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

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Chapter 4

Human circumsporozoite-specific effector-memory CD8$^+$ T-cells recognize and kill *Plasmodium falciparum*-infected hepatocytes

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Abstract

Infected *Anopheles* mosquitoes transmit *Plasmodium falciparum* (*Pf*) sporozoites (SPZ) to humans by inoculation in the skin. From the skin, SPZ will migrate to the liver where they will multiply inside hepatocytes over 7 days. *In vivo* models suggested that CD8$^+$ T-cells play an important role in recognizing and killing *Pf* infected hepatocytes, but whether this is true for human malaria remains elusive. In this study, we aimed to unravel if human memory CD8$^+$ T-cells specific for the *Pf* circumsporozoite protein (CSP) epitope YLNKIQNSL can be activated *in vitro* to specifically recognize and kill *Pf* infected hepatocytes. Here, we show that HLA-A*02-restricted YLNKIQNSL-specific CD8$^+$ T-cells can be activated by HLA-A*02 antigen-presenting cells resulting in increased CD137 cell surface expression, and elevated production of IFN-γ, granzyme B and perforin. YLNKIQNSL-specific CD8$^+$ T-cells kill 98% of hepatocytes if stimulated with the cognate antigen while a-specific epitopes do not elicit cytotoxicity. Furthermore, we demonstrate that the YLNKIQNSL-specific CD8$^+$ T-cells are able to kill 45% of *Pf* infected hepatocytes, when added to parasite cultures 24 hours post infection and 55% when added 48 hours post infection. Thus, human CD8$^+$ T-cells specific for the CSP epitope YLNKIQNSL can specifically recognize and kill *Pf* infected hepatocytes. This finding not only provides a method to test the killing capacity of malaria-specific CD8$^+$ T-cells *in vitro*, but also underlines the importance of discovering novel T-cell epitopes expressed by *Pf* parasites in the liver stage to be included in the next generation vaccines.

Author summary

Malaria, caused by *Plasmodium falciparum* (*Pf*), is transmitted to humans through the bite of infected *Anopheles* mosquitoes introducing sporozoites (SPZ) into the skin. These SPZ migrate to the liver, where they replicate inside hepatocytes. Although the importance of CD8$^+$ T-cells in recognizing and eliminating infected hepatocytes has been put forward in animal models, their relevance for eliminating human malaria-infected hepatocytes remains unclear. In this study we demonstrate that human memory CD8$^+$ T-cells specific for the *Pf* circumsporozoite protein (CSP) epitope YLNKIQNSL, can be activated to specifically target and kill human malaria *Pf* infected hepatocytes *in vitro*. As a first formal proof that human CD8$^+$ T-cells are involved in the detection and elimination of *Pf* infected hepatocytes, these findings have the potential to inform the development of new malaria vaccines targeting liver-stage parasites.

Introduction

Global deaths due to malaria are increasing each year and have now crossed 600,000 (WHO 2023). Severe and fatal malaria is predominantly caused by *Plasmodium falciparum (Pf)* [1]. After inoculation by *Anopheles* mosquitoes, *Pf* sporozoites *(*SPZ) find their way to the human liver, where they reside intracellularly for 7 days. The efficiency with which *Pf* infections occur is remarkable, as in any malaria-naïve individual 5 bites of infected mosquitoes will almost always lead to patent infection [2, 3, 4]. Because the number of infected hepatocytes is considered low and parasites persist intrahepatic for a lengthy period, this life stage seems a likely target for immune attack.

Recently, we provided unequivocal evidence for the central role of liver-stage malaria parasites as a target for immune attack by demonstrating that late-liver-stage-arresting SPZ-based vaccines much more efficiently induce protection to *Pf* as compared to early-arresting counterparts (ref GA2, submitted). These experimental medicine studies were the first to demonstrate the importance of protection based on liver-stage antigenic exposure in human malaria. However, if human CD8$^+$ T-cells play a role in this immune protection, the antigens they recognize and the mechanism by which these cells can kill infected hepatocytes in humans remain elusive.

In rodent and non-human primate models, the importance of CD8$^+$ T-cells in recognizing malaria-infected hepatocytes has been demonstrated in CD8$^+$ T-cell depletion experiments whereby animals without T-cells were not protected against malaria [5, 6, 7]. Importantly, with the use of a T-cell receptor CSP-specific transgenic system, clustering of antigen-specific CD8$^+$ T-cells around infected mouse hepatocytes *in vivo* suggested a role for cytotoxic T-cells in detecting infected hepatocytes [8]. Recently, it has been discovered that tissue-resident memory CD8$^+$ T-cells (T_{RM}), a subset of CD8$^+$ T-cells, which are confined to the liver, are required for sterilizing immunity to liver-stage malaria in radiation-attenuated sporozoites (RAS) immunized mice [9]. However, if human hepatocytes present malaria antigens in the context of MHC class I and whether these can be recognized by memory CD8$^+$ T-cells remains unclear.

Once *Pf* SPZ invade and infect hepatocytes, they form a parasitophorous vacuole membrane (PVM), which separates the parasite from the host cytoplasm [10, 11]. Interestingly, parasite modification of the PVM allows for the passage of small molecules from the hepatocyte cytoplasm to feed the growing liver-stage parasite [12]. However, for antigen presentation via MHC class I [13, 14], such antigens should pass reversely through the PVM and enter the cytoplasm, a process seen as highly complex

[15, 16, 17]. The most studied and abundant pre-erythrocytic stage antigen of *Pf* is circumsporozoite protein (CSP) [18, 19], which is expressed during invasion and early liver stage [20, [20, 21]. CSP has several CD8$^+$ T-cell epitopes, of which epitope YLNKIQNSL has been best described [22, 23, 24].

To better understand the role of CD8$^+$ T-cells in killing of liver-stage malaria parasites, we investigated activation of YLNKIQNSL-specific CD8$^+$ T-cells and tested their capacity to recognize *Pf* infected hepatocytes *in vitro* (Fig 1).

