Vaccine 41 (2023) 1265–1273

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Immunodominant T cell peptides from four candidate malarial antigens as biomarkers of protective immunity against malaria

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ARTICLE INFO

Article history:
Received 7 June 2022
Received in revised form 5 January 2023
Accepted 7 January 2023
Available online 13 January 2023

Keywords:
FluoroSpot
Malaria
IFN-γ
Granzyme B
T cells
Epitope

ABSTRACT

A malaria vaccine with high efficacy and capable of inducing sterile immunity against malaria within genetically diverse populations is urgently needed to complement ongoing disease control and elimination efforts. Parasite-specific IFN-γ and granzyme B-secreting CD8 + T cells have been identified as key mediators of protection and the rapid identification of malaria antigen targets that elicit these responses will fast-track the development of simpler, cost-effective interventions. This study extends our previous work which used peripheral blood mononuclear cells (PBMCs) from adults with life-long exposure to malaria parasites to identify immunodominant antigen-specific peptide pools composed of overlapping 15mer sequences spanning full length proteins of four malarial antigens. Our current study aimed to identify CD8 + T cell epitopes within these previously identified positive peptide pools. Cryopreserved PBMCs from 109 HLA-typed subjects were stimulated with predicted 9-11mer CD8 + T cell epitopes from *P. falciparum* circumsporozoite protein (CSP), apical membrane antigen 1 (AMA1), thrombospondin related anonymous protein (TRAP) and cell traversal for ookinetes and sporozoites (CelTOS) in FluoroSpot assays. A total of 135 epitopes out of 297 tested peptides from the four antigens were experimentally identified as positive for IFN-γ and/or granzyme B production in 65 of the 109 subjects. Forty-three of 135 epitopes (32 %) were promiscuous for HLA binding, with 31 of these promiscuous epitopes (72 %) being presented by HLA alleles that fall within at least two different HLA supertypes. Furthermore, about 52 % of identified epitopes were conserved when the respective sequences were aligned with those from 16 highly diverse *P. falciparum* parasite strains. In summary, we have identified a number of conserved epitopes, immune responses to which could be effective against multiple *P. falciparum* parasite strains in genetically diverse populations.

1. Introduction

An efficacious and broadly effective malaria vaccine is highly sought after as an additional intervention for malaria control, especially in areas that have high levels of transmission and mortality [1,2]. Developing such a vaccine has, however, been hampered by the malaria parasite's complexity. Currently, the most advanced malaria subunit vaccine, RTS,S, is an adjuvanted formulation of the repeat and C-terminal regions of the *P. falciparum* circumsporozoite protein (CSP) fused with the hepatitis B surface antigen and

Abbreviations: AMA1, Apical membrane antigen 1; CelTOS, cell traversal for ookinetes and sporozoites; CSP, Circumsporozoite protein; HLA, Human leukocyte antigen; IFN-γ, Interferon-gamma; MHC, Major histocompatibility complex; NMRC, Naval Medical Research Center; NMIMR, Noguchi Memorial Institute for Medical Research; PBMC, Peripheral blood mononuclear cell; TCR, T cell receptor; TRAP, thrombospondin related anonymous protein; WHO, World Health Organisation.

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https://doi.org/10.1016/j.vaccine.2023.01.016
0264-410X/

shows modest efficacy of up to 50 % against clinical malaria over one year in children from malaria endemic areas [3,4]. Preliminary data from the pilot rollout of RTS,S/AS01 in Ghana, Kenya and Malawi shows that three doses of the vaccine reduced severe malaria by 30 %, confirming findings from the phase 3 trials of the same vaccine [5]. This vaccine, despite the limited efficacy, has completed pilot testing in three African countries and has been recommended by the WHO for inclusion in childhood immunization programs in regions with moderate to high malaria transmission [5]. A similar protein-based vaccine candidate known as R21, also a fusion of a portion of the CSP antigen with the hepatitis B surface antigen, has completed phase 2b trials and shown to have 77 % efficacy against clinical malaria over a one-year period in 5–17 month old children living in a seasonal transmission region [6]. This is expected to undergo further testing and assessment of efficacy and the durability of protective responses.

The search for new ways of making more efficacious malaria vaccines, however, continues and another class of vaccines that show promise are the attenuated parasite vaccines. Attenuated whole sporozoite vaccines have been shown to provide sterile protection of up to 100 % in malaria-naïve vaccinees under experimental infection conditions for up to a year [7–9]. The protection offered by these experimental models is believed to be mediated mainly by antigen-specific CD8 + T cells that identify and kill malaria-infected hepatocytes through recognition of immunodominant HLA class I-restricted peptides [9–11] from multiple antigens. Identification of the antigenic targets of protection-associated immune responses in these whole organism models is expected to significantly enhance our chances of developing relatively simpler subunit, multi-epitope vaccines with similar efficacy.

