A plasmodium falciparum sporozoite's journey: through organs and across CD8+ T-cell challenges

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Chapter 3

Human *Plasmodium falciparum* sporozoite effector-memory CD8$^+$ T cells exhibit circumsporozoite protein-related antigen precursor epitope-specific activation and display distinct T cell receptor clustering

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Abstract

Malaria, caused by *Plasmodium falciparum* (*Pf*), remains a leading cause of morbidity and mortality globally. Despite manyfold evidence for the promising role of $CD8^+$ T cells in protective immunity, the specific mechanisms by which these cells target particularly malaria-infected liver cells in humans remain unclear. In this study, we investigate the activation and effector functions of human *Pf* sporozoite (SPZ)-specific memory $CD8^+$ T cells. Using an *in vitro* antigen-presenting cell (APC) co-culture model, we demonstrate that repeated stimulation with *Pf* SPZ induces the differentiation of highly activated effector-memory (Tem) $CD8^+$ T cells expressing CD137, IFNγ, and Perforin. T cell receptor (TCR) sequencing of the activated $CD137^+$ *Pf* SPZ memory T cell population reveals distinct TCR clusters, suggesting clonal expansion of several *Pf* epitope-specific T-cells. Additionally, when stimulated with several well-known liver-stage specific epitopes, including circumsporozoite protein (CSP), thrombospondin-related anonymous protein (TRAP), and liver-stage antigen-1 (LSA-1), only circumsporozoite protein-related antigen precursor (CRA) epitope GLLGNVSTV elicits significant activation of *Pf* SPZ memory $CD8^+$ T cells. This epitope induces upregulation of key activation markers (CD137, IFNγ) and cytotoxic molecules (Perforin, Granzyme A and Granzyme B), evidencing strong recognition by the *Pf* SPZ memory T cells. This study advances our understanding of human *Pf* SPZ memory $CD8^+$ T cell responses and provides a novel approach for dissecting antigen specificity and memory development, which could inform the design of more effective malaria vaccines.

Introduction

Malaria causes around 600,000 deaths yearly (WHO 2023) [1]. Severe and fatal malaria is primarily caused by *Plasmodium falciparum* (*Pf*) [2]. After being transmitted by infected *Anopheles* mosquitoes, *Pf* sporozoites (the infective form, SPZ) quickly migrate to the liver, where they reside within liver cells for around 7 days [3]. This process is remarkably efficient, where malaria-naïve individuals can develop patent infections after only five infectious mosquito bites [4]. The relatively lengthy developmental period in the liver makes this stage a prime opportunity for immune intervention [5]. We demonstrated that vaccines based on late-liver-stage-arresting SPZ induce significantly better protection against *Pf* compared to those that arrest early [6]. These findings were the first in human experimental medicine to emphasize the necessity of antigen exposure during liver-stage development for robust immunity. However, although CD8+ T cells likely contribute to this protection [7], the specific antigens they recognize and the mechanisms by which they eliminate malaria-infected hepatocytes in humans remain largely undefined.

In both rodent and non-human primate models, CD8+ T cells have been shown to play a crucial role in protection, where depleting these cells abolishes immunity [8]. In mice, CD8+ T cells specific for *Pf* circumsporozoite protein (CSP) have revealed that CD8+ T cells cluster around infected hepatocytes in an antigen-specific manner, supporting a cytotoxic mechanism of action [9]. Additionally, tissue-resident memory CD8+ T cells (Trm), which permanently reside in the liver, are crucial role in achieving sterilizing immunity after immunization with radiation-attenuated sporozoites (RAS) in mice [10, 11, 12]. Nevertheless, it remains unclear how effective human hepatocytes present liver-stage malaria antigens via MHC class I, which antigens they present and whether this presentation is sufficient for recognition by memory CD8+ T cells.

Once *Pf* SPZ infect hepatocytes, they develop within a parasitophorous vacuole membrane (PVM), which serves as a barrier between the parasite and the host cytoplasm [13]. The PVM is selectively permeable, allowing nutrients to pass from the host cytoplasm to support the parasite's development. However, for antigen presentation via MHC class I to occur, parasite-derived antigens must be exported from the vacuole into the host cytosol [14]. This is where they can be processed and presented to CD8+ T cells, a process that is still not fully understood. While CSP is the most widely studied pre-erythrocytic antigen and contains well-characterized CD8+ T cell epitopes [15, 16], it represents only a small fraction of the antigens expressed during liver-stage development [17]. To fully assess the role of CD8+ T cells in targeting

liver-stage malaria, it is essential to investigate T cell responses that extend beyond CSP and include recognition of a broader array of pre-erythrocytic expressed antigens. Other pre-erythrocytic proteins, such as thrombospondin-related anonymous protein (TRAP), sporozoite surface protein 2 (SSP2/UIS3), liver-stage antigen-1 (LSA-1), merozoite surface protein-1 (MSP-1), exported protein 1 (EXP1), circumsporozoite protein-related antigen precursor (CRA) and heat shock protein 70 (HSP70) are expressed at various stages during SPZ migration, hepatocyte invasion, and intrahepatic development [18, 19, 20, 21, 22]. Many of these antigen's harbour $CD8^+$ T cell epitopes and have been associated with protective immune responses in both preclinical and clinical studies [23, 24].