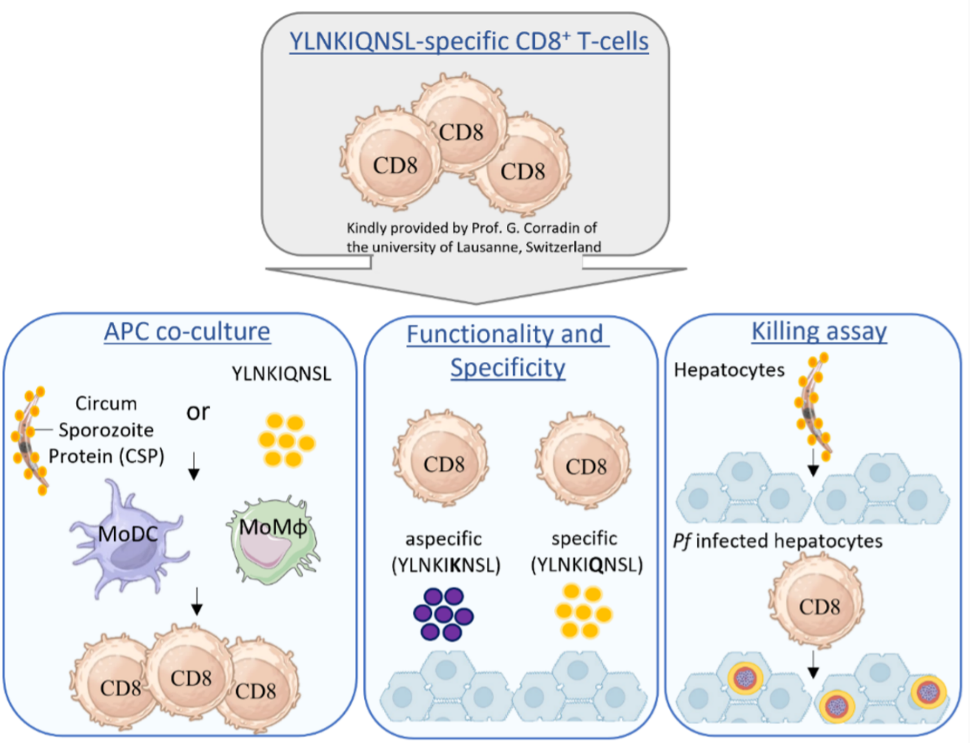

Figure 1. Experimental set up. YLNKIQNSL-specific CD8$^+$ T-cells were co-cultured with antigen presenting cell (APC) and stimulated with CSP epitope YLNKIQNSL or *Pf* SPZ. Different epitopes were used, YLNKIKNSL (purple dots) and YLNKIQNSL (yellow dots), to investigate activation specificity of the CD8$^+$ T-cells. *Pf* SPZ were used to infect HC-04.j7 cells followed by co-culture with YLNKIQNSL-specific CD8$^+$ T-cells to analyze recognition and killing.

Materials and Methods

Parasite culture

Anopheles stephensi mosquitoes were infected with *Pf* wildtype NF54 infected blood through membrane feeding as previously described [32, 33, 34]. Salivary glands of infected and uninfected mosquitoes were dissected 14 days post blood-meal [25] in RPMI + 10% fetal calf serum (FCS), and homogenized to extract Pf SPZ from the glands [35].

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.

YLNKIQNSL-specific CD8$^+$ T-cell expansion

YLNKIQNSL specific CD8$^+$ T-cells (kindly provided by Prof. G. Corradin, University of Lausanne, Switzerland) were cultured as described previously [25, 31]. Briefly, YLNKIQNSL specific CD8$^+$ T-cells were thawed and cultured with irradiated Human Leukocyte Antigens a2 (HLA a2) positive Peripheral Blood Mononuclear Cells (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% heath inactivated human serum and cultured 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 YLNKIQNSL-specific CD8$^+$ T-cells

YLNKIQNSL-specific CD8$^+$ T-cells were harvested after expansion and directly stained with markers (table 1-2) at 4°C for 30 min. For tetramer staining the cells were pre incubated with Dasatinib (Sigma-Aldrich) for 15 min at 37°C + 5% CO_2, stained with YLNKIQNSL HLA-A*02 tetramer (APC) or CMV HLA-A*02 tetramer (PE) at 4°C for 30 min followed by surface marker staining at 4°C for 30 min (table 3). 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 YLNKIQNSL specific CD8⁺ T-cells.

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 YLNKIQNSL-specific CD8⁺ T-cells.

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 tetramer staining YLNKIQNSL specific CD8$^+$ T-cells.

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 YLNKIQNSL-specific 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 MoMϕs using 20 ng/mL macrophage colony stimulating factor (M-CSF ; Biolegend, San Diego, CA, USA) [36] or 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 [37]. On day 6, MoMϕs or 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 or MoDCs were stimulated with 100,000 day 14 post blood meal *Pf* SPZ, equal amounts of salivary gland extract (SGE), 5 µg/ml CSP epitope YLNKIQNSL (kindly provided by Prof. G. Corradin, University of Lausanne, Switzerland) 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 counted and 10,000 *Pf* SPZ stimulated MoMϕs and 10,000 *Pf* SPZ stimulated MoDCS were co-cultured with 40,000 YLNKIQNSL specific CSP CD8$^+$ T-cells in a U bottom 96 wells plate and incubated o/n at 37°C + 5% CO_2. For indirect contact 40,000 YLNKIQNSL specific CSP CD8$^+$ T-cells were cultured in a 96-wells transwell plate (Corning, USA). 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 APC co-culture with YLNKIQNSL specific CSP CD8$^+$ T-cells.