We have previously investigated the presence of immunodominant T cell regions within four *P. falciparum* antigens, including CSP, Apical membrane antigen 1 (AMA1), Thrombospondin related anonymous protein (TRAP), and Cell Traversal for Ookinetes and Sporozoites (CelTOS). CSP is the most abundant antigen on the sporozoite surface and is essential for sporozoite invasion of liver cells [12]. Portions of this antigen are the key components of the two most advanced subunit malaria vaccines, RTS,S and R21 [6,13,14]. AMA1 is a highly immunogenic antigen present in the sporozoite, liver and blood stage parasites [15] and hence a good multistage subunit vaccine candidate [10]. It is very well characterized in the blood stage parasite, where it is involved in tight junction formation at the red cell surface, prior to parasite invasion of the red cell [16,17], but plays a protective role through cell mediated immunity against the pre-erythrocytic stages of the parasite [15,18]. TRAP and CelTOS are both expressed in sporozoites, are involved in sporozoite motility and hepatocyte invasion [19–21] and have both been implicated in protective roles against pre-erythrocytic stage immunity [22,23]. Pools of overlapping 15mer peptides that span the entire sequences of these four antigens were assessed for their potential to induce IFN-ɣ and granzyme B responses using FluoroSpot assays with peripheral blood mononuclear cells (PBMCs) from 300 HLA-typed malaria-experienced subjects. From the earlier assays, we identified a total of 127 subjects whose PBMCs recalled IFN-ɣ and/or granzyme B responses [24] upon stimulation with antigen-specific peptide pools. The aim of the present study is therefore to delineate peptides that are responsible for the identified antigen pool-specific IFN-ɣ and/or granzyme B responses and identify minimal CD8 + T cell epitopes predicted to bind to globally prevalent HLA types. We will further identify and select immunodominant peptides that are conserved across different parasite variants for further development. Identified conserved and immunodominant peptides with wide population coverage will be relevant as biomarkers of protective immunity.

2. Methods

2.1. Ethics

This study was conducted in the laboratories of the Naval Medical Research Center (NMRC) in Silver Spring, MD, USA, using PBMCs collected from study subjects in a malaria endemic community in Southern Ghana. The samples were collected under an approved human research protocol by the Institutional Review Board at the Noguchi Memorial Institute for Medical Research (NMIMR), University of Ghana. The NMRC study with de-identified samples was conducted under an approved determination of non-human subject research project. Both approvals were subsequently reviewed and approved by the Human Research Protections Office of the United States Army Medical Research and Development Command (USAMRDC). NMIMR (FWAA00001824) and NMRC (FWA00000152) both hold a United States Government Federalwide Assurance from the Office for Human Research Protections. NMRC also holds a Department of Navy Addendum to the FWA for human subject protections (DoDI 3216.02). The protocol was conducted in accordance with the principles in The Belmont Report and federal regulations regarding the protection of human subjects in research including 32 CFR 219 (The Common Rule), and all regulations pertinent to the Department of Defense, the Department of the Navy, the Bureau of Medicine and Surgery of the United States Navy and internal NMRC policies for human subject protections and responsible conduct of research. All NMIMR and NMRC personnel contributing to or performing human research (involving the initial part of the study done at NMIMR) or non-human subject research (done at NMRC) were certified as having completed human research ethics education and training. Written informed consent was sought from all study subjects who willingly agreed to be part of the study and met the inclusion criteria.

2.2. Study area and participants

Study subjects were male and female adults between 18 and 50 years who were part of a larger cohort of 300 study subjects recruited from southern Ghana and whose PBMCs were previously used for FluoroSpot assays with pooled 15mer peptides from the four study antigens. In this previous study, 127 subjects from a total of 252 subjects who met our positivity criteria as previously described made positive responses to one or more peptide pools [24]. Samples from 109 subjects who expressed at least one of the five HLA supertypes of interest (A01, A02, A03, B07, and B44) and met the inclusion criteria were used in the current study. Study subjects HLA allele types were subsequently used to predict the binding capacities for peptides that were within antigen pools that they tested positive against in the previous study [24], and this was basis for selecting and synthesizing the peptides tested in the current study. As done for the earlier study, eligibility criteria for re-sampling of the 109 subjects were as follows: males or females who were not pregnant or nursing; normal screening medical history and physical examination; hemoglobin > 10 g/dl and subjects with at least one positive peptide pool response from the previous assays. All participants who were re-sampled for this follow up study generally had a normal medical history at screening and physical examination. PBMCs were processed and stored at NMIMR and later shipped to the NMRC labs in the USA for the performance of FluoroSpot assays to identify positive epitopes.

2.3. Sample collection and processing

Sixty milliliters (60 ml) of venous blood were collected from each subject into heparinized tubes. PBMCs were isolated from

blood by gradient centrifugation using Ficoll-Paque gradient centrifugation. After washing and counting, freshly isolated cells were suspended in fetal bovine serum (FBS) with 10 % dimethyl sulfoxide (DMSO) and transferred to a −80 °C freezer overnight. Cells were subsequently cryopreserved by transfer to liquid nitrogen until required for assays. When required, PBMCs were thawed and rested overnight in a humidified incubator with 5 % CO_2 at 37 °C. PBMCs were afterwards washed, enumerated, and plated at between 300,000 and 400,000 PBMCs per well for *ex vivo* FluoroSpot assays.

2.4. Synthetic peptides

We previously identified positive 15mer peptide pools of the study antigens, which for each antigen consisted of a pool of overlapping 15mer peptides spanning specific portions of an antigen [24]. A computerized algorithm, NetMHC, was then used to predict class 1 restricted 9–11 epitopes within individual 15mer peptide components of the previously identified positive 15mer peptide pools. Commercially synthesized 9-11mer peptides from the four study antigens, predicted based on subjects' HLA types and present in previously positive peptide pools were used for FluoroSpot assays. Peptides were synthesized (Mimotopes, VIC, Australia) to a purity of > 90 % and transported in lyophilized form. In all, a total of 297 9-11mer peptides (50 CSP, 108 AMA1, 59 TRAP and 80 CelTOS) were predicted and synthesized.