In this study, we explore the activation and effector function of polyclonal $CD8^+$ T cells capable of recognizing whole *Pf* SPZ instead of a single epitope. We assess their ability to recognise antigens in an antigen-presenting cell (APC) and $CD8^+$ T cell co-culture model *in vitro*. This model, together with T cell receptor analysis, will help to better understand which epitopes are being recognized by *Pf* SPZ memory $CD8^+$ T cells and can be used to investigate the presentation and recognition of *Pf*-infected hepatocytes.

Methods

Anopheles rearing

Mosquitoes from a colony of *Anopheles stephensi* (line Nijmegen SDA500) were used. Larval stages were reared in water trays at a temperature of 28 ± 1°C and a relative humidity of 80%. Adult females were transferred to incubators with a temperature of 28 ± 0.2°C and a relative humidity of 80%. For all the experiments, 3- to 5-day old mosquitoes were used for feeding.

Parasite culture

Anopheles stephensi mosquitoes were infected with *Plasmodium falciparum* (*Pf)* wildtype NF54 infected blood through membrane feeding as previously described [38]. Salivary glands of infected and uninfected mosquitoes were dissected 14 days post blood-meal in RPMI + 10% fetal calf serum (FCS) and homogenized to extract *Pf* SPZ from the glands [38].

Differentiation from naïve into SPZ memory $CD8^+$ T cells

From a Human Leukocyte Antigens a2 (HLA-A*0201) positive malaria naïve Dutch female, 150,000 peripheral blood mononuclear cells (PBMC) in IMDM + 10% heat-inactivated human serum supplemented penicillin/streptomycin were plated per well in a U-bottom plate (Corning) and incubated at 37°C + 5% CO_2. The following day,

150,000 *Pf* sporozoites were added to each well. After 5 days of incubation with *Pf* SPZ, 50 U/mL human rIL-2 (Miltenyi Biotec) was added to each well. After 14 days incubation, the cells were beads sorted with the human aCD8 beads according to protocol (Miltenyi). 200,000 isolated 1-time stimulated $CD8^+$ T cells were cultured in IMDM + 10% heat-inactivated human serum supplemented penicillin/streptomycin and 50 U/mL human rIL-2 per well.

After 13 days incubation with *Pf* SPZ, PBMCs pooled from four HLA-A*0201 positive malaria naïve Dutch volunteers (Sanquin, Amsterdam) were stimulated 1:1 with *Pf* SPZ and incubated at 37°C + 5% CO_2. After 24 hours, the *Pf* SPZ-stimulated PBMCs were irradiated with 3000 RAD and used as feeder cells. 500,000 feeders were added to the isolated 1-time stimulated $CD8^+$ T cells. The cells were cultured at 37°C + 5% CO_2 for 14 days. After 14 days incubation, the $CD8^+$ T cells with feeders were beads sorted with human aCD137 labelled with PE followed by anti-PE beads according to protocol (Miltenyi). 200,000 isolated 2-time stimulated $CD137^+$ $CD8^+$ T cells were cultured in IMDM + 10% heath-inactivated human serum supplemented penicillin/streptomycin and 50 U/mL human rIL-2 per well.

The day prior to $CD137^+$ isolation, PBMCs pooled from four HLA-A*0201 positive malaria naïve Dutch volunteers (Sanquin, Amsterdam) were stimulated 1:1 with *Pf* SPZ and incubated at 37°C + 5% CO_2. After 24 hours, the *Pf* SPZ-stimulated PBMCs were irradiated with 3000 RAD and used as feeder cells. 500,000 feeders were added to the isolated 2-time stimulated $CD137^+$ $CD8^+$ T cells and cultured in cultured in IMDM + 10% heat-inactivated human serum supplemented penicillin/streptomycin and 50 U/mL human rIL-2 per well for 14 days at 37°C + 5% CO_2. After 14 days, the 3 times stimulated $CD137^+$ $CD8^+$ T cells (SPZ memory $CD8^+$ T cells) were frozen in vials of 5.10^6 cells per vial.

Expansion of the *Pf* SPZ memory $CD8^+$ T cells

Pf SPZ memory $CD8^+$ T cells were cultured as described previously [38, 39]. Briefly, *Pf* SPZ memory $CD8^+$ T cells were thawed and cultured with irradiated HLA-A*0201 positive PBMCs (Sanquin, Amsterdam), 5 ng/ml human rIL-15 (Miltenyi Biotec), 5 ng/ml human rIL-7 (Miltenyi Biotec) and aCD3/28 Dynabeads (Thermo Fisher Scientific) in IMDM + 10% heat-inactivated human serum supplemented penicillin/streptomycin at 37°C + 5% CO_2. After two days, 50 U/ml human rIL-2 (Miltenyi Biotec) was added. When needed the cells were split and 100 U/ml rIL-2 was added. The cells were used 10 days after expansion.

Phenotype determination of *Pf* SPZ memory CD8$^+$ T cells

Pf SPZ memory CD8$^+$ T cells were harvested after expansion and directly stained with markers (table 1-3) for 30 minutes. The cells were measured by flow cytometry using Aurora 5 laser (Cytek Bioscience B.V., Amsterdam) and analyzed by Spectroflow (Cytek Bioscience B.V., Amsterdam), Flowjo version 10.8 (FlowJo LLC, Ashland, OR, USA) and R-studio version 1.4.1717.

