restricted CD4+ T cell clones recognizing the T* epitope were isolated from the peripheral blood of immunized donors after in vitro stimulation with the T* peptide (36). Analysis of the IL-2

### Table 3

Recognition of T* peptides by human T cell clone 92E10

| Peptide | Mean Proliferation | IL-2 production |
|---------|-------------------|----------------|
| T*      | 131,569           | >10,000        |
| T*-1    | 133,737           | >10,000        |
| T*-2    | 37,307            | 0.108          |
| T*-3    | 135,901           | >10,000        |
| T*-4    | 599               | <0.001         |
| T*-5    | 922               | 1.3            |
| T*-6    | 543               | 0.8            |

IL-2 production (S.I.)

| T cell clone | 92E10 | 5 | 6 | 19 |
|--------------|-------|---|---|----|
| T*           | 266   | 267 | 266 |
| T*           | 435   | 435 |
| T*           | 830   | 830 |

### Table 4

Identification of residues in the T*-1 peptide that are critical for MHC and TCR binding by alanine-scanning mutagenesis

A series of T*-1 analogues with single alanine substitutions were synthesized and tested for their ability to bind to HLA-DR4 and to stimulate CS-specific T cell clones (92E10, 5, 6, and 19). Relative binding was measured by an inhibition binding assay as in Table 2 and Fig. 1. T cell activation was measured by IL-2 bioassay and expressed as a stimulation index (S.I.). S.I. considered as significant (>10% response with T*) are shown in boldface. Clone 19 reacts with a C-terminal T* epitope not present in T*-1 (36).5

### Table 5

Recognition of L328 DR4 binding register by human T cell clones

T cell clones isolated from volunteers 09 and 10 (5, 36) were stimulated with autologous antigen-presenting cells pulsed with the indicated T*-truncated peptides. Activation was measured as stimulation index (S.I.) (fold increase upon background stimulation), using a bioassay for secreted IL-2. Positive results are highlighted in boldface.
response of the clones from volunteer 09 (DRB1*0401) and volunteer 10 (DRB1*0403) upon stimulation with T*-truncated peptides demonstrates that these clones recognize the minimal peptide sequence LNKIQNSLST (CS-(328–337)) (Table 5). Amino acid deletions at the N or C terminus of this register abolished antigen recognition (Table 5 and data not shown), suggesting that they recognize nine amino acids comprising the predicted Leu^{328} DR4 binding register (Table 1). The similarity in the recognition of the T*-1 epitope by the DRB1*0401 and
DRB1*0403 clones is consistent with the similarity in the overall primary sequence of these molecules. DRB1*0403 differs from DRB1*0401 in only three positions: Kb71R, Ab74R, and Gβ86V; these substitutions are predicted to lead to only minor changes in the specificity of the P1 and P4 pockets (30, 34).