2.5. Ex vivo FluoroSpot assays

Cells that secreted IFN-γ, granzyme B, or a combination of the two in response to 9-11mer peptide stimulation were enumerated using dual-color FluoroSpot assays with pre-coated plates (FSP-0110–10, Mabtech) according to the manufacturer's instructions. Briefly, subject PBMCs were retrieved from liquid nitrogen storage, thawed, washed, and rested overnight in a humidified 37 °C incubator with 5 % CO_2 feed. PBMCs were afterwards washed, enumerated, and brought to the desired cell concentration for the assay. Pre-coated PVDF microplates were removed from their sealed packages and washed four times with 200 µl/well of sterile phosphate buffered saline (PBS) solution. Plates were blocked by incubation with 200 µl/well of cold 10 % Human AB serum in Penicillin streptomycin, L-Glutamine, MEM NEAA and RPMI for 1 h at room temperature after which, 100 µl of subject-specific pre-selected 9-11mer peptides (final well concentration of 10 µg/ml) from the *P. falciparum* 3D7 clone CSP, AMA1, TRAP, and CelTOS antigens based on previous peptide pool positivity and bioinformatic predictions were added in duplicates to wells. Leucoagglutinin PHA-L (Sigma-Aldrich, St. Louis, MO) at 0.625 µg/ml was used as a positive control to stimulate 200,000 cells per well in duplicates on each plate. For each subject, 100 µl/well PBMC suspension at a concentration of 4 million cells/ml were added to plates and incubated for 40–42 h in a humidified 37 °C 5 % CO_2 incubator. After harvesting and washing five times with 300 µl/well PBS, a diluted mixture of detection antibodies (anti-IFN-γ mAb-BAM, at 1:200 dilution of stock, and anti-granzyme B mAb-biotin at 1:500 dilution) was added at 100 µl/well and the plates incubated for 2 h at room temperature and in the dark. Plates were subsequently washed five times with PBS and incubated with a diluted mixture of fluorophore-conjugated reagents (anti-BAM-490 and SA-550, each at 1:200 dilution of stock) for 1 h at room temperature in the dark. Unbound fluorescent conjugates were removed by washing five times with PBS and the plates incubated with 50 µl of fluorescence enhancer solution for 15 min in the dark. After this final incubation, the enhancer solution was emptied by flicking the plate and then tapping against clean paper towel. Underdrain was removed and the plate allowed to dry overnight in the dark. The spots in each well were then visualized and counted using the automated FluoroSpot reader (AID iSpot, Autoimmun Diagnostika GmbH, Strassberg, Germany).

2.6. Data analyses

FluoroSpot assays were used to enumerate the numbers of IFN-γ and granzyme B secreting cells in PBMCs and assays were qualified as passed based on positive IFN-γ and/or granzyme B responses in wells that had PBMCs stimulated with PHA (evidence of cell viability). IFN-γ and granzyme B secretion activities were calculated as spot forming cells per million PBMCs (sfc/m) as detected using the different fluorophores for the two analytes. For either analyte, peptide stimulations were considered positive if there was (i) at least a doubling of sfc/m in test wells relative to medium control wells, and (ii) a difference of at least 10 spots between test and control wells. This definition has been adapted for use in our previous studies [25,26]. For the current study, peptides that met these criteria were classified as peptides with "high response", peptides that made spots but not enough to meet these criteria were classified as peptides with "low response" and peptides that yielded no spots were classified as "no response". Spot forming cells per million (sfc/m) data for both IFN-γ and granzyme B were compared either by Mann-Whitney tests (between two groups) or by Kruskal-Wallis test, followed by Bonferroni post-hoc tests where appropriate (amongst multiple groups). All graphics and tables were created in Microsoft Excel (Microsoft Office 2016) while statistical analyses were performed using the R statistical software, version 4.1.2 (R Development Core Team).

3. Results

A total of 297 chemically synthesized 9-11mer peptides were used to stimulate cryopreserved PBMC samples from 109 matched subjects in FluoroSpot assays. These included 50 peptides from the CSP antigen, 108 peptides from AMA1, 59 peptides from TRAP and 80 from CelTOS. Peptides were selected based on MHC binding predictions [27] of the likely minimal epitopes contained in overlapping 15mer peptide pools identified in the first round of FluoroSpot assays that has been reported previously. One hundred and thirty-five (135) 9-11mer epitopes from all four study antigens, restricted by one or more of the five HLA supertypes of interest (A01, A02, A03, B07, and B44), elicited positive immune responses and have been experimentally identified as being immunodominant. Thirty-four (34) of the 135 epitopes identified in this study population have been previously reported by us and others (Table S1) while the other 101 epitopes are, to the best of our knowledge, could represent novel and are undergoing additional characterization. Sixty-four (64) of the 135 peptides were 9mers, 62 were 10mers and nine were 11mers. A total of 60 peptides elicited IFN-γ responses against PBMCs from different subjects and 130 peptides made granzyme B responses with 55 peptides eliciting both IFN-γ and granzyme B responses. Of the 55 peptides that elicited both IFN-γ and granzyme B responses, 11 peptides elicited dual IFN-γ/granzyme B responses in the same cells (those of the previously reported epitopes are presented in Table S1).

In all, PBMCs from 65 of the 109 subjects (60 %) made positive responses upon stimulation with 9-11mer peptides; 34 of the 65 subjects (52 %) made positive IFN-γ responses, 60 subjects (92 %) made granzyme B responses and 11 of the 65 subjects (17 %) made dual IFN-γ/granzyme B responses.