Table 1. Markers and fluorochromes used for phenotype determination.

Marker	Fluorochrome	Supplier
CD3	BUV395	BD Bioscience
CD8	BUV805	BD Bioscience
CD56	BV750	BD Bioscience
CD45RA	BUV496	BD Bioscience
CD197	BV785	Biolegend
CD69	BV605	Biolegend
CD27	APC-Fire810	Biolegend
CD28	BV480	BD Bioscience
CD137	PE-Cy5	Biolegend
CD278	PerCP-Cy5.5	Biolegend
CD279	BUV615	BD Bioscience
HLA-DR	BV711	BD Bioscience
CD127	PE-Fire700	Biolegend
CD25	BV421	Biolegend
TCF-1	PE	Biolegend
T-Bet	KB520	Biolegend
Eomes	PE-eFluor610	invitrogen
CD71	APC-Fire710	Biolegend
Live/Dead	Zombie Nir	Biolegend

Table 2. HLA-A*02 staining.

Marker	Fluorochrome	Supplier
CD3	FITC	Biolegend
HLA-A*02	BV450	Biolegend
Live/Dead aqua	amycan	Biolegend

Table 3. Markers and fluorochromes used for phenotype determination.

Marker	Fluorochrome	Supplier
CD56	BV786	Biolegend
CCR7	AF700	Biolegend
CD3	APC-ef780	invitrogen
CD4	PE-Cy7	Biolegend
CD45RA	BV480	invitrogen
CD8	FITC	Biolegend
Live/Dead	Zombie Nir	Biolegend

Antigen presenting cells and *Pf* SPZ memory CD8+ T cell co-culture

Monocytes were isolated from whole blood of HLA-A*02 donors using CD14+ MACS isolation (miltenyi Biotec, Bergisch Gladback, Germany), and differentiated into monocyte-derived macrophages (MoMϕs) using 20 ng/mL macrophage colony-stimulating factor (M-CSF; Biolegend, San Diego, CA, USA) or monocyte-derived dendritic cells (MoDCS) using 20 ng/mL granulocyte-macrophage colony-stimulation factor (GM-CSF; Biosource/invitrogen, Carlsbad, CA, USA) and 0.86 ng/mL human rIL4 (R&D systems, Minneapolis, MN, USA) in RPMI + 10% FCS supplemented with penicillin/streptomycin. On day 6, MoMϕs and MoDCs were harvested, counted and 100,000 cells/well were plated in flat bottom 96 wells plate and rested for overnight at 37°C + 5% CO_2. The following day the MoMϕs and MoDCs were stimulated with 100,000-day 14 post blood meal *Pf* SPZ, equal amounts of salivary gland extract (SGE), 5 µg/mL epitope or RPMI + 10 % FCS supplemented with penicillin/streptomycin, spun down for 3 min at 1200 rpm and incubated at 37°C + 5% CO_2 for 24 hours. After 24 hours the cells were harvested and counted and 10,000 stimulated MoMϕs and 10,000 stimulated MoDCS were co-cultured with 40,000 *Pf* SPZ memory CD8+ T cells (naïve-3 times stimulated) in a U bottom 96 wells plate and incubated o/n at 37°C + 5% CO_2. After 4 hours 3 µg/mL brefeldin A (Thermofisher) was added to the culture. The following day, the cells were harvested and washed with PBS and stained with aqua live/dead (Thermofisher) for 20 min at RT. The cells were washed and fixed with 3.7% paraformaldehyde (PFA; Sigma Aldrich, St Louis, MO, USA) in PBS for 15 min at RT. The

cells were washed twice and stained (table 4) for 30 min at 4°C and measured by flow cytometry using LSR Fortessa (BD bioscience, San Jose, CA, USA) and analyzed by FlowJo version 10.8 (FlowJo LLC, Ashland, OR, USA).

Table 4. Markers and fluorochromes used for APCs co-culture with SPZ memory $CD8^+$ T cells.

Marker	Fluorochrome	Supplier
CD137	PE	Biolegend
CD3	APC-ef780	eBioscience
IFNγ	HV450	Biolegend
Perforin	PERCP-ef710	eBioscience
Granzyme A	PECy7	Biolegend
Granzyme B	APC	Biolegend
Live/Dead	Amcyan	Biolegend

T cell receptor sequencing

After the SPZ or SGE-stimulated APC co-culture with *Pf* SPZ memory $CD8^+$ T cells the cells were live stained according to table 5 and sorted at $CD137^+$ $CD3^+$ live single cells at 4°C. After sorting the RNA was isolated according to the manufacturer's protocol (Qiagen). TCRβ repertoires were amplified using a two-step nested PCR approach with primers targeting conserved V and J gene segments. In the first round, cDNA was amplified using a multiplex primer set targeting all functional TRBV gene segments and a universal reverse primer. A second round of PCR added sequencing adaptors and sample-specific barcodes. PCR products were purified using AMPure XP beads (Beckman Coulter) and quantified using a Qubit fluorometer and TapeStation (Agilent), followed by T cell receptor (TCR) bulk sequencing. The data was compared with cluster analysis between SGE and SPZ stimulated conditions.

Table 5. Markers and fluorochromes used for sorting.