**MHC Oligomers Containing DR4 Bound to T* Peptide—**As a step toward developing reagents to easily follow the T cell response to T*-derived antigens in vaccinees, we prepared fluorescent oligomers ("tetramers") (11) of DR4-T* complexes, and we evaluated their use in staining and activating malaria-specific T cell clones. For MHC tetramer formation, we used DR4 constructions that contained an unpaired cysteine at the C terminus of the α subunit, which we derivatized with biotin using a polyethylene glycol linker (44). We attempted to make DR4 complexes for each of the T*-derived peptides in Table 2 by using a standard peptide loading protocol (24). However, based on the binding results described above and the characterization of the peptide binding affinity for DR4 (Table 2), we expected that only peptides T*, T*-1, T*-5, and T*-6 (IC50 = 0.1, 0.2, 0.9, and 3.0 μM, respectively) would be able to produce stable complexes. The yields of monomers of DR4-T*-2 and DR4-T*-3 complexes (IC50 > 30 μM) after gel filtration were 10-fold lower compared with the yields of DR4/T*, DR4/T*-1, and DR4/T*6 monomers (data not shown). (R)-Phycocerythrin-labeled, streptavidin-linked tetramers of these complexes were incubated with clone 92E10 (Fig. 2A) and clones 5, 6, 19, and 64 (Fig. 2B) before washing and analysis by flow cytometry. As expected, DR4/T* and DR4/T*-1 but not DR4/T*-2 or DR1/HA tetramers were able to stain the 92E10 T cell clone (Fig. 2A). Despite the low yield of DR4/T*-2 and DR4/T*-3, we produced tetramers with these complexes, which were not capable of staining the 92E10 T cell clone (not shown). In order to test the specificity of the DR4/T*-1 tetramers, four additional DRB1*0401-restricted T cell clones were tested for staining to T*-1 tetramers (Fig. 2B). Staining by the T*-1 tetramer was also observed for clones 5 and 6 that recognize a core epitope in T*-1 (Table 5). In contrast, clones 19 and 64 that recognize a different epitope in T* (36)5 did not stain with the T*-1 tetramer. Interestingly, CD4 down-regulation was sometimes observed together with tetramer binding, as seen for DR4/T*-1 staining for clones 92E10 and 6 (Fig. 2). The significance of this phenomenon is not clear together with tetramer binding, as seen for DR4/T*-1 staining for clones 92E10 and 6 (Fig. 2). The significance of this phenomenon is not clear.

In addition to their utility as detection/staining reagents, the MHC peptide tetramers can be bound to a solid support and used for the evaluation of functional T cell responses (46). Fig. 3 shows the results of a representative experiment on the functional response of T*-1-specific CD4⁺ T cell clones upon stimulation with immobilized DR4-T*-1 complexes. Stimulation of T*-1-specific cells with bound complexes elicited CD3 (TCR) down-regulation (Fig. 3, A and C), CD25 (IL2Rα) up-regulation (Fig. 3, B and D), CD69 (Leu93) up-regulation (Fig. 3E) and also induced IL-2 production (Fig. 3F). These are all known as conventional T cell activation responses (47). The results of these experiments confirmed the functional specificity of the MHC tetramers.

**T*-1-specific T Cells Elicited Upon Vaccination with a Subunit Vaccine Containing T* Cross-react with Natural Variants of This Epitope—**The amino acid sequence of T*1 epitope is highly polymorphic within the otherwise relatively conserved T* sequence (Fig. 4). Although this polymorphism has been hypothesized to play a role in parasite immune evasion (48), individuals vaccinated with one T* sequence exhibit significant cross-reactivity with variants of the CS protein present in other Plasmodium strains (2, 36). We used the detailed information on expected MHC and TCR contacts of the T*-1 epitope determined above to help interpret the pattern of cross-reactivity of the T*-1-specific T cell clones with the natural variants of the T*-1 sequence. Analyses of the known CS sequence variants show that the key residues for T*-1 binding to DR4, Leu328 and Asn333 predicted to bind in the major P1 and P6 pocket, and also Ile311 and Ser326 predicted to bind in the minor P4 and P9 pockets, all are highly conserved in the known Plasmodium variants, despite significant variation at the intervening positions (Fig. 4A). We evaluated the degree of cross-reactivity of the vaccine-induced T cell clones 92E10, 5, and 6 with a set of natural peptide variants selected to be representative of the full T* variability observed for all strains (Fig. 4A). All three clones were broadly cross-reactive with sequence variants harboring changes at Glu126 (to Gln or Lys) and Asn129 (to Thr, Lys, or Gln), and clone 92E10 also was cross-reactive with a sequence variant at Lys310 (to Arg). However, all three clones were sensitive to a single amino acid substitution of the TCR contact residue Gln332 (to Lys). This pattern of cross-reactivity was not limited to these particular clones, as eight additional DR4-restricted T cell clones presented a similar pattern of cross-reactivity (Fig. 4B). Additional clones from the same volunteers but sensitive for T*1 binding to DRB1*0401 replaced with eight different core epitopes within the T* sequence also cross-reacted with numerous strain-specific peptides (36). These findings suggest a significant recognition of T* variants by T cells elicited upon vaccination with (T1BT)₄-P3C (5, 36).

