INFECTION AND IMMUNITY, Apr. 2001, p. 2766–2771 Vol. 69, No. 4
0019-9567/01/$04.00+0 DOI: 10.1128/IAI.69.4.2766–2771.2001

HLA-A*01-Restricted Cytotoxic T-Lymphocyte Epitope from the *Plasmodium falciparum* Circumsporozoite Protein

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Received 1 August 2000/Returned for modification 7 September 2000/Accepted 18 January 2001

Here, we report the identification of a novel CD8$^+$ cytotoxic T-lymphocyte epitope on the *Plasmodium falciparum* circumsporozoite protein (3D7; amino acids 310 to 319 [EPSDKHIKEY]) that is restricted by HLA-A*01 and is recognized by human volunteers immunized with irradiated *P. falciparum* sporozoites. HLA-A*01 is the second most common HLA allele among Caucasians.

Sterile protective immunity against malaria in humans is induced by immunization with irradiated *Plasmodium falciparum* sporozoites (1, 2, 7, 9, 19), is dependent on CD8$^+$ T cells (5, 20, 27), and is presumed to be directed against antigens expressed by irradiated sporozoites in infected hepatocytes (10, 11). Accordingly, a major approach to developing a malaria vaccine that duplicates the excellent protection induced by the irradiated sporozoite vaccine is to identify CD8$^+$ T-cell epitopes on parasite proteins expressed by irradiated sporozoites in hepatocytes (10, 17). To date, 32 *P. falciparum* CD8$^+$ T-cell epitopes derived from five proteins known to be expressed in infected hepatocytes have been reported (6; Table 1).

One potential challenge to the development of epitope-based vaccines is the polymorphism of major histocompatibility complex (MHC) class I molecules. Many HLA-A molecules can be grouped into different HLA supertypes which are characterized by largely overlapping peptide-binding repertoires and are present in high frequencies, irrespective of the particular ethnicity considered. Focusing on the major HLA supertypes generally simplifies the process of development of epitope-based vaccines (21, 22, 24). HLA-A1 is one of five HLA antigens (A1, A2, A3, A11, and A24) expressed in a high proportion of different populations (12, 24) and is the second most common antigen expressed by Caucasians (26 to 36.5%), after HLA-A2 (42 to 64%) (6; Table 2). Recent data also suggest that HLA-A*01 might represent a prototype allele of an HLA-A1 supertype composed of several alleles with similar peptide-binding motifs (22). Thus, HLA-A*01-restricted epitopes should be considered for inclusion in multiepitope peptide-based vaccines.

Accordingly, we searched the *P. falciparum* circumsporozoite protein (PfCSP) 3D7 sequence for sequences containing potential HLA-A*01 binding motifs, specifically a threonine (T), serine (S), or methionine (M) at position 2 or an aspartic acid (D), glutamic acid (E), serine (S), or threonine (T) at position 3 and a tyrosine (Y) at the C terminus (3, 13–15). Two peptides, amino acids (aa) 31 to 40 (NTRVLNELNY) and aa 310 to 319 (EPSDKHIKEY), predicted to contain an HLA-A*01 peptide-binding motif were synthesized and analyzed for their affinity of binding (23, 25) to HLA-A*01. Peptides which bound to HLA-A*01 with high affinity (50% inhibitory concentration [IC_{50}], 500 nM) were then assessed for their capacity to induce peptide-specific recall cytotoxic T-lymphocyte (CTL) responses from peripheral blood mononuclear cells (PBMC) from three volunteers expressing the HLA-A*01 molecule (Table 3) (7) and immunized with *P. falciparum* irradiated sporozoites.

Generation of effector cells. PBMC from irradiated sporozoite-immunized volunteers were infected with recombinant vaccinia virus expressing the entire PfCSP 3D7 sequence at 10 PFU/cell (16) or stimulated with 10 μg of synthetic PfCSP peptide (aa 310 to 319 [EPSDKHIKEY]) per ml for 6 days as described previously (16). In some experiments effector cell populations were depleted of CD8$^+$ T cells using anti-CD8$^+$-coated Dynabeads.

CTL assay. CTL assays and transient transfection of autologous and HLA-mismatched lymphoblastoid B-cell lines (B-LCL) were performed as described previously (16) using plasmid DNA expressing the 3D7 PfCSP gene (VR2510) or the same plasmid without the PfCSP insert (VR1020) (8) or autologous and HLA-mismatched B-LCL pulsed at 10 μg/ml with peptide PfCSP 3D7 (aa 310 to 319) or control peptide from *P. falciparum* sporozoite surface protein 2 (PfSSP2 [RRHNW VNHA]) (28).