Marker	Fluorochrome	Supplier
CD137	PE	Biolegend
CD3	APC-ef780	eBioscience
Live/Dead	Amcyan	Biolegend

Results

Phenotype of *Pf* SPZ memory CD8$^+$ T cells

After generating *Pf* SPZ memory CD8$^+$ T cells from repeated stimulation of PBMCs from a naïve Dutch HLA-A*0201 positive donor with *Pf* SPZ, we characterized the phenotype of this polyclonal T-cell population (Fig. 1A). We observed that the *Pf* SPZ memory CD8$^+$ T cell pool exist of 93,6% CD8$^+$ T cells (Fig. 1B), of which 95,9% categorized as effector-memory cells (Tem, CD45RA$^-$CCR7$^-$, Fig. 1C). We subsequently characterized Tem cells on expression of CD28 and CD27 and categorized 40.38% as Tem1 (CD45RA$^-$CCR7$^-$ CD28$^+$CD27$^+$) and 28.50% as Tem4 (CD45RA$^-$CCR7$^-$CD28$^+$CD27$^-$, Fig. 1D). We investigated of the total Tem, Tem1 and Tem4 population the expression of multiple markers. Here we observed that the most abundant Tem1 population highly expressed markers related to expansion (CD127), activation (HLA-DR, CD69, CD56) and differentiation (Eomes) compared with the Tem4 population. Moreover, the Tem1 population showed reduced expression of markers related to regulation (CD25, ICOS), immune checkpoint (PD-1) and differentiation (T-Bet, TCF-1) compared to Tem4. Consequently, we conclude that the most abundant Tem1 population is highly activated with limited regulation capacities.

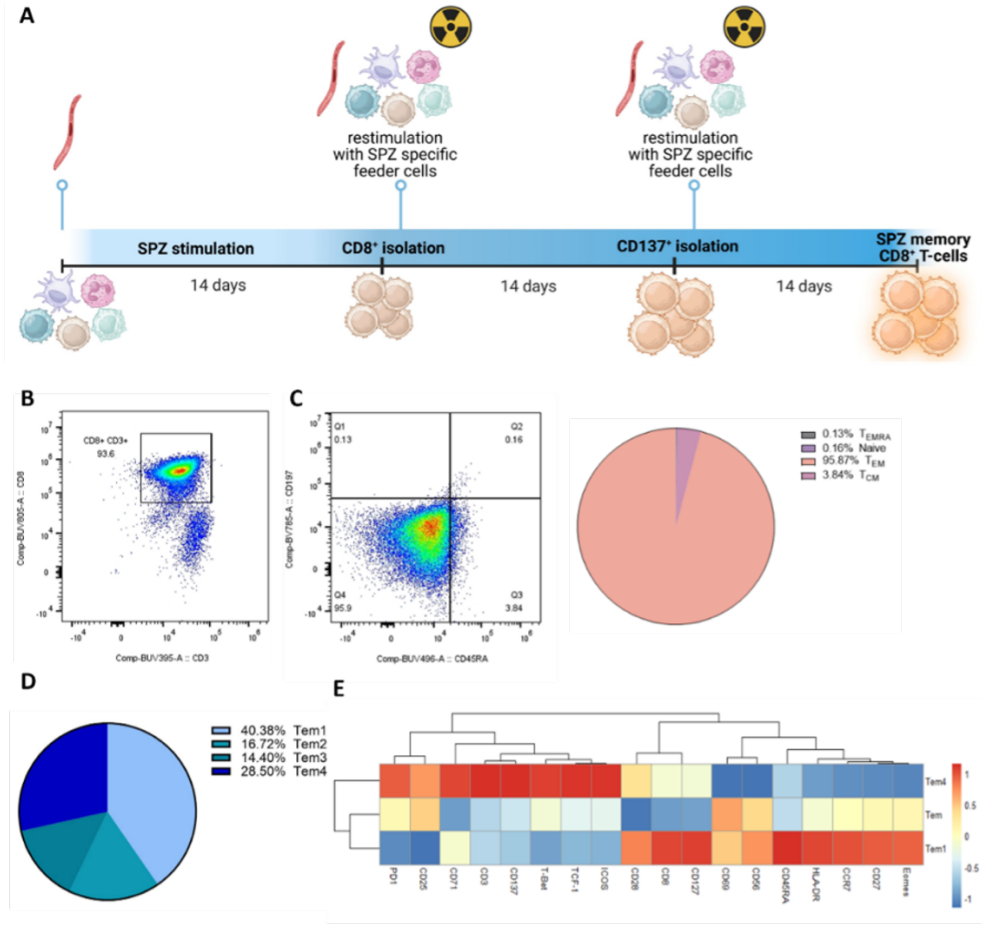

Figure 1. Phenotype SPZ memory CD8⁺ T cells. A. Timeline of the differentiation from naïve CD8⁺ T cells into *Pf* SPZ memory CD8⁺ T cells. **B.** Gating of the *Pf* SPZ memory CD8⁺ T cells based on CD3 and CD8 expression. **C.** Gating and population percentages of Tem (orange), naïve (purple), Tcm (pink) and Temra (grey) based on the expression of CCR7 and CD45RA. **D.** Population percentages of Tem1 (light blue), Tem2 (light green), Tem3 (dark green), Tem4 (dark blue) based on expression of CD28 and CD27. **E.** Heatmap of expression of multiple markers on Tem4, Tem1 and Tem cells.