3.1. Antigen-specific peptide responses

CSP: A total of 34 HLA class1-restricted CSP epitopes (68 % of predicted, synthesized and tested CSP peptides) belonging to one

or more of the HLA supertypes of interest (A01, A02, A03, B07 and B44) were identified (Fig. 1A) in assays with PBMCs from 44 subjects. Twenty (20) of the 34 epitopes identified in this study population have been previously reported while the other 14 could represent novel epitopes and undergoing further characterization. Amongst the five HLA supertypes, those with the highest frequencies were the A02 (29 %) and B07 (25 %) supertypes (Fig. 1B). The CSP-specific 15mer peptide pools that contained the highest number of 9-11mer epitopes were the Cp1 and Cp9 pools, representing the N- and C-terminal regions of CSP respectively (Fig. 1).

AMA1: 57 HLA class 1-restricted AMA1 epitopes (53 % of predicted, synthesized and tested peptides) belonging to one or more of five HLA supertypes of interest were identified (Fig. 2A) in assays with PBMCs from 28 subjects. Seven of the 57 epitopes identified in this study population have been previously reported while the other 50 could represent novel epitopes and undergoing further characterization. The HLA supertypes with the highest frequencies were B07 (35 %) and A03 (25 %) (Fig. 2B). The AMA1-specific 15mer peptide pools that contained the highest number of 9-11mer epitopes were the Ap3 and Ap4 pools (*N*-term), but some were also found in the C-term regions of AMA1 (Fig. 2).

TRAP: A total of 25 HLA class 1-restricted TRAP epitopes (42 % of predicted, synthesized and tested peptides) belonging to one or more of the five HLA supertypes of interest were identified (Fig. 3A) in assays with PBMCs from 11 subjects. Seven of the 25 epitopes identified in this study population have been previously reported while the other 18 could represent novel epitopes and undergoing further characterization. The HLA supertypes with the highest frequencies were A02 (41 %) and B07 (29 %) (Fig. 3B). Although epitopes were found across the antigen's sequence, there was a

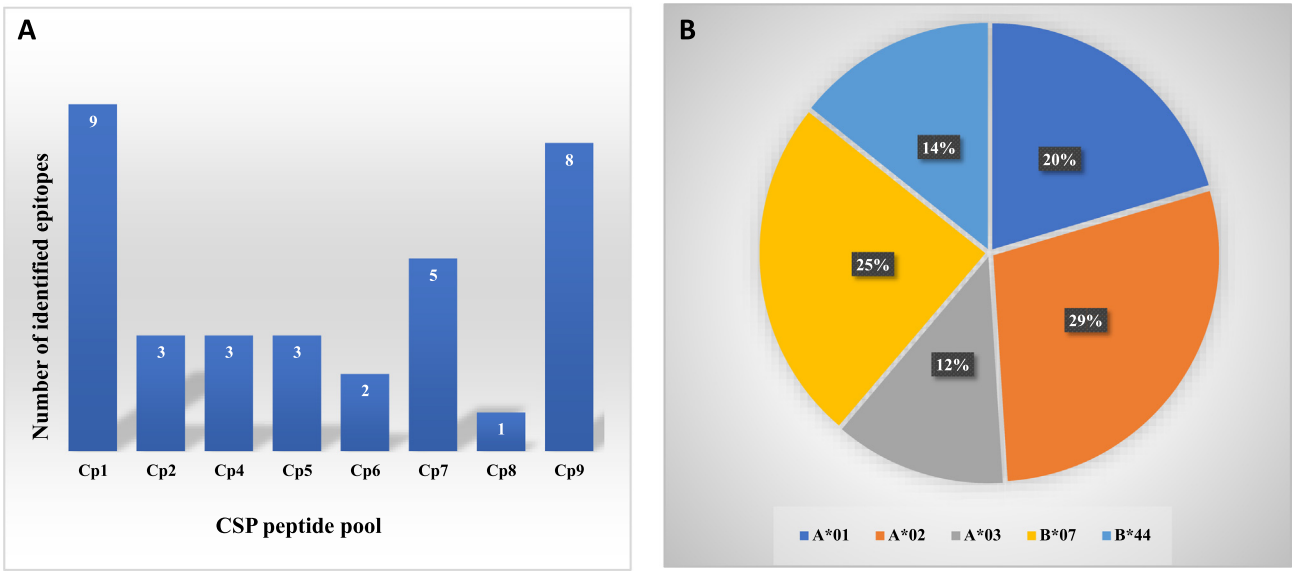

Fig. 1. Distribution of A) experimentally identified CSP 9-11mer epitopes within 15mer peptide pools and B) matching HLA supertypes. Data presented are only for peptides that were recognized by the five HLA supertypes of interest in this study (A01, A02, A03, B07 and B44).