The studies reported herein were conducted in accordance with U.S. Navy regulations governing the protection of human subjects in medical research. The research protocols employing

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TABLE 1. $CD8^+$ CTL epitopes on *P. falciparum* preerythrocytic-stage proteins recognized by T cells from volunteers immunized with radiation-attenuated *P. falciparum* sporozoites

Peptide no.	Protein	HLA	Residues	Sequence	Reference
1	CSP		368–390	KPKDELDYENDIEKKICKMEKCS	16
2	SSP2[a]	A2	1–15	MNHLGNVKYLVIVFL	28
3	SSP2	A2	46–60	EVDLYLLMDCSGSIR	28
4	SSP2	A2	121–135	LLSTNLPYGKTNLTD	28
5	SSP2	A2	126–140	LPYGKTNLTDALLQV	28
6	SSP2	A2	131–145	TNLTDALLQVRKHLN	28
7	SSP2	A2	136–150	ALLQVRKHLNDRINR	28
8	SSP2	A2	221–235	ENVKNVIGPFMKAVC	28
9	SSP2	A2	281–295	CEEERCLPKREPLDV	28
10	SSP2	A2	286–300	CLPKREPLDVPDEPE	28
11	SSP2	A2	521–535	ALLACAGLAYKFVVP	28
12	SSP2	A2	546–560	APFDETLGEEDKDLD	28
13	SSP2	A2	551–565	TLGEEDKDLDEPEQF	28
14	SSP2	B8	107–115	ASKNKEKAL	29
15	SSP2	B8	109–117	KNKEKALII	29
16	SSP2	A2	14–23	FLIFFDLFLV	4
17	EXP1	A2	80–88	VLAGLLGNV	4
18	CSP	A2	394–402	GLIMVLSFL	4
19	EXP1	A2	2–10	KILSVFFLA	4
20	EXP1	A2	83–91	GLLGNVSTV	4
21	EXP1	A2	91–100	VLLGGVGLVL	4
22	CSP	A2	7–6	ILSVSSFLFV	4
23	LSA1	A3	94–102	QTNFKSLLR	4
24	SSP2	A3	523–531	LACAGLAYK	4
25	CSP	A3	344–353	VTCGNGIQVR	4
26	EXP1	A3	10–18	ALFFIIFNK	4
27	SSP2	A3	522–531	LLACAGLAYK	4
28	LSA1	A3	105–113	GVSENIFLK	4
29	LSA1	A3	59–68	HVLSHNSYEK	4
30	LSA1	A3	11–20	FILVNLLIFH	4
31	PfS16	B7	77–85	MPLETQLAI	4
32	SSP2	B7	539–548	TPYAGEPAPF	4

[a] Some epitopes reported on *P. falciparum* sporozoite surface protein 2 (SSP2) were identified as overlapping epitopes (28).

human subjects in this study were reviewed and approved by the Naval Medical Research Institute's Committee for the Protection of Human Subjects and the Walter Reed Army Institute of Research Human Use Committee.

TABLE 2. Prevalence of HLA-A*01 allele in various populations[a]

Population	Frequency (%)
U.S. Whites	30.94
Australians	36.48
British	27.75
Swedish	34.55
Cornish	31.61
Germans	32.76
Greeks	17.55
Italians	26.04
North American Blacks	10.32
North American Amerinds	9.56
North African Blacks	7.07
South African Blacks	4.74
West African Blacks	10.32
Japanese	1.40
Northern Han	9.18
Southern Han	0.80
Thai-Chinese	5.52
Javanese	3.17
Papua New Guinea highlanders	0

[a] Reproduced from Doolan et al. (6).