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

Hepatocyte and YLNKIQNSL specific CD8$^+$ T-cell co-culture

A 96-wells optical glass bottom plate was coated with 50 µg/mL collagen type I (Corning, USA) in PBS for 30 min at RT. The wells were gently washed with PBS and 40,000 HC-04.j7 cells per well (kindly provided by Dr. Rhoel Dinglasan) were plated in 200 µl DMEM + 5% FCS supplemented with 1% penicillin/streptomycin and incubated overnight at 37°C + 5% CO_2. The following day, the medium was refreshed with 100 µl DMEM+ 5% FCS supplemented with 1 % penicillin/streptomycin, 15 mM glucose (Thermo Fisher Scientific, Waltham, MA, USA) and 0.01 mg/mL insulin, 5,5 µg/mL transferrin and 0.67 µg/mL selenium (Thermo Fisher Scientific, Waltham, MA, USA). *Pf* SPZ and uninfected salivary gland extract (SGE) were extracted from salivary glands as previously described [35] and diluted to 60,000 *Pf* SPZ/200 µl or equal number SGE/200 µl in DMEM + 5% FCS supplemented with 1% penicillin/streptomycin, 15 mM glucose (Thermo Fisher Scientific, Waltham, MA, USA) and 0.01 mg/mL insulin, 5.5 µg/mL transferrin and 0.67 µg / mL selenium (Thermo Fisher Scientific, Waltham, MA, USA). Medium was removed from the HC-04.j7 cells and 200 µl *Pf* SPZ (60,000), SGE, medium or 5/0,5/0,1/0,01 µg/ml CSP epitope YLNKIQNSL (kindly provided by Prof. G. Corradin) or 5 µg/ml CSP peptide epitope YLNKIKNSL (peptides&elephants GmbH, Henningsdorf) were added. The plate was centrifuged for 3 min at 1200 rpm and incubated at 37°C + 5% CO_2. At 24 hours or 48 hours post infection the wells were gently washed with 37°C IMDM + 10% and 60,000 YLNKIQNSL specific CD8$^+$ T-cells were added direct to the hepatocytes or above for indirect contact in a 96-wells transwell plate (Corning, USA) in 200 µl 37 °C IMDM + 10 % heath inactivated human serum and incubated overnight at 37°C + 5% CO_2. The following morning, the cells were fixed with 3.7% paraformaldehyde (PFA; Sigma Aldrich, St Louis, MO, USA) in PBS for 15 min at RT. After the permeabilization with 0.5 % Triton (Thermo Fisher Scientific, Waltham, MA, USA) in PBS for 20 min, the cells were washed and blocked with 10% FCS in PBS for 30 min, and stained with primary antibodies HSP70 1:500 (Thermo Fisher Scientific, Waltham, MA, USA) and GAPDH 1:1000 (The European malaria reagent repository, Edinburgh) in PBS overnight at 4°C. The following day the cells were washed 5 times with PBS and stained with secondary antibodies anti-rabbit AF594 (Invitrogen) 1:500 and anti-mouse AF488 (Invitrogen) 1:500 in PBS for 1 hour in the dark. The nuclei of the cells were stained with Hoechst 33342 (Sigma) 1:200 for 30 min in the dark. The cells were washed 3 times and 100 µl PBS was added to each well. The wells were analyzed by ImageXpress confocal (9 fields per well automatically selected, Molecular Devices, LLC, San Jose USA). The HSP70 and GAPDH positive cells and cell nucleus were automatically counted by high-content image acquisition and analysis software MetaXpress (Molecular Devices, LLC, San Jose USA).

Statistical analysis

Data was analyzed using Spectroflow (Cytek Bioscience B.V., Amsterdam), FlowJo version 10.8 (FlowJo LLC, Ashland, OR, USA), Graphpad version 9.3.1, analysis software MetaXpress (Molecular Devices, LLC, San Jose USA), R-studio version 1.4.1717, and SPSS version 24.0.0.0. Comparison between two independent data groups were made by Mann Whitney U test (for nonparametric data) or paired T-test (for paired parametric data). $P<0.05$ was considered statistically significant.

Graphical Fig 1, Fig 2D and Fig 4A have been developed in BioRender.

Results

Effector-memory phenotype of YLNKIQNSL-specific CD8$^+$ T-cells

We characterized the phenotype of the YLNKIQNSL-specific CD8$^+$ T-cell clone after expansion by tetramer staining and phenotyping by flow cytometry. We observed that >98% of cells uniformly expressed high levels of CD8, and consistently 97.2% of the cells were positive for HLA-A*02 tetramer YLNKIQNSL (Fig 2A+B+C, S1). The YLNKIQNSL-specific CD8$^+$ T-cells showed expression of CD71, HLA-DR, CD25, CD69, CD27 and CD28 (Fig 2D). Based on expression of CD45RA and CCR7, 98.7% of the cells were categorized as effector-memory cells (T$_{EM,}$ CD45RA$^-$CCR7$^-$). We subsequently characterized T$_{EM}$ cells on expression of CD28 and CD27 and categorized them as T$_{EM1}$ (CD45RA$^-$CCR7$^-$ CD28$^{+,}$CD27$^+$) 41.40%, T$_{EM2}$ (CD45RA$^-$CCR7$^-$CD28$^{-,}$CD27$^+$) 7.75% , T$_{EM3}$ (CD45RA$^-$CCR7$^-$ CD28$^{-,}$CD27$^-$) 9.25% and T$_{EM4}$ (CD45RA$^-$CCR7$^-$CD28$^{+,}$CD27$^-$) 41.60% (Fig 2E, S1). The T$_{EM1}$ population showed a high expression of different activation (HLA-DR, CD69, CD56), differentiation (EOMES, T-Bet, TCF-1), expansion (CD127) and regulation (CD25) markers, whereas T$_{EM4}$ showed a high expression of immune checkpoint CD279 (PD-1), regulation (CD278), differentiation into the memory phenotype (CD28) and activation (CD137) markers (Fig 2F).

In conclusion, the YLNKIQNSL-specific CD8$^+$ T-cells are HLA-A*02 positive and form a heterogeneous T_{EM} population.