Specificity of *Pf* SPZ memory CD8⁺ T cells

To assess the specificity of *Pf* SPZ memory CD8⁺ T cells, we first generated three distinct CD8⁺ T cell populations from the same HLA-A*0201 positive donor: naïve CD8⁺ T cells, CD8⁺ T cells stimulated once with *Pf* SPZ (1x), and CD8⁺ T cells stimulated three times with *Pf* SPZ (3x). These T cell populations were then co-cultured *in vitro* with HLA-A*0201 positive APCs that had been stimulated with *Pf* SPZ to model antigen-specific

reactivation. Following co-culture, we observed no upregulation of activation markers CD137 or IFNγ in either the naïve or 1x SPZ-stimulated CD8⁺ T cells (Fig. 2B+C), suggesting low-level antigen recognition. However, the 3x SPZ-stimulated CD8⁺ T cells showed clear signs of antigen-specific activation, with increased frequencies of CD137⁺ (14%), IFNγ⁺ (6%), Perforin⁺ (5%), and Granzyme B⁺ (6%) CD8⁺ T cells compared to the medium control (Fig. 2A+D+E+F). These findings demonstrate that repeated stimulation with *Pf* SPZ is necessary to generate a sufficient number of functional memory CD8⁺ T cells capable of responding to antigen presentation by SPZ-stimulated APCs. Thus, 3x SPZ-stimulated CD8⁺ T cells acquire an effector memory phenotype and exhibit *Pf* SPZ-specific reactivation in a co-culture model with significant increase in perforin and CD137 expression. Some background activation was observed across all groups due to SGE exposure.

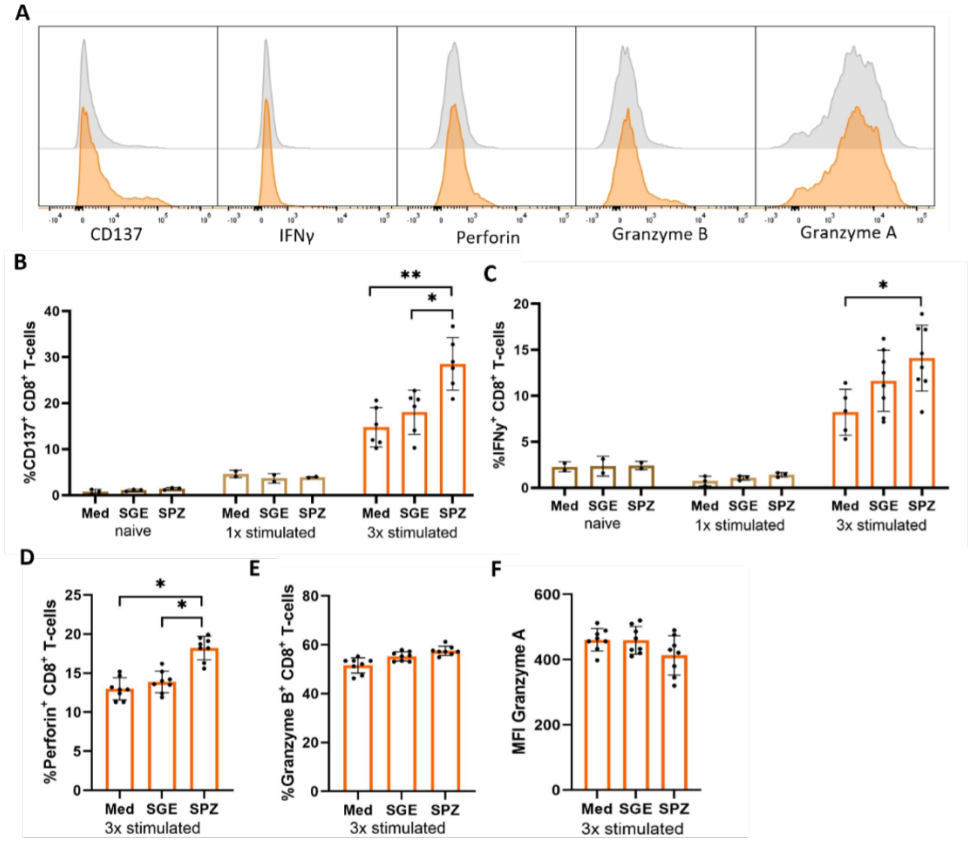

Figure 2. Activation of SPZ memory CD8⁺ T cells. A. Histogram of the expression of CD137, IFNγ, Perforin, Granzyme B and Granzyme A after *Pf* SPZ memory CD8⁺ T cells co-culture with APC stimulated with medium (grey) or SPZ (orange). **B.** Number of CD137⁺ CD8⁺ T cells after co-culture with medium, salivary gland extract (SGE) or SPZ-stimulated APC for naïve

(brown), 1-time stimulated (light brown) or 3 times stimulated (orange) CD8⁺ T cells. **C.** Number of IFNγ⁺ CD8⁺ T cells after co-culture with medium, salivary gland extract (SGE) or SPZ-stimulated APC for naïve (brown), 1-time stimulated (light brown) or 3 times stimulated (orange) CD8⁺ T cells. **D.** Number of Perforin⁺ CD8⁺ T cells after co-culture with medium, salivary gland extract (SGE) or SPZ with 3 times stimulated memory CD8⁺ T cells. **E.** Number of Granzyme B⁺ CD8⁺ T cells after co-culture with medium, salivary gland extract (SGE) or SPZ with 3 times stimulated memory CD8⁺ T cells. **F.** Expression of Granzyme A on CD8⁺ T cells after co-culture with medium, salivary gland extract (SGE) or SPZ with 3 times stimulated memory CD8⁺ T cells. Analysis using Mann-Whitney U test, Fisher's exact *: P=<0.05, **: P= <0.005, ***: P=<0.0005 and ****: P=<0.0001