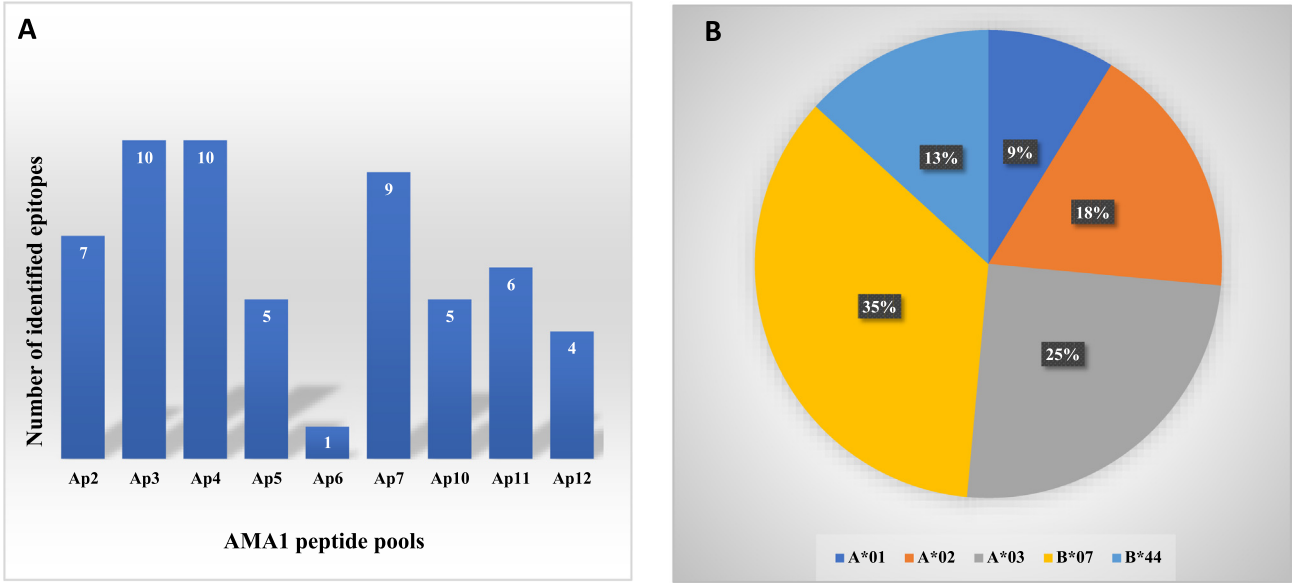

Fig. 2. Distribution of A) experimentally identified AMA1 9-11mer epitopes within 15mer peptide pools and B) matching HLA supertypes. Data presented are only for peptides that were recognized by the five HLA supertypes of interest in this study (A01, A02, A03, B07 and B44).

tendency of concentration of these epitopes at the *N*-terminal end, with the TD3, TD4 and TD5 peptide pools not having any epitopes (Fig. 3).

CelTOS: A total of 19 HLA class 1-restricted epitopes (24 % of predicted, synthesized and tested peptides) belonging to one or more of five HLA supertypes of interest were identified (Fig. 4A) in assays with PBMCs from 11 subjects. All 19 epitopes identified in this study population, to the best of our knowledge, could represent novel epitopes and undergoing further characterization. The HLA supertypes with the highest frequency were, as seen with CSP, AMA1 and TRAP, were B07 (38 %) and A02 (29 %) (Fig. 4B). The highest number of 9-11mer epitopes was identified in the CelTOS-specific 15mer peptide pool Cel3 pool (Fig. 4).

For all peptides, background responses to unstimulated cells were subtracted from the corresponding stimulated cells for the same subject prior to analyses. Peptide-specific responses per subject were averaged and expressed as spot forming cells per million (sfc/m) PBMCs. Overall, specific epitopes induced IFN-γ alone, granzyme B alone, or IFN-γ + granzyme B. The magnitude of both IFN-γ and granzyme B positive responses for those epitopes identified in this study population which have also been previously reported in various study populations are presented in Fig. 5. For all epitopes identified within all four antigens in our study, the magnitudes of IFN-γ and granzyme B positive responses were not statistically different, with the exception of CelTOS, where IFN-γ responses were statistically significantly higher (84 sfc/m)

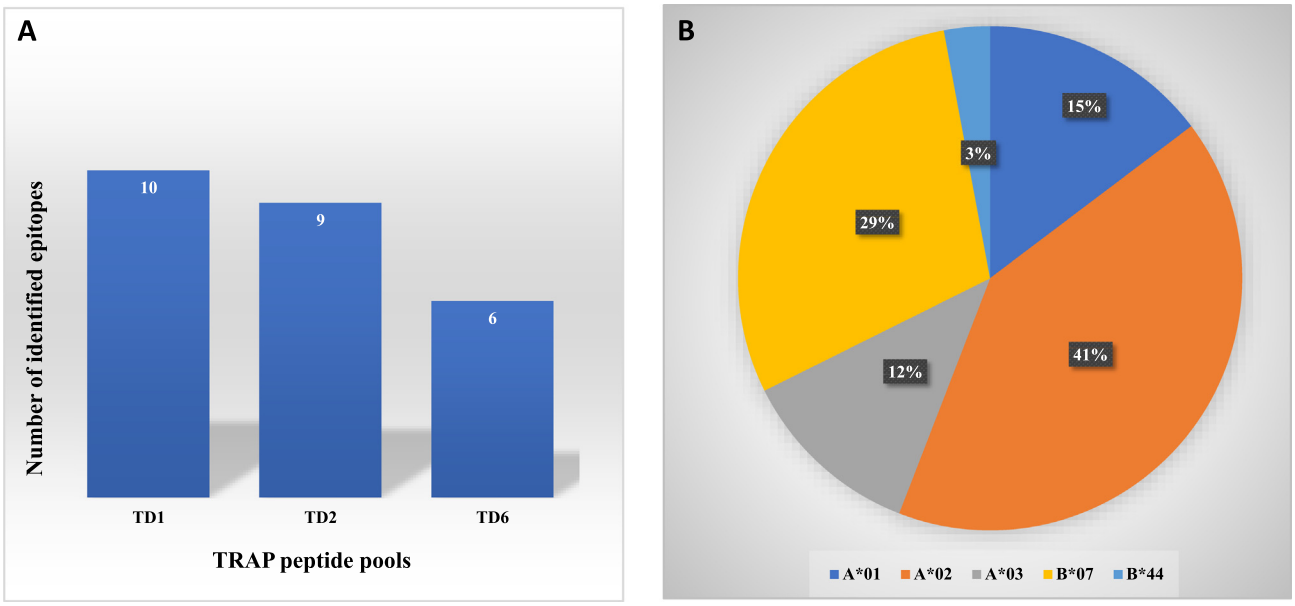

Fig. 3. Distribution of A) experimentally identified TRAP 9-11mer epitopes within 15mer peptide pools and B) matching HLA supertypes. Data shown are for TRAP epitopes recognized by the five HLA supertypes of interest in this study.