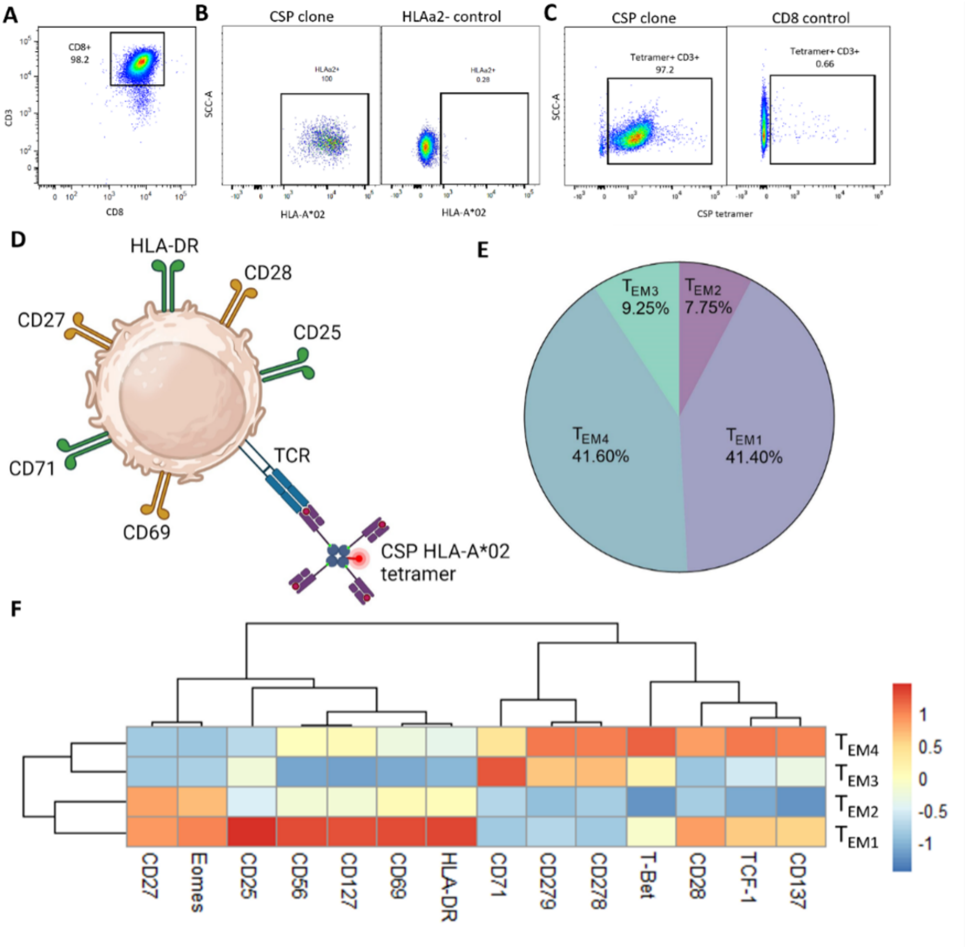

Figure 2. Effector-memory phenotype of YLNKIQNSL-specific CD8$^+$ T-cell clone prior to co-culture. A Expression of CD8. B Expression of HLA-A*02. C TCR staining with the use of YLNKIQNSL epitope HLA-A*02 tetramer. D Profiling of expression of different cell surface markers. E Percentages of T_{EM1}, T_{EM2}, T_{EM3} and T_{EM4}. F Heatmap of MFI expression log transformed of different markers on T_{EM1}, T_{EM2}, T_{EM3} and T_{EM4} cells.

Activation of YLNKIQNSL-specific CD8$^+$ T-cells by antigen-presenting cells

Next, we analyzed the activation of the YLNKIQNSL-specific CD8$^+$ T-cells in co-culture with antigen presenting cells (APCs) derived from monocytes. For these experiments, monocyte-derived macrophages (MoMϕs) and monocyte-derived dendritic cells (MoDCs) were stimulated with the peptide epitope YLNKIQNSL. Following co-culture, we found a ~ 7 fold increase in activated CD137$^+$ CD8$^+$ T-cells (p=0.008, mean 70.4%; SD 22%) (Fig 3A). This was substantiated with an equal increase in IFN-γ$^+$ CD8$^+$ T-cells (p= 0.03, mean 53.1%; SD 40% Fig 3A). In parallel, we found an increase in granzyme B$^+$ (p= 0.03, mean 66%; SD 15%) and perforin$^+$ CD8$^+$ T-cells (p=0.03, mean 19.2%; SD 12%) (Fig 3A). In contrast, co-culturing led to a loss of granzyme A expression in CD8$^+$ T cells (MFI 1150; SD 2200, Fig 3A). Whereas increased expression of cytotoxic markers was clear after co-culture with APCs that had been stimulated with the peptide epitope YLNKIQNSL, we only found an increase in CD137$^+$ CD8$^+$ T-cells, but not in IFN-γ, granzyme B or perforin after whole *Pf* SPZ APC co-culture, which contain the CSP epitope YLNKIQNSL albeit in much lower quantities (Fig 3A).

Similarly, we found 43.3% of YLNKIQNSL-specific CD8$^+$ T-cells co-expressing IFN-γ and CD137, and 15.4% co-expression of perforin and granzyme B after stimulation with YLNKIQNSL, but not *Pf* SPZ (Fig 3B). The activation of CD8$^+$ T-cells was cell-contact-dependent, as it was abrogated when CD8$^+$ T-cells were not in direct contact with the APCs (Fig 3C).

Collectively, we show that YLNKIQNSL-specific CD8$^+$ T-cells specifically recognize epitope YLNKIQNSL and express the cytotoxic molecules perforin and granzyme B ,but not granzyme A, after co-culture with APCs presenting the YLNKIQNSL epitope. Activation of YLNKIQNSL-specific CD8$^+$ T-cells was much lower once co-cultured with APCs stimulated with *Pf* SPZ as compared with peptide epitope YLNKIQNSL stimulated APCs.