Pf SPZ memory CD8⁺ T cell receptor sequencing

Next, we analyzed the T cell receptor (TCR) repertoire of the activated CD137⁺ CD8⁺ T cell population derived from 3x *Pf* SPZ-stimulated memory CD8⁺ T cells following co-culture with either SGE- or SPZ-stimulated APCs. This analysis revealed distinct TCR profiles between the two stimulation conditions. Cluster analysis identified two TCR clusters that were predominantly present in the SPZ-stimulated APC condition (blue circles), but largely absent in the SGE-stimulated group. These findings suggest that repeated *Pf* SPZ stimulation induce memory CD8⁺ T cells bearing unique, SPZ-specific TCRs, potentially recognizing epitopes exclusive to *Pf* SPZ.

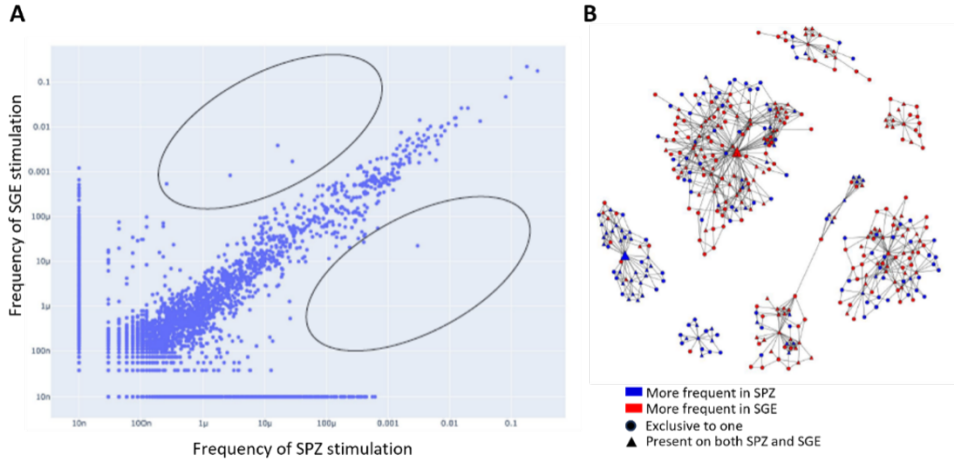

Figure 3. TCR sequencing. A. Frequency of different T cell receptors found after SGE or SPZ APC stimulation. **B.** Cluster analysis of the different TCRs found after SGE or SPZ APC stimulation. Clusters based on the frequency of TCR expressed between SPZ, SGE or combined.

Pf SPZ memory CD8⁺ T cell epitope specificity

Having shown SPZ-specific activation with distinct TCR clusters of the *Pf* SPZ memory CD8⁺ T cells, we next investigated epitope-specific activation. Following stimulation of APCs with epitopes from CSP, TRAP, MSP-1, SSP2/TRAP, LSA-1, EXP1, CRA and HSP70 (Supp. Table 1), we observed that the majority of parasite-derived epitopes did not specifically activate *Pf* SPZ memory CD8⁺ T cells when compared to the medium control (Fig. 4). Notably, although CSP-derived epitopes were of particular interest, *Pf* SPZ memory CD8⁺ T cells exhibited minimal activation in response to them, with only low-level expression of IFNγ and Perforin alongside CD137, and no significant upregulation of Granzyme B or Granzyme A. Interestingly, restimulation with the CRA epitope GLLGNVSTV led to increased expression of all activation and functional markers, not only when APCs were matched to the original donor (Fig. 4), but also with APCs from other HLA-A*0201-matched donors (Supp. Fig. 1). This finding shows that *Pf* SPZ memory CD8⁺ T cells specifically recognize the GLLGNVSTV epitope.

Collectively, our data reveal distinct TCR clusters in *Pf* SPZ memory CD8⁺ T cells following stimulation with SPZ-loaded APCs and demonstrate specific activation upon co-culture with the CRA epitope GLLGNVSTV, indicating antigen-specific memory recognition by *Pf* SPZ memory CD8⁺ T cells.