**DISCUSSION**

The T* sequence from the CS protein of *P. falciparum* has been described as a universal T cell epitope because it is recognized by T cells from individuals of different class II genotypes immunized with irradiated sporozoites or subunit vaccines (1, 2, 5, 6). The experiments reported here identify two regions within the overall T* sequence T*1 and T*5 that bind to DRB1*0401, a common human class II MHC protein. The immunogenicity of the C-terminal region, T*-5, has been described elsewhere (36).4 The N-terminal region, T*1 also is highly immunogenic, being recognized by T cell clones elicited by vaccination of DRB1*0401 and DRB1*0403 individuals with a prototype peptide-based vaccine (Table 5). More importantly, immunization of human volunteers with irradiated sporozoites elicits T*-specific T cells restricted by DR4, DR7, and DR9 that recognize a core epitope overlapping with T*-1 (2).

Apparent MHC and TCR contacts within the T*-1 sequence were mapped by truncation and alanine-scanning mutagenesis in an effort to understand the biochemical basis for the immunogenicity of this sequence, the effect of the genetic variability in the recognition by T cells, and as a guide for subsequent vaccine development. All of the data obtained on MHC and T cell interactions of the T*-1 peptide are consistent with the T*-1 sequence YLNKIQNSLSTE (CS-327–338) binding to DRB1*0401 in a standard polyproline type II-like conformation, placing T*-1 residues Leu238 and Asn333 into the P1 and P6 pockets of the DRB1*0401 peptide-binding site. T*-1 residues between and flanking these pockets are important in interactions with one or more T*-specific, DRB1*0401-restricted T cell clones. To visualize these effects, we have constructed a hypothetical model of the central region of the T*-1 peptide bound to DRB1*0401 based on the standard conformation of DR4-bound peptides as observed in the DRB1*0401-collagen II crystal structure (42), by substituting T*-1 for collagen II peptide side chains and carrying out simple minimization of the entire binding site. Although this simple hypothetical model (Fig. 5) remains to be confirmed by direct structural characterization, it is useful as a tool to interpret the experimental data and to predict the effect of T* variation on immunogenicity. According to this model the side chains of Leu238 and Asn333 observed as the major sites of MHC-peptide interaction in the

5 C. Parra-Lopez, manuscript in preparation.
model, are largely buried in the P1 and P6 pockets. Side chains of Asn\textsuperscript{329}, Lys\textsuperscript{330}, and Gln\textsuperscript{332} are exposed for potential interaction with TCR, whereas Ile\textsuperscript{331} and Ser\textsuperscript{334} are partially buried in the P4 and P7 pockets with a portion of each exposed for interaction with T cell receptors. This model is in agreement with the effect in the T cell responses by the three T cell clones presented in Table 4. Ile\textsuperscript{331} seems to play a more critical role because T cell activation is affected completely in two of the clones and almost totally in the third clone. The fact that mutation of Ser\textsuperscript{334} completely abolished recognition of the peptide by the clone 92E10 but only marginally reduced recognition by the other two clones suggests significant clone-to-clone differences in the manner in which the T*-1 epitope is recognized.