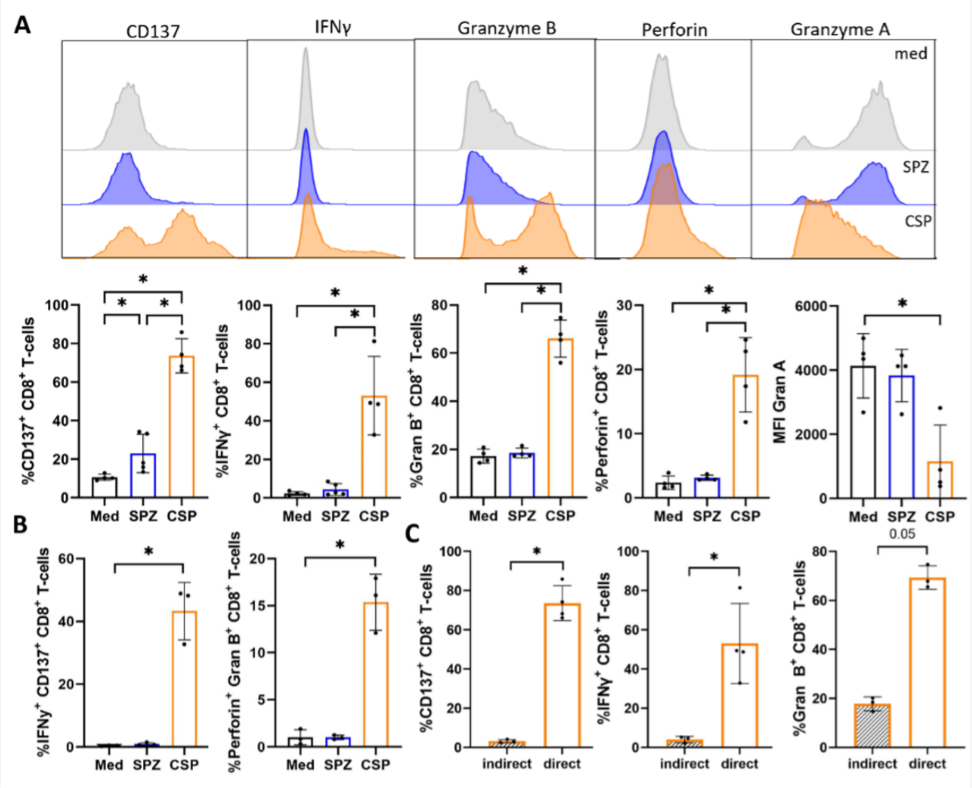

Figure 3. YLNKIQNSL-specific CD8⁺ T-cells in APC co-culture. A. Histogram overlay and bar graph show gated percentage (%) of YLNKIQNSL-specific CD8⁺ T-cells positive for CD137, IFN-γ, granzyme B, perforin and MFI of granzyme A after co-culture with APC stimulated with medium (grey), *Pf* SPZ (blue) or CSP epitope YLNKIQNSL (orange), n=2 different mosquito batches, 4-5 donors. B. Percentage (%) of IFN-γ⁺ CD137⁺ and perforin⁺ granzyme B⁺ YLNKIQNSL-specific CD8⁺ T-cells after co-culture with APC stimulated with medium (grey), *Pf* SPZ (blue) or CSP epitope YLNKIQNSL (orange), n=2 different mosquito batches, 3-4 donors C. Percentage (%) of CD137⁺, IFN-γ⁺ or granzyme B⁺ YLNKIQNSL specific CD8⁺ T-cells after co-culture with APCs presenting the CSP epitope YLNKIQNSL with indirect (striped) or direct (non-filled) contact with the YLNKIQNSL specific CD8⁺ T-cells, n=2, 4 donors. Analysis using Mann Whitney U test, Fishers exact. *: P=<0.05, **: P= <0.005, ***: P=<0.0005 and ****: P=<0.0001

CD8$^+$ T-cells effectively and specifically kill hepatocytes presenting the YLNKIQNSL epitope

Next, we investigated whether YLNKIQNSL-specific CD8$^+$ T-cells are able to kill hepatocytes that present the YLNKIQNSL epitope. When the YLNKIQNSL epitope was added to hepatocytes at a concentration of 0.5 µg/ml, we found that YLNKIQNSL-specific CD8$^+$ T-cells kill all hepatocytes. We thus decreased the epitope concentration to find the minimum concentration of YLNKIQNSL required for hepatocyte killing. A rapid decrease in killing capacity was observed from 0.1 µg/ml YLNKIQNSL onwards (Fig 4A). We then investigated peptide recognition specificity by stimulating the YLNKIQNSL-specific CD8$^+$ T-cells with a mutant epitope with one amino acid difference (change from YLNKIQNSL to YLNKIKNSL). Notably, we found no killing when this altered CSP epitope was added to the hepatocyte-CD8$^+$ T-cell co-culture (mean 14200; SD 1200, Fig 4B), highlighting that the CD8$^+$ T-cell mediated killing is specific and strictly dependent on cognate antigen recognition.

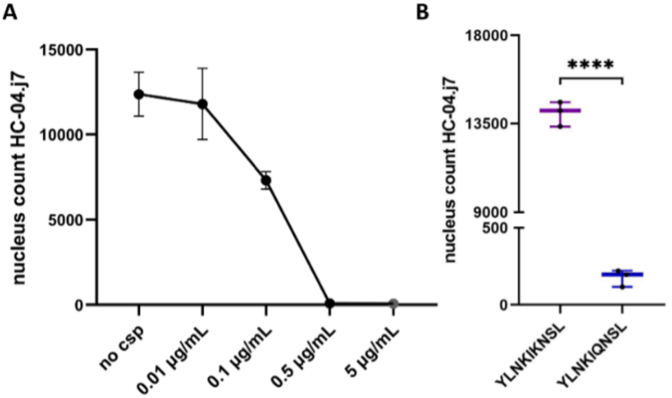

Figure 4. YLNKIQNSL-specific CD8$^+$ T-cells killing ability and specificity. A. CD8$^+$ T-cells killing at a concentration range of CSP epitope YLNKIQNSL ranging from 5 µg/ml to 0.01 µg/ml, n=1, total of 9 automatically selected fields per well, average of 2-3 wells. B Specificity of YLNKIQNSL-mediated killing of CD8$^+$ T-cells by stimulation with CSP epitope YLNKIKNSL and YLNKIQNSL, n=3, total of 9 automatically selected fields per well, average of 2-3 wells. Analysis using Mann Whitney U test, Fishers exact. *: P=<0.05, **: P= <0.005, ***: P=<0.0005 and ****: P=<0.0001