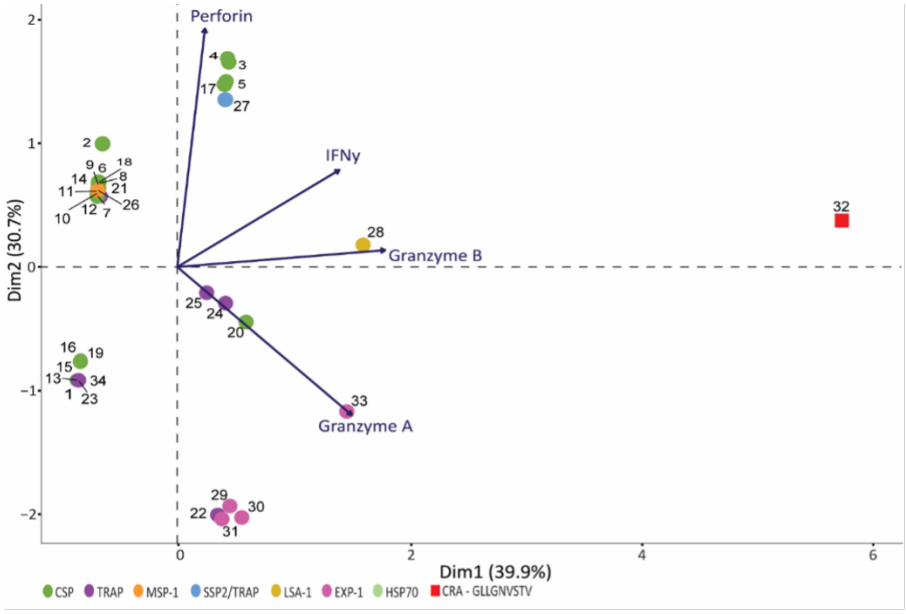

Figure 4. PCA of the correlation between IFNγ, Perforin, Granzyme A, Granzyme B with CD137. T-cell Activation after stimulation with epitopes from different pre-erythrocytic proteins in fold change relative to medium. Epitope used for each number can be found in Supp. Table 1.

Discussion

Using a co-culture model with APCs, we demonstrate that repeated stimulation with *Pf* SPZ leads to the generation of a highly activated population of Tem CD8$^+$ T cells. These cells exhibit robust functional markers including CD137, IFNγ, and Perforin upon re-stimulation with distinct TCR clusters, suggesting antigen-specific recognition and cytolytic potential. After stimulation with different pre-erythrocytic specific epitopes, we found activation of the SPZ memory CD8$^+$ T cells for CRA epitope GLLGNVSTV indicating that this epitope is being processed and presented by *Pf* SPZ-stimulated APCs.

The predominance of Tem CD8$^+$ T cells within the Pf SPZ memory CD8$^+$ T cell population reflects their differentiation following multiple SPZ stimulations. Notably, the Tem1 subset (CD28$^+$CD27$^+$) constituted the largest fraction and was marked by elevated expression of activation (HLA-DR, CD69), proliferation (CD127), and effector (Eomes, CD56) markers, indicating readiness for rapid effector function [25, 26, 27]. The relatively lower expression of regulatory markers such as PD-1, TCF-1, and ICOS further supports the conclusion that these cells are highly activated with reduced susceptibility to inhibition[28, 29]. This phenotype is consistent with a previously described phenotype associated with protective responses in rodent malaria models and other human vaccine studies [8, 30], yet their antigen specificity remains unknown.

To address this, we evaluated CD8$^+$ T cell responses against multiple pre-erythrocytic epitopes to determine which antigens drive activation within this memory subset. Despite testing epitopes from proteins including CSP, TRAP, LSA-1, EXP1, HSP70 and MSP-1, only CRA epitope GLLGNVSTV elicited upregulation of activation markers CD137, IFNγ and killing pathway markers Perforin, Granzyme B and Granzyme A [25]. CRA is a less-studied pre-erythrocytic antigen, which is known to be expressed during intrahepatic parasite development and has been shown to result in CD8$^+$ T cell activation of PBMC isolated from RAS immunized volunteers restimulated with GLLGNVSTV *ex vivo* [31]. Interestingly, GLLGNVSTV has been described as a potential supertype epitope that can fit in the three most abundant HLA types A2, A3 and B7 [32, 33]. Interestingly, we observed a comparable activation of Pf SPZ memory CD8$^+$ T cells in co-culture with APC used from the same donor as for two other HLA-A*02-APC matched donors, suggesting immunodominance and broad MHC presentation potential. However, extending these findings by investigating epitope-specific activation in Pf SPZ memory CD8$^+$ T cells derived from individuals with HLA types beyond HLA-A*0201 will be crucial for developing protective responses across a broader population. Moreover, CD8$^+$ T cell activation was absent in both naïve and one-

time SPZ-stimulated CD8⁺ T cells, reinforcing the notion that protective CD8⁺ T cell memory responses in malaria require repeated or prolonged antigenic exposure, likely mimicking natural infection [34].

To further understand the underlying mechanisms, we investigated the TCR repertoire of Pf SPZ memory CD8⁺ T cells. TCR repertoire analysis revealed distinct TCR clusters predominantly enriched in SPZ-stimulated samples, indicative of clonal expansion in response to SPZ antigens. This observation is consistent with findings from murine models, where antigen-specific CD8⁺ T cells undergo clonal expansion and localize to hepatocytes in response to defined epitopes [35]. Whether the CRA-derived epitope GLLGNVSTV is represented within these expanded TCR clusters remains unclear, as current data are insufficient to definitively link specific TCRs to this epitope. A potential strategy to address this would involve repeated restimulation of donor-derived T cells with GLLGNVSTV, followed by sequencing to map TCR-epitope specificity. This underscores the need for refined epitope-TCR mapping to better understand antigen-specific T cell responses.