Antigen-specific $CD8^+$ CTL against endogenously synthesized PfCSP are induced in HLA-A*01-irradiated sporozoite-immunized volunteers. To determine whether immunization with radiation-attenuated *P. falciparum* sporozoites could generate anti-PfCSP CTL responses in HLA-A*01 individuals (Table 3), and if so, whether such CTL would recognize endogenously synthesized antigen, immune PBMC from irradiated sporozoite-immunized volunteers (4, 16, 17) were stimulated in vitro with autologous PBMC infected with recombinant PfCSP-expressing vaccinia virus. Cytolytic activity was assessed against autologous or HLA-mismatched B-LCL transiently transfected with PfCSP-encoding DNA (VR2510) or control DNA (VR1020). Effector cells from all three volunteers lysed

TABLE 3. HLA phenotypes of volunteers immunized with *P. falciparum* irradiated sporozoites (NF54 or 3D7)

Volunteer no.	HLA type	
	Class I	Class II
4	A1, A28, B44, Bw57, Cw6	DR7, DRw11, DQw2, DQw7, DRw52, DRw53
16	A1, A24, B8, B38, Bw4	DR11, DR5, DQw2, DRw52, DRw53
17	A1, A3, B7, B8, Bw6	DR17, DR3, DQw2, DQw3, DRw52, DRw53