Several aspects of the DRB1\textsuperscript{*0401}-T*-1 interaction reported herein, although consistent with the model, were not expected from previous
work on class II MHC-peptide complexes. First, the weak binding of the
T*-3 peptide is surprising (IC$_{50}$ = 30 M; Table 2) given that it differs
from the tight binding T*-1 peptide (IC$_{50}$ = 0.2 M) only by loss of the
T*-1 C-terminal residue Glu338 at the P11 position and replacement of
Thr337 at the P10 position by aspartic acid. These residues were not
included in the hypothetical model of Fig. 5, because the conserved
polyproline II-like conformation diverges after the P9 position, and thus
the structure in this region cannot be confidently modeled (49). The side
chain of the peptide P11 residue has not been observed to make sub-
stantial contact with the MHC protein, in MHC-peptide crystal struc-
tures, and truncation studies routinely do not observe a significant effect
on MHC-peptide interaction by deletion of this residue. The side chain
at the P10 position can make substantial contact with the MHC protein,
and recently we have mapped DRB1*0101-peptide preferences at this
position (50). DRB1*0401 preferences would be expected to be similar,
as all of the residues lining the P10 pocket are identical in DRB1*0101
and DRB1*0401. In that study, Asp and Thr at the P10 position were
found to have similar contributions to the observed binding (50). Here the
T337D substitution (together with truncation of Glu338) blocked bind-
ing (Table 2), and alanine substitution of either Thr337 or Glu338 did not
lead to substantial decreases in binding affinity (Table 4). Thus, the basis
for the contribution of these residues to the observed binding affinity of
the T*-1 peptide is not clear and may result from cooperative effects (51)
at odds with the independent side-chain contribution analysis or from
idiosyncratic aspects of the interaction of T*-1 with DRB1*0401. Sec-
ond, the inability to identify the P4 and P9 positions by alanine-scanning
mutagenesis was unexpected. In a previous study of alanine-based
model peptides, Ile at P4 and Ser at P9 were found to increase binding in
a competition assay similar to that employed here (6- and 15-fold,

![FIGURE 4. Effect of sequence variation of P. falciparum in the T* region on T cell recognition. A, relative amount of IL-2 production by T cell clones stimu-
lated with antigen-presenting cells pulsed with T*-1 sequence variants. B, T cell clones isolated from volunteer 09 and 10 (5, 36) were stimulated as above, and T cell activation was measured as stimu-
lation index (S.I.) (fold increase upon stimulation), using a bioassay for secreted IL-2. Positive results are highlighted in boldface.](image)

![FIGURE 5. Hypothetical mode of T*-1 peptide bound to DR4. Predicted molecular surface of peptide binding domain of DR4 is shown in white, and the T*-1 peptide colored by residue. Only residues predicted to occupy the canonical P1–P9 pocket region were included in the model. Arrowheads below the peptide sequence indicate peptide side chains with large effects on MHC-peptide interaction; Leu$_{328}$ and Asn$_{333}$ are sug-
gested to fit into P1 and P6 pockets, respectively. Arrowheads above the peptide indicate peptide side chains with large effects on TCR interactions; Lys$_{330}$, Ile$_{331}$, and Gln$_{332}$ (large arrowheads), which are important in interactions with all T*-1-specific clones tested, and
Y$_{327}$ and S$_{334}$ (small arrowheads), which are important in interactions with some but not all clones.](image)
residually) (32), but we did not observe a significant effect on substitution of either Ile331 or Ser336 by alanine (Table 4). Alanine-scanning mutagenesis has been useful in other efforts to identify MHC contacts for DR-binding peptides (49, 52–54). Although it is possible that the lack of effect observed upon substitution of alanine for Ile331 indicates a noncanonical binding mode for the T*-1 peptide, the effects on T cell recognition upon substitution of Ile331, Gln332, and Ser334 and the effects on MHC-peptide interaction upon substitution of Asn333 suggest that these residues adopt the canonical conformation at the P3, P5, P6, and P7 positions, and thus constrain possible alternate conformations at P4. Third, we observed robust T cell activation induced by the T*-3 peptide in cellular proliferation and IL-2 secretion assays (Table 3) despite its drastically reduced ability to bind to DRB1*0401 in competition and native gel peptide binding assays (Fig. 1 and Table 2). Most likely this was because of the higher sensitivity of the cellular assays. Finally, the very weak binding of the T*-4 peptide (Table 2) was at odds with the prediction of tight binding for the Ile331 binding frame (Table 1); however, the motif-based prediction algorithms, such as employed here (32–34), have a substantial false-positive rate. This suggests caution in pre-screening potential T cell epitopes based on MHC-peptide binding only. For example, in a recent comprehensive study of T cell epitopes presented by several human class I and class II MHC proteins, Doolan et al. (35) observed a positive predictive value of ~70% for HLA-DR1.