While these TCR repertoire data provide valuable insights into clonal expansion and potential antigen specificity, they do not confirm the functional capacity of these T cells to recognize and eliminate infected hepatocytes. Our data indicate that our model is effective for dissecting epitope-specific CD8⁺ T cell responses to *Pf* SPZ. However, even though CRA epitope GLLGNVSTV seems to be presented by SPZ-stimulated APCs, it will remain unclear if CRA GLLGNVSTV-specific CD8⁺ T cells can recognize and kill *Pf*-infected hepatocytes. To date, an *in vitro* model to investigate liver-stage-specific CD8⁺ T cell responses has been lacking, primarily due to nonspecific bystander T cell activation [36, 37]. To address this limitation, our model could be adapted to culture *Pf* liver-stage memory CD8⁺ T cells instead of *Pf* SPZ memory CD8⁺ T cells. Upon epitope stimulation, these liver-stage memory CD8⁺ T cells may reveal which antigens are expressed during the less-characterized liver stage, which could inform strategies to prime CD8⁺ T cells with enhanced specificity, ultimately improving their ability to recognize and eliminate malaria-infected hepatocytes. However, before this approach can be realized, effective strategies to increase antigen load during the liver stage, given the low infectivity of sporozoites, must first be developed.

Collectively, our data reinforce the concept that effective malaria vaccines must induce robust and functionally competent memory CD8⁺ T cells capable of recognizing antigens beyond CSP. The identification of CRA as a target of CD8⁺ T cell responses

introduce a new candidate for inclusion in next-generation multi-antigen vaccines. Furthermore, our study highlights the utility of human *in vitro* co-culture systems combined with TCR sequencing to dissect antigen specificity and memory development, complementing *in vivo* human experimental medicine approaches.

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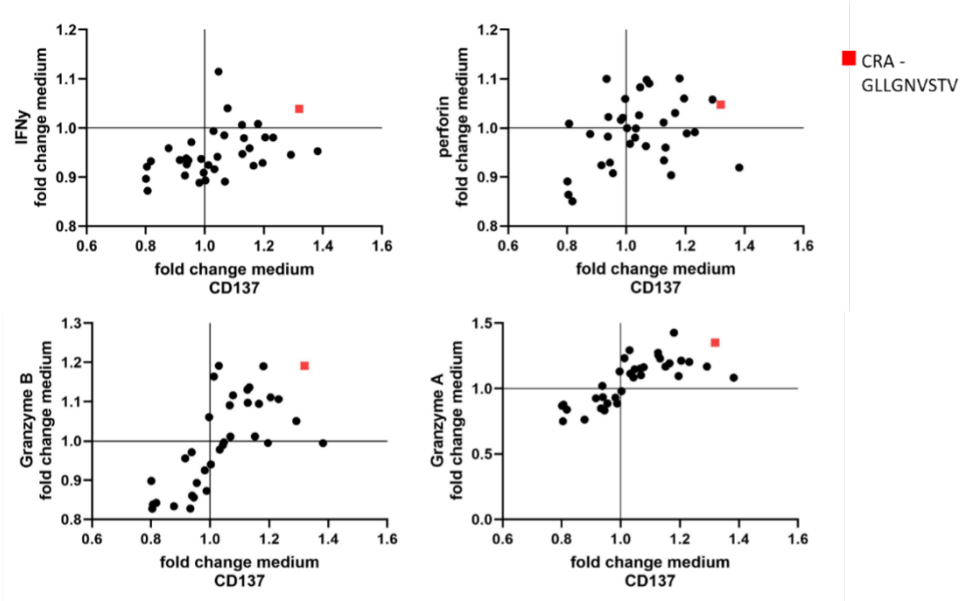
Supplementary

Supplementary table 1. Proteins and epitope sequences used for APC co-culture with SPZ memory CD8+ T cells.

Number	Protein	Epitope
1	CSP	NYDNAGTNL
2	CSP	NANPNVDPNANP
3	CSP	GLIMVLSFL
4	CSP	ILSVSSFLFV
5	CSP	YLNKIQNSL
6	CSP	MMRKLAILSV
7	CSP	FLFVEALFQEY
8	CSP	SVFNVVNSSI
9	CSP	ALFQEYQCYG
10	CSP	YQCYGSSSNT
11	CSP	LIMVLSFLFL
12	CSP	IMVLSFLFL
13	CSP	SVSSFLFVEA
14	CSP	HIKEYLNKI
15	CSP	IQNSLSTEW
16	CSP	MPNDPNRNV
17	CSP	SSFLFVEAL
18	CSP	STEWSPCSV
19	CSP	VTCGNGIQVR
20	CSP	YANDIEKKI
21	TRAP	YLLMDCSGSI
22	TRAP	NLTDALLQV
23	TRAP	ALLQVRKHL
24	TRAP	HLGNVKYLV
25	TRAP	GIAGGLALL
26	MSP-1	YLFKNLNL
27	SSP2/TRAP	KLKQPNTTL
28	LSA-1	YLNRVALYL
29	EXP1	LLMDCSHFL
30	EXP1	VLAGLLGNV
31	EXP1	KILSVFFLA
32	CRA	GLLGNVSTV
33	EXP1	VLLGGVGLVL
34	HSP70	AIKFVENDL
35	SSP2	FLIFFDLFLV



Supplementary figure 1. Correlation activation markers three HLA-A*0201 donors.
Correlation of expression of IFNγ, perforin, Granzyme B and Granzyme A with CD137 for epitopes from different pre-erythrocytic proteins in fold change relative to medium. Mean of three HLA-A*0201 donors.