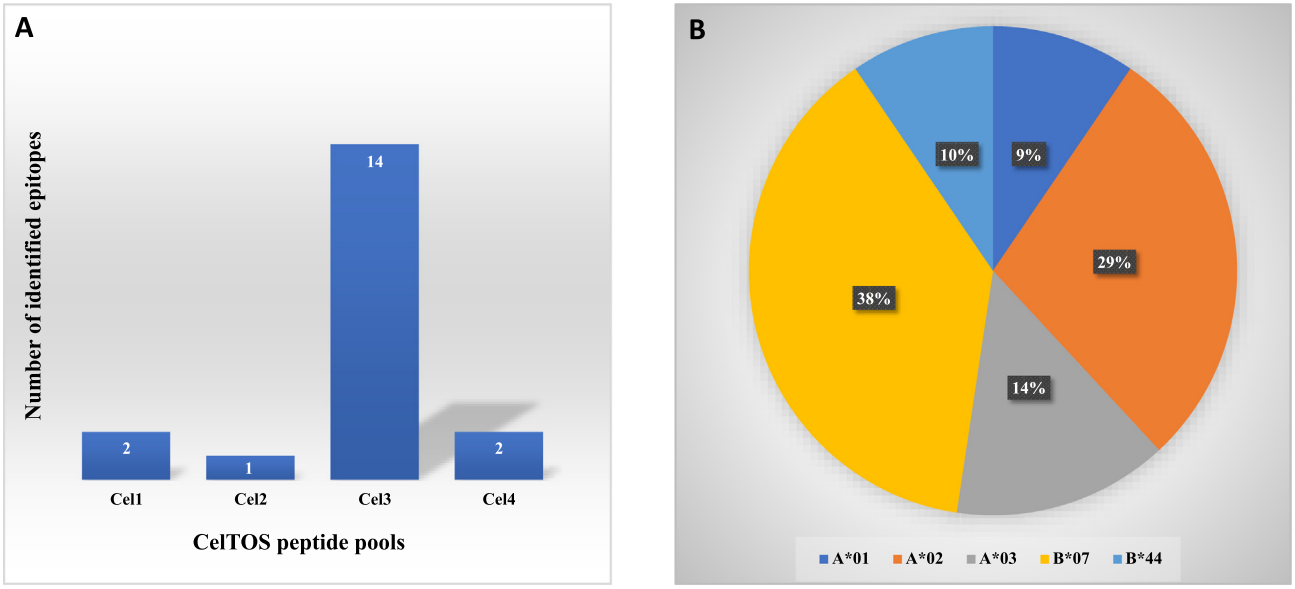

Fig. 4. Distribution of A) experimentally identified CelTOS 9-11mer epitopes within 15mer peptide pools and B) matching HLA supertypes. Data shown are for CelTOS epitopes recognized by the five HLA supertypes of interest in this study.

compared to granzyme B responses (53 sfc/m, p = 0.002, Mann-Whitney *U* test). Further analyses also showed no differences in both IFN-γ and granzyme B responses across the five HLA supertypes of interest. Thus, the propensity for a particular peptide to be immunodominant was neither dependent on the antigen (with the exception of CelTOS) source of the peptide nor the HLA type that was presenting the peptide. Response magnitudes were also not dependent on the peptide length as there were no differences in response magnitude amongst 9mers, 10mers and 11mers, irrespective of the antigen (data not shown).

3.2. Some identified epitopes show promiscuity in HLA binding

A total of 43 peptides out of the total 135 epitopes identified (32 %) were shown to be presented by more than one HLA supertype, and 16 of the 43 identified in this study population which have also been previously reported in various study populations (Table 1) while the remaining 26 are undergoing further characterization. Among the 43 promiscuous epitopes identified to be presented by our five HLA supertypes of interest (A01, A02, A03, B07, and B44), 22 (51 %) were also found to be presented by other HLA supertypes including HLA A24, B27, B58, B62, A01A03 and unclassified HLA types. Overall, 31 of the epitopes were presented by HLAs belonging to two different supertypes, eight were shown to be presented by HLAs from three supertypes, two were shown to be presented by HLAs from four supertypes and one epitope each was shown to be presented by HLAs from five and seven supertypes, respectively. Fifteen of the 43 promiscuous epitopes were from the CSP antigen, 17 were from AMA1, nine were from TRAP and two from CelTOS. The most promiscuous epitope was IQNSLSTEW (CSP), presented by HLA types present in four of the five supertypes of interest (A01, A02, B07 and B44) (Figure S1 A), as well as HLAs within supertypes A24, B27 and B58. This epitope yielded positive responses in a total of 18 study subjects.

Comparison of responses between promiscuous and non-promiscuous epitopes did not reveal any statistical differences for both IFN-γ and granzyme B responses.