YLNKIQNSL-specific CD8$^+$ T-cells kill *Pf* infected hepatocytes

Having shown the ability of YLNKIQNSL-specific CD8$^+$ T-cells to be activated through presentation of epitope YLNKIQNSL by APC and hepatocytes in co-culture, we next investigated their ability to kill *Pf* infected hepatocytes. Co-culture of YLNKIQNSL-specific CD8$^+$ T-cells with *Pf* infected hepatocytes did not result in a reduction of the overall number of hepatocytes (24 hours (h) post infection (p.i.) mean 8047; SD 1543, 48 h p.i. mean 9375; SD 1810) as compared with no CD8$^+$ T-cells (mean 8345; SD 1703). In contrast, addition of the YLNKIQNSL peptide epitope to the culture as a positive control resulted in a significant overall decrease in hepatocytes (mean 2830; SD 1080, Fig 5C). However, when specifically investigating the number of *Pf* infected hepatocytes (exoerythrocytic forms, EEFs), we found that 45% of infected hepatocytes were killed after addition of YLNKIQNSL-specific CD8$^+$ T-cells 24 h p.i. and overnight (o/n) co-culture (mean 45%; SD 30%, Fig 5B+D+E). Additionally, o/n co-culture of YLNKIQNSL-specific CD8$^+$ T-cells with *Pf* infected hepatocytes 48 h p.i. showed a further increase (55%) in killing of *Pf* infected hepatocytes ($p = 0.0521$, mean 55%; SD 23,9, Fig 5D+E). Killing did not occur without direct contact between the YLNKIQNSL-specific CD8$^+$ T-cells and the *Pf* infected or CSP epitope YLNKIQNSL-stimulated hepatocytes in a transwell setup (mean 2,8% SD 19,5% Fig 5D, S2). To confirm that YLNKIQNSL-specific CD8$^+$ T-cells kill infected hepatocytes exclusively, we investigated YLNKIQNSL-specific CD8$^+$ T-cell killing with salivary gland extract (SGE) stimulated hepatocytes. Here, we did not find any differences in nucleus count when YLNKIQNSL-specific CD8$^+$ T-cells are added (mean 7873 ; SD 750, S3A) compared with uninfected hepatocytes (mean 8147 ; SD 250, S3). Furthermore, we observed YLNKIQNSL-specific CD8$^+$ T-cells clustering with *Pf* infected or CSP epitope YLNKIQNSL stimulated hepatocytes, but not for SGE stimulated hepatocytes (S3B), which is in line with earlier described CD8$^+$ T-cells clustering around *Plasmodium yoelli* murine infected liver cells [8].

In conclusion, we demonstrate that YLNKIQNSL-specific CD8$^+$ T-cells are able to specifically kill the *Pf* infected hepatocytes, and this killing requires direct cell-cell contact.

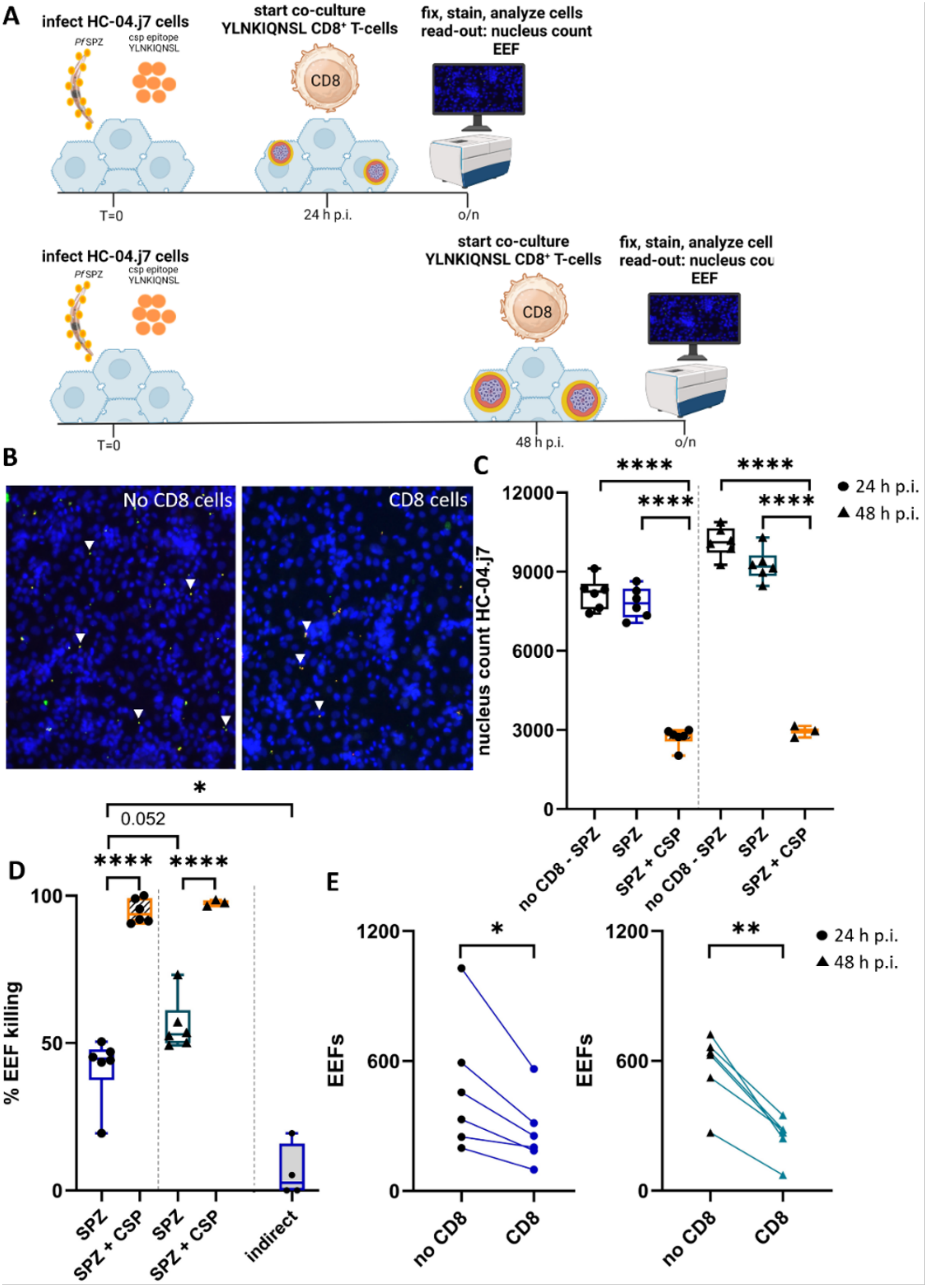

Figure 5. *Plasmodium falciparum* (*Pf*) infected hepatocyte killing by YLNKIQNSL specific CD8$^+$ T-cells. A. Experimental overview: Infection/stimulation of HC-04.j7 cells with CSP