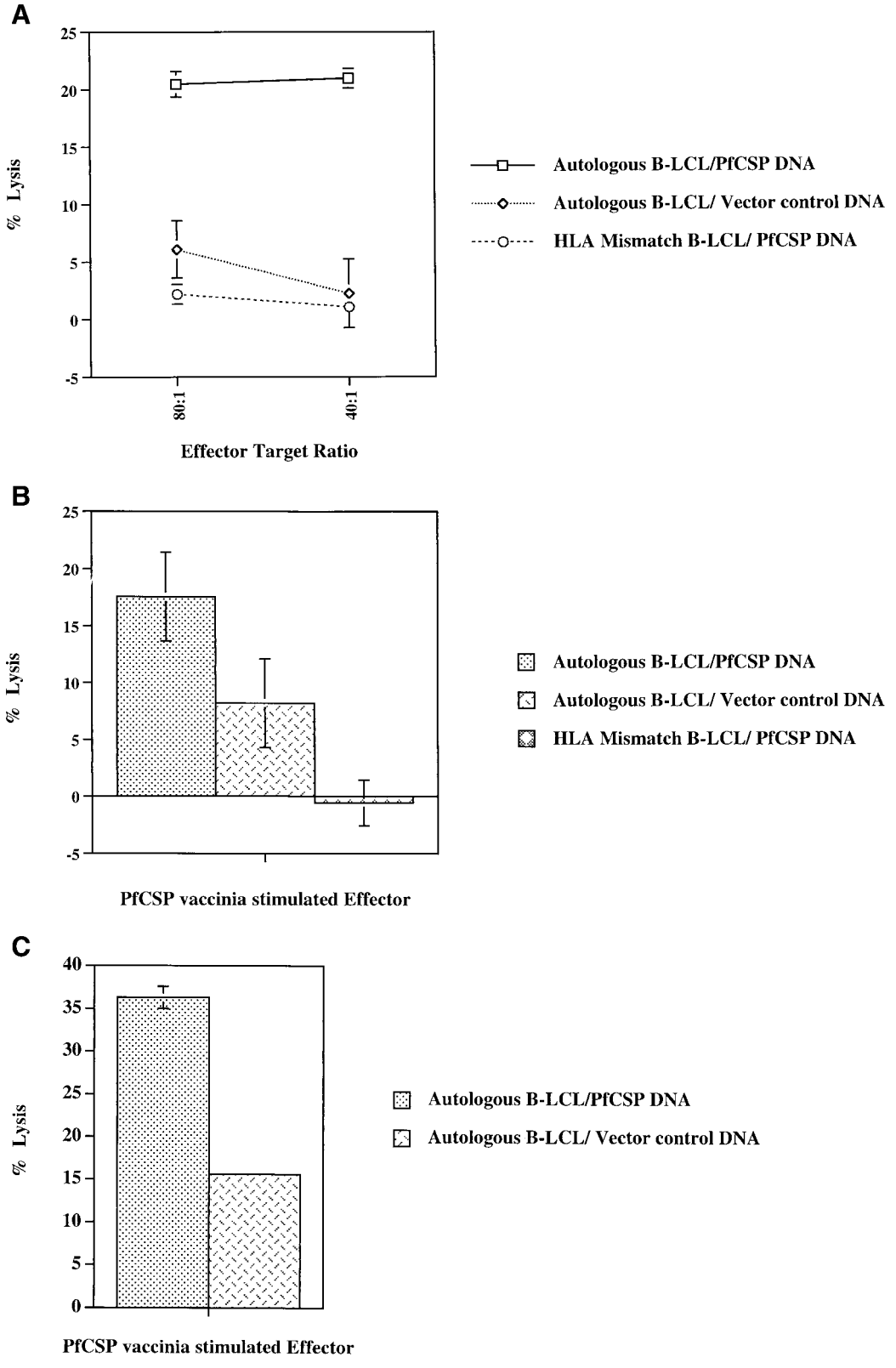

FIG. 1. CTL lysis of target cells expressing endogenously synthesized PfCSP. Immune PBMC from volunteer 16 (A), volunteer 4 (B), and volunteer 17 (C) were stimulated with autologous PBMC infected with recombinant vaccinia virus expressing the entire gene of PfCSP 3D7 (PfCSP vaccinia). Cytotoxicity was assessed in a CTL assay against autologous and HLA-mismatched B-LCL transiently transfected with PfCSP-encoding plasmid DNA (2510) or vector control DNA (1020) at an 80:1 effector/target ratio.