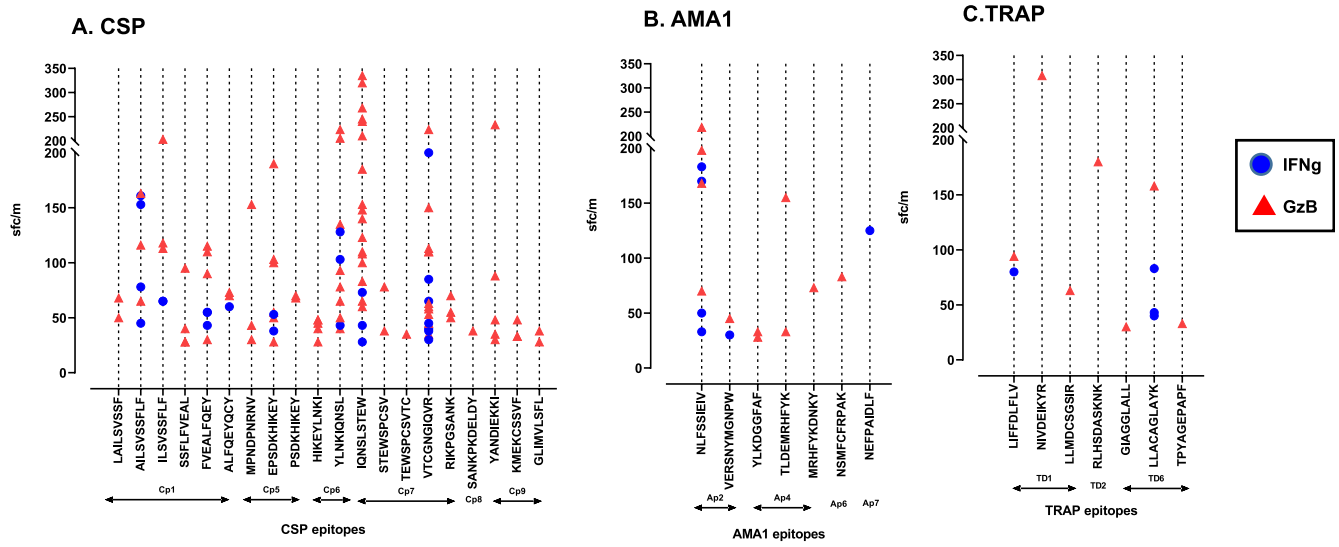

Fig. 5. Magnitude of IFN-g and Granzyme B responses against minimal epitopes of CSP, AMA1, and TRAP. The presented data are for identified epitopes that have also been previously reported by us and others.

Table 1
CSP, AMA1, and TRAP epitopes that show HLA binding promiscuity.

Antigen	Epitope	Presenting HLA supertypes	Number of responding subjects	Previously Published [Ref.]
CSP	ALFQEYQCY	**A01, A03**	3	[28]*
	FVEALFQEY	**A01, B07**, B27, **B44**	6	[28,29]
	HIKEYLNKI	**A02, B07,** B62	5	[26]
	ILSVSSFLF	**A01**, A24	3	[28,30,31]
	IQNSLSTEW	**A01, A02,** A24, **B07,** B27, **B44**, B58	18	[26,32,33]
	KMEKCSSVF	**A01**, A24	3	[34]
	MPNDPNRNV	**A02, B07**, *unclass*	3	[26,28,35]
	SANKPKDELDY	**A01,** B58	1	[36]
	SSFLFVEAL	**A02,** B27	4	[28,29]
	STEWSPCSV	**A01, A02**	2	[32,33]
	VTCGNGIQVR	**A02, A03**	12	[30,31,34]
	YANDIEKKI	**A02, B07**, B62	5	[34]
	YLNKIQNSL	**A02, B07**, B27	9	[26,28,31–33]
AMA1	TLDEMRHFYK	**A03, B44**	2	[28,37]
	YLKDGGFAF	**B07, B44**	2	[28,37]
TRAP	LLACAGLAYK	**A01, A03, B07**	3	[30]

The five HLA supertypes of interest in this study are in bold font. The presented epitopes are those that have also been previously reported. ***Unclass*** refers to an HLA allele that does not as yet have a supertype classification.

* Ref [28] describes predicted epitopes that have not been experimentally tested.

3.3. Over half of the experimentally identified epitopes are conserved

For all identified antigen-specific epitopes, peptide sequences were compared with those of the corresponding antigen sequences from 16 highly diverse laboratory parasite strains (Table S2). Among the 135 positive epitopes, 70 (23 CSP, 28 AMA1, 12 TRAP and 7 CelTOS) were completely conserved within all 16*P. falciparum* strains, 3 epitopes (one each of CSP, AMA1 and CelTOS) were conserved within 15 strains and 8 epitopes (5 AMA1 and one each of CSP, TRAP and CelTOS) were conserved within 14 strains. The remaining epitopes were conserved within varying number of strains while 2 out of the 135 positive epitopes (one each for CSP and TRAP) cannot be considered as conserved since they were found within the Pf3D7 strain only. A summary of the data for the 34 out of the 70 conserved epitopes identified in this study population which have been previously reported in various study populations is presented in Table S2.

4. Discussion

Whole organism approaches to malaria vaccine design have provided significant insight into the T cell mediated mechanisms of sterile protective immunity against malaria. Antigen-specific CD8 + T cells have been directly shown, at least in animal models, to be key in pre-erythrocytic stage malaria immunity [38–40]. Picking out the epitope targets of protective T cells from immunodominant antigens is therefore key for the future design of simpler subunit malaria vaccines. These protection-associated epitopes will also have broad applicability as they can elicit the appropriate immune responses in populations with high HLA diversity. We have previously identified portions of four malarial antigens that contain immunodominant peptides recognized by T cells from persons with repeated natural exposure to diverse malaria parasites [24]. This earlier study tested 15mer overlapping peptide pools from the parasite antigens CSP, AMA1, TRAP and CelTOS and identified 127 out of 252 subjects whose T cells were reactive against different portions of the four antigens. In the current study, we proceed to identify specific immunodominant 9-11mer peptides using PBMCs from 109 of the 127 previously positive subjects. The 9-11mer peptides were selected on the basis of their presence within the previously positive peptide pools and their bioinformatic prediction as epitopes with high binding affinity to subject HLA types.