epitope YLNKIQNSL or *Pf* SPZ followed by YLNKIQNSL specific CD8$^+$ T-cells co-culture after 24 or 48 hours (h) post infection (p.i.). After overnight (o/n) co-culture, cells are fixed, stained and analyzed for nucleus count or exoerythocytic forms (EEF). B Immunofluorescence assay (IFA) of *Pf* infected hepatocytes 24h p.i. EEFs (yellow) stained with GAPDH and HSP70, nucleus (blue) stained with hoechst, without YLNKIQNSL-specific CD8$^+$ T-cells added or with YLNKIQNSL-specific CD8$^+$ T-cells. C Nucleus count of *Pf* infected hepatcoytes after co-culture without YLNKIQNSL-specific CD8$^+$ T-cells (black), with YLNKIQNSL-specific CD8$^+$ T-cells added after 24 h p.i. (blue), with YLNKIQNSL-specific CD8$^+$ T-cells added after 48h p.i. (green) and hepactoytes stimulated with 5 µg/ml CSP eptiope YLNKIQNSL and co-cultured with YLNKIQNSL-specific CD8$^+$ T-cells (blue striped), n=4-5, total of 9 automatically selected fields per well, average of 2-3 wells. D Percentage (%) of EEFs killing of *Pf* infected hepatcoytes after co-culture with YLNKIQNSL specific CD8$^+$ T-cells added after 24h p.i. (blue), YLNKIQNSL-specific CD8$^+$ T-cells added after 48 h p.i. (green), hepatoytes stimulated with 5µg/ml CSP epitope YLNKIQNSL and co-cultured with YLNKIQNSL-specific CD8$^+$ T-cells (blue striped) and *Pf* infected hepatocytes after co-culture with indirect contact of YLNKIQNSL-specific CD8$^+$ T-cells added after 24 h p.i. (blue bar, grey filled) n=4-6, total of 9 automatically selected fields per well, average of 2-3 wells. E Paired trend between the addition of YLNKIQNSL-specific CD8$^+$ T-cells and without YLNKIQNSL-specific CD8$^+$ T-cells 24 h p.i. (blue) and 48h p.i. (green) n=6, total of 9 automatically selected fields per well, average of 2-3 wells. Analysis using paired T-test, *: P=<0.05, **: P= <0.005, ***: P=<0.0005 and ****: P=<0.0001

Discussion

Despite growing interest in CD8$^+$ T-cell mediated protection during *Pf* liver stage infection, little is known about the role of human CD8$^+$ T cells and their response to *Pf* infected hepatocytes as well as the particulars of antigen presentation and recognition of *Pf* antigens by APCs. Here we provide evidence that human CD8$^+$ T-cells specific for the CSP epitope YLNKIQNSL can recognize and kill around 45% of *Pf* infected hepatocytes *in vitro*. This data provides unequivocally proof that hepatocytes can present *Pf* CSP epitope sequence YLNKIQNSL during *Pf* liver stage infection and as a consequence are targets for CD8$^+$ T-cell mediated killing.

The demonstration of specific killing of *Pf* infected hepatocytes is, as far as we are aware, the first proof of CSP processing and presentation in the context of MCH class I on human *Pf* infected hepatocytes *in vitro*. The potential for hepatocytes to process and present CSP in the context of MHC class I has been highly debated given the fact that sporozoites form a hepatocyte-derived membrane (PVM) after hepatocyte invasion, which was thought to shield parasites from the hepatocyte intracellular environment

[10, 12, 16]. Here we observed an increase in YLNKIQNSL-specific CD8$^+$ T-cell mediated killing of more mature liver schizonts (48 h p.i.). This could be related to an increase in volume of epitope presented on the hepatocytes as a consequence of increased schizont size. Nonetheless, killing of *Pf* infected hepatocytes did not reach 100%, despite the high ratio of YLNKIQNSL-specific CD8$^+$ T-cells to *Pf* infected hepatocytes. During natural infection, the number of *Pf* infected hepatocytes is much lower and infected cells are more dispersed throughout the liver. As a consequence, it remains unknown how *in vitro* killing efficiency translates to *in vivo* efficacy of CD8$^+$ T-cell mediated protection given the differences between *in vitro* and *in vivo* settings. For instance, the differentiation status of HC-04.j7 hepatocytes used in this study differs from that of primary human hepatocytes, which may influence antigen processing capacity of hepatocytes. In addition, liver stage development of *Pf* parasites *in vivo* takes much longer (up to 7 days [12, 25]), whilst development of *Pf* in hepatocytes in HC-04.j7 cell lines is abrogated. Moreover, *in vivo* liver T_{RM} have been suggested to have a central role in identifying infected hepatocytes [9, 26, 27]. These cells may be more efficient in recognition and/or display different effector mechanisms as compared to *in vitro* T-cell lines. Moreover, these CD8$^+$ T-cells should theoretically be able to recognize different epitopes beyond CSP [27]. Given our results showing the recognition and killing of *Pf* infected hepatocytes by antigen-specific CD8$^+$ T cells *in vitro*, this opens up avenues to explore the presentation and recognition of different T-cell epitopes from other known liver stage antigens and/or in the context of different HLA types. A better understanding of *Pf* antigen presentation *in vitro* is critical to explore the possibility of broadly protective long-lasting memory CD8$^+$ T-cell responses in the liver and may pave the way for combinatorial liver-stage antigen based vaccines.