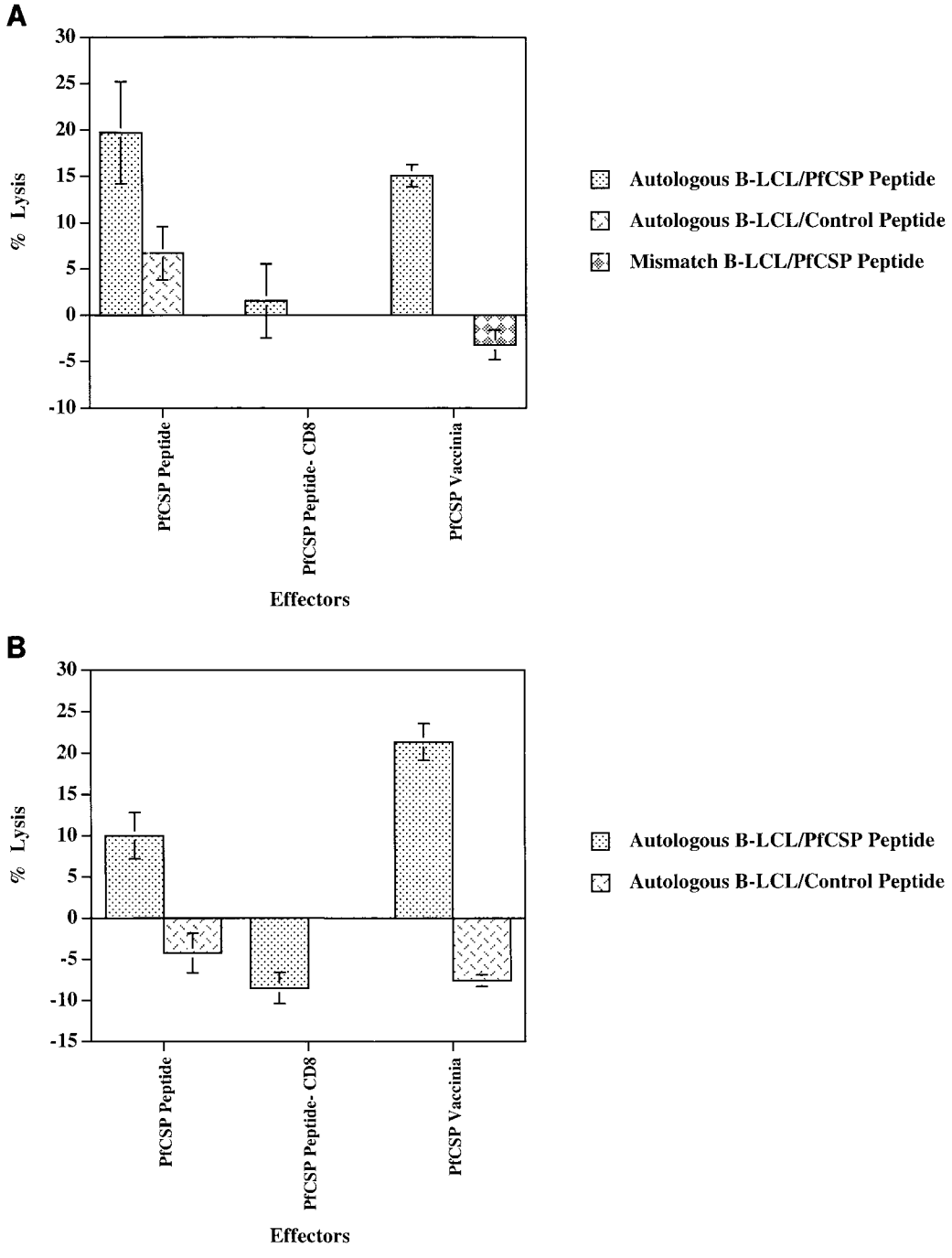

FIG. 2. Antigen-specific, MHC-restricted, $CD8^+$ T-cell-dependent CTL activity in HLA-A*01 volunteers. Immune PBMC from volunteer 4 (A) and volunteer 17 (B) were stimulated with peptide PfCSP 3D7 310–319 (EPSDKHIKEY) (PfCSP peptide) or PfCSP-expressing vaccinia virus (PfCSP vaccine). Cytotoxicity was assessed in a CTL assay at an 80:1 effector/target ratio. Autologous or HLA-mismatched B-LCL were pulsed with peptide PfCSP 3D7 310–319 or a control peptide from the PfSSP2 sequence. Where indicated, cells were depleted of $CD8^+$ T cells using Dynabeads (PfCSP peptide-CD8).

autologous B-LCL transiently transfected with PfCSP-encoding DNA (Fig. 1) but not autologous B-LCL transfected with control DNA or HLA-mismatched B-LCL targets transfected with PfCSP-encoding DNA. These data demonstrated that an antigen-specific, HLA-restricted CTL response against endogenously presented PfCSP was induced in all three HLA-A*01-positive irradiated sporozoite-immunized volunteers.

Identification of an HLA-A*01-restricted epitope on PfCSP 3D7. We next determined whether these effector cells could recognize either of two predicted HLA-A*01 epitopes from PfCSP (aa 31 to 40 [NTRVLNELNY] and aa 310 to 319 [EPSDKHIKEY]). Two peptides containing these epitopes were synthesized, and their HLA-A*01 binding capacity was estimated to be 2,604 and 147 nM, respectively, as described

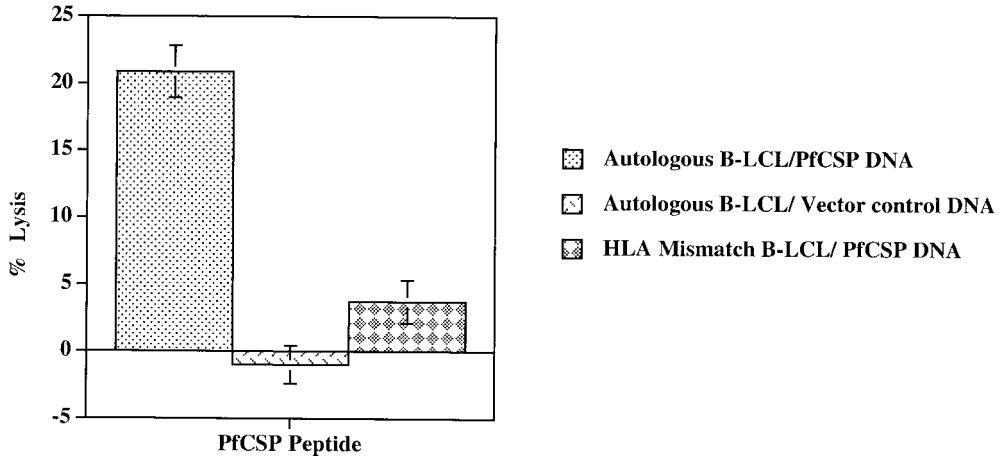

FIG. 3. Antigen-specific, MHC-restricted CTL response in volunteer 16 (HLA-A*01). Immune PBMC from volunteer 16 were stimulated with peptide PfCSP 3D7 310-319. Cytolytic activity was then assessed in a CTL assay at an 80:1 effector/target ratio using autologous and HLA-mismatched B-LCL transiently transfected targets with a plasmid expressing PfCSP-encoding DNA (2510) or vector control DNA (1020) as a negative control.