The fine mapping of MHC and TCR contacts for the T* peptide provided an opportunity to address the impact of polymorphism of the CS protein of *P. falciparum* on its recognition by MHC proteins and by T cells. The CS protein is a relatively conserved protein with significant variation restricted to regions of the protein in which T cell epitopes have been defined. Variability is postulated to be the product of selective pressure by the immune system of the host and is considered as a major limitation to the development of subunit vaccines based on T cell responses (55, 56). The sequence of the T* epitope is not an exception to the rule (Fig. 4). However, the T* epitope is unique in this respect, because variability is limited to the first half of the sequence. The second half of T* is highly conserved, even between species of malaria parasites, and seems to have a central role in the targeting of sporozoites to host cells (57–59). There are over 20 variants reported for the T* sequence, with variation restricted to positions Gln326, Asn329, Lys330, Gln332, Gln333, Asn333, and Leu335 of the T* sequence (NF54). Despite the relatively high number of variants, there is a limited variability at each position in the sequence (Fig. 4). According to the model presented in Fig. 5, the impact of variation in the T* sequence, at least in the case of DRB1*0401, will be mostly in the potential TCR contacts, whereas conserved residues have a critical role stabilizing the peptide-MHC interaction. This model is supported by the observation that the relative binding of T* variants to DR1 and DR4 molecule is highly similar among T* variants (2). T*-specific T cell clones from sporozoite- and peptide vaccine-immunized volunteers recognized a substantial fraction of peptides representing variants of the T* sequence (2, 36) (Fig. 4). This degree of cross-reactivity could be partially explained by the model presented in Fig. 5. In this model MHC contacts involve conserved residues distributed within the otherwise polymorphic region. Moreover, even among the polymorphic T cell contacts many of the amino acid substitutions are conservative.

The observed unresponsiveness of T*-1-specific T cell clones to some peptide variants might cast doubt on the ability of the T*-1 peptide sequence to protect against a large number of parasite strains. However, Riley et al. (60) have shown that T cells reactive to peptides overlapping the T* sequences can recognize polymorphic variants in the field, similarly to the cross-reactivity of (T1BT*)-P3C-elicited T cells with T* variants as we observed here.

Finally, fluorescent oligomers of DR4 bound to T* and T*-1 peptides were able to detect and activate antigen-specific T cells elicited by vaccination of a DR4+ individual. The availability of DR4/T*-1 fluorescent oligomers should prove useful in the continuing studies of anti-malaria immunity. Several such applications of these reagents can be envisioned, including (i) comparison of the prevalence of T*-specific T cells in vaccinees and in individuals living in highly endemic areas who become immune after multiple episodes of malaria (61); (ii) monitoring the appearance, expansion, and persistence in peripheral blood of T* and T*-1–specific cells in individuals exposed to this epitope; and (iii) examination of the T helper status of T*-specific cells in individuals immune to malaria as a result of vaccination with candidate peptide- or subunit-based vaccines or with irradiated sporozoites (62). Peptides overlapping the T*-1 sequence have proven to be highly immunogenic in different malaria vaccine trials and the cytokine response induced suggests an important role in resistance to malaria challenge (1, 2, 63–66). For these reasons it is expected that T* and T*-1–MHC oligomers might be a useful reagent in the study of responses to malaria CS-based vaccines and in the immunity elicited by natural exposure.

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