We report here that PBMCs from 65 of 109 subjects made positive T cell responses and that overall, we have identified 135 epitopes from the four study antigens that elicit either IFN-γ and/or granzyme B responses. Thirty-four of these identified epitopes have also been previously reported by us [26,29,37,41] and others [28,30–36,42–44], using samples from subunit vaccine and/or whole organism clinical trials as well as samples from different malaria endemic areas, while the remaining 101 epitopes are, to the best of our knowledge, could represent novel epitopes revealed in this particular malaria endemic area with specific transmission pattern upon further characterization. Of the four antigens, AMA1 had the highest number of epitopes (57 peptides, representing 53 % of tested peptides, with 50 peptides being novel), followed by CSP (34 peptides, representing 68 %, with 14 peptides being novel), TRAP (25 peptides, representing 42 %, with 18 peptides being novel) and CelTOS (19 peptides, representing 24 % of peptides, all of which are novel) in that order. This outcome therefore confirms our initial report that AMA1 and CSP contained significantly higher numbers of epitopes, especially when compared to CelTOS [24].

Also in agreement with our previous findings [24], the HLAs B07 (31 %), A02 (27 %) and A03 (18 %) supertypes were the most frequent supertypes that matched the 135 identified epitopes. These three HLA supertypes are known to be expressed by a significant proportion of the global population [45]. We also found that 43 of the 135 epitopes (32 %) are promiscuous for HLA binding, with the majority of epitopes being presented by HLA alleles within two different supertypes. A single CSP epitope (IQNSLSTEW) was presented by alleles in up to seven different HLA supertypes, yielding positive responses in up to 18 study subjects. We have previously shown that this CSP epitope has been predicted to be an HLA class I epitope [26]. Finally, up to 52 % of the 135 epitopes are conserved relative to the aligned antigen-specific sequences of at least 16 highly diverse parasite strains. These findings collectively affirm our strategy of being able to elicit protection-associated immune responses against these epitopes in populations with high HLA diversity. A number of the 135 epitopes identified here have already been experimentally described previously by us and others [20,26,29,37,41].

A significantly high proportion of subjects made granzyme B responses compared to those that made IFN-γ responses. The importance of CD8 + T cells that perform cytolytic effector function in the elimination of liver stage parasites is well known [46,47]. The reverse of this observation was however found in the earlier testing of pooled peptides [24]. It is important to note that the previous testing was with pools of 15mer peptides, while the current study has used 9-11mer peptides, sequences of each of which falls within one or two of the earlier 15mer peptides. These differences in peptide length means that only CD8 + T cell-specific responses are expected to be detected in the current study as 9-11mer peptides are the preferred lengths for HLA class I antigen presentation [48,49], and HLA class II molecules do not efficiently bind or present peptides of that length. CD4 + T cells have been shown to generally make higher proportions of IFN-γ responses compared to CD8 + T cells, while the opposite is true for granzyme B [50]. These arguments may therefore explain the observation of a higher proportion of granzyme B responses to the 9-11mer peptides in the current study.

Analyses of positive responses for both IFN-γ and granzyme B showed no effect of factors such as the antigen source, HLA supertype that presented positive peptides and positive peptide length on the magnitude of the response. There was also no evidence that epitope promiscuity for HLA binding affected the magnitudes of immune responses. Ultimately, the magnitude of responses may be more associated with amino acid residue positions and the peptide antigen sequence interaction with both MHC and TCR [51–54].

For some of the antigens, epitopes were mostly concentrated at the N and/or C terminal regions and this has significant implications for selection of epitope with significant protective value. As an example, CSP epitopes were found mostly in the Cp1 and Cp9 peptide pools. The CSP component of the RTS,S vaccine is the C terminal (with both antibody and T cell epitopes) and central repeat region (rich in antibody epitopes) of the antigen [55,56], and our findings suggest that an incorporation of some components of the CSP *N*-terminal into RTS,S may further improve upon the immunogenicity of the vaccine, especially the capacity to elicit potent T cell responses.

In summary, we have identified 135 epitopes that are presented by some of the most globally predominant HLA supertypes in assays with PBMCs from 65 persons with repeated natural exposure to malaria. Over 50 % of these epitopes are conserved, while another 32 % of identified epitopes show HLA binding promiscuity. We therefore demonstrate an experimental strategy for identifying

conserved immunodominant peptides that elicit T responses in human populations with high HLA type diversity.

Funding

This work was funded by the Congressionally Directed Medical Research Program (CDMRP; CDMRPL-19-0-PR180876 and W81XH-19-1-0020).

CRediT authorship contribution statement

Martha Sedegah: Conceptualization, data curation, funding acquisition, investigation, methodology, project administration, supervision, writing-original draft, writing-review and editing

Data availability

Data will be made available on request.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Kwadwo Asamoah Kusi reports financial support was provided by United States Department of Defense. Martha Sedegah reports financial support was provided by United States Department of Defense.

Acknowledgements

We are grateful to the study participants from the study Community for agreeing to be part of this study. Funding for this work was from the Congressionally Directed Medical Research Program (CDMRP; CDMRPL-19-0-PR180876 and W81XH-19-1-0020)

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2023.01.016.

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