previously (14, 23). Only one peptide, PfCSP 310-319, bound with an IC_{50} of less than 500 nM. Based on our previous work we consider a peptide that binds with an affinity between 50 and 500 nM to be a high binder (23), and studies have established a correlation between high binding affinity and immunogenicity (23). Accordingly, PBMC from the three HLA-A*01 volunteers were stimulated with peptide PfCSP 3D7 310-319. In parallel, PBMC cultures were also stimulated with PBMC infected with recombinant PfCSP-expressing vaccinia virus. Autologous B-LCL were pulsed with either the PfCSP 3D7 310-319 peptide or with a control peptide or were transiently transfected with PfCSP-encoding DNA (VR2510) or control DNA (VR1020). The CTL response elicited by peptide PfCSP 3D7 310-319 was peptide specific, since effectors stimulated with peptide PfCSP 3D7 310-319 lysed targets pulsed with peptide PfCSP 3D7 310-319 but only minimally lysed or failed to lyse targets pulsed with the control peptide (Fig. 2). Furthermore, the CTL response was genetically restricted, since no significant CTL activity was detected when HLA-mismatched targets were pulsed with peptide PfCSP 3D7 310-319 (Fig. 2A and 3). The CTL response was dependent on $CD8^+$ T cells, since depletion of $CD8^+$ T cells from the effector cells eliminated the CTL activity (Fig. 2).

Recognition of endogenously synthesized PfCSP by peptide PfCSP 3D7 310-319 stimulated immune effectors. Next, we wanted to assess whether effector cells induced by the PfCSP 3D7 310-319 peptide could recognize target cells that endogenously synthesized the PfCSP antigen. Accordingly, PBMC from volunteer 16 were stimulated with the PfCSP 3D7 310-319 peptide, and autologous B-LCL and HLA-mismatched B-LCL were transiently transfected with PfCSPs-encoding DNA (VR2510) or control DNA (VR1020). Data presented in Fig. 3 establish that peptide-stimulated effectors could recognize endogenously processed PfCSP, since they lysed autologous B-LCL targets transfected with the PfCSP gene but not autologous B-LCL transfected with control vector VR1020. Furthermore, this CTL response was genetically restricted, since CTL activity could not be detected using HLA-mismatched B-LCL transfected with the PfCSP gene (Fig. 3).

In summary, our data demonstrate for the first time that HLA-A*01 volunteers immunized with *P. falciparum* irradiated sporozoites generate a specific $CD8^+$ CTL response which recognizes a 10-amino-acid peptide, PfCSP 3D7 310-319 (EPSDKHIKEY). The peptide contains an HLA-A*01 motif and binds to the purified HLA-A*01 molecule with high affinity. This is the first HLA-A*01-restricted CTL epitope identified on PfCSP or on any malaria protein.

It has been reported that $CD4^+$T cells from malaria-exposed individuals may respond to stimulation with synthetic peptides but not to native parasite (18), suggesting that endogenously produced protein may be processed differently than synthetic peptides. Our data demonstrate that the PfCSP 3D7 310-319 epitope is recognized by irradiated sporozoite-immunized volunteers following stimulation with endogenously synthesized PfCSP as a result of infection with recombinant vaccinia or with PfCSP presented on the surface of the target cells by transient transfection. Thus, our data indicate that the PfCSP 3D7 310-319 epitope is generated by natural processing of the PfCSP antigen. Recently, it has also been shown that $CD8^+$ T cells from volunteers immunized with a plasmid DNA vaccine expressing PfCSP (26) recognize this epitope.

Given the high prevalence of HLA-A*01 and related alleles (HLA-A1 supertype) in the Caucasian population (26 to 36.5%; Table 2) and in other ethnic groups worldwide (22) and the fact that this epitope is the only reported HLA-A*01-restricted epitope identified on PfCSP or on any malaria protein, we believe that this epitope should be included in all PfCSP-containing vaccines. The fact that the HLA-A*01 allele is found in low prevalence in Africa (4 to 10%) does not undermine the importance of this epitope from a vaccine perspective. Identification and incorporation of such epitopes in a multiepitope-based vaccine are necessary to protect tens of thousands of individuals bearing this particular HLA type who annually visit areas where malaria is endemic. Furthermore,

the peptide containing this epitope will be important for the routine evaluation of the efficacy of experimental PfCSP malaria vaccines in immunized volunteers.

We thank the volunteers who participated in this study and Denise Doolan for critically reviewing the manuscript.

This work was funded by NMRC work unit numbers 61102A.00101-BFX.1431 and 6287A00101EFX.1432 and in part by a grant from the Belgian Walloon Region to SmithKline Beecham Biologicals.

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Editor: J. M. Mansfield