During APC co-culture we found that activation of YLNKIQNSL-specific CD8$^+$ T-cells was highly effective when APCs were exposed to the epitope, but almost absent when APCs were co-cultured with *Pf* SPZ. This could be related to the low natural concentration of CSP epitope YLNKIQNSL on *Pf* SPZ, and the requirement of antigen processing prior to presentation. Yet, the co-culture experiment with hepatocytes proved that even a low concentration of infected hepatocytes (3-5% *in vitro* [28]) are effectively activated and killed by YLNKIQNSL-specific CD8$^+$ T-cells. Thus, processing and presentation of antigen from *Pf* SPZ by APCs seems to be a rather ineffective process for activating YLNKIQNSL-specific CD8$^+$ T-cells. This could be related to the preference of APC to present exogenous antigens in the context of MHC class II as opposed to MHC class I or it could represent the lack of co-stimulatory signals expressed by APC [29, 30]. In line with this, we previously have shown that particularly macrophages adopt a regulatory phenotype after phagocytosis of *Pf* SPZ [31], which provides a potential explanation for the ineffective activation of CD8$^+$ T-cells by *Pf* SPZ APCs.

In summary, our platform assay for investigating the activation of CD8$^+$ T-cells and their subsequent functional killing activity towards *Pf* infected hepatocytes *in vitro* can aid the discovery of novel T-cell epitopes expressed by *Pf* parasites in the liver stage. This may pave the way for the identification of promising immunogenic *Pf* epitopes to be included in the next generation of vaccines.

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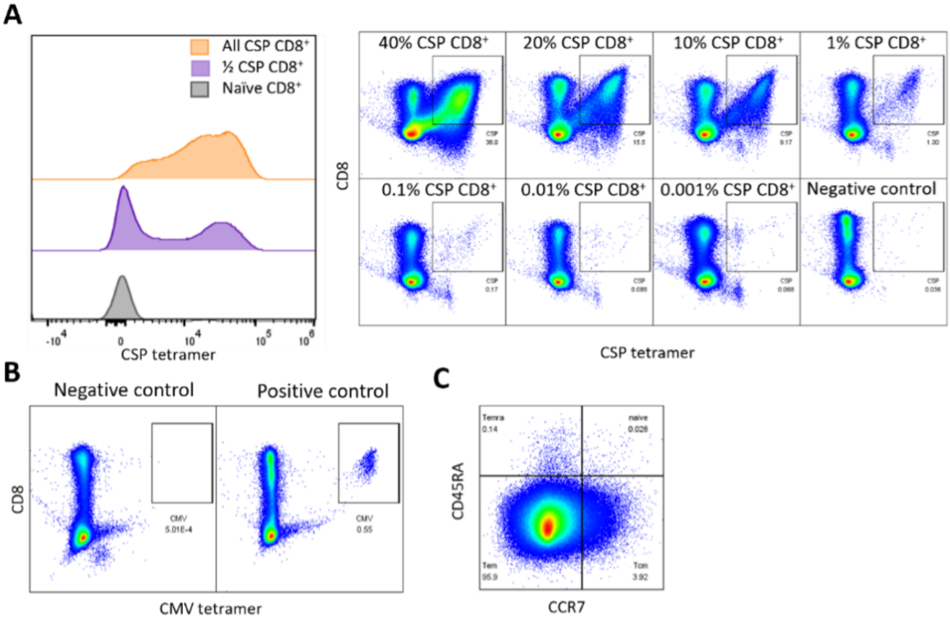
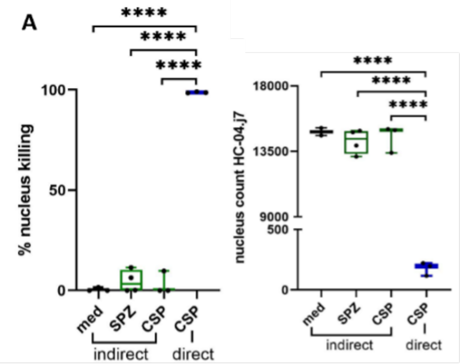
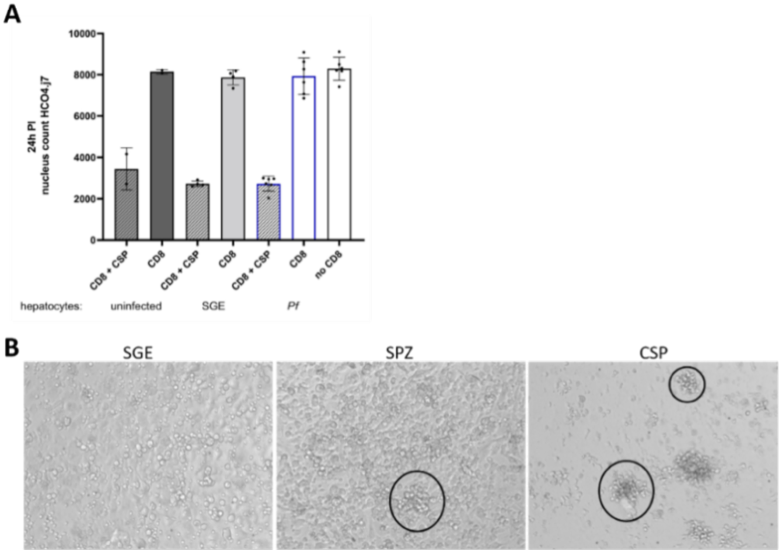
Supplementary



Supplementary Figure 1. HLA-A*02 tetramer YLNKIQNSL staining. A. titration of YLNKIQNSL-specific $CD8^+$ T-cells stained with HLA-A*02 tetramer YLNKIQNSL. B. tetramer staining methodology confirmed by the use of HLA-A*02 tetramer CMV with naïve cells (negative control) and CMV positive cells (positive control). C. Gating of YLNKIQNSL-specific $CD8^+$ T-cells stained with CD45RA and CCR7 to classify them as $T_{EM,}$ T_{emra}, T_{cm} or naïve cells.



Supplementary Figure 2. Direct and indirect contact. A. Percentage of nucleus killing and nucleus count of medium (black), sporozoite (*Pf* SPZ, green), CSP epitope YLNKIQNSL (green stiped) stimulated HC-04.j7 cells after co-culture with YLNKIQNSL-specific CD8$^+$ T-cells via direct or indirect contact (blue) with the use of a transwell plate. n=3-4, total of 9 automatically selected fields per well, average of 2-3 wells. Analysis using paired T-test, *: P=<0.05, **: P= <0.005, ***: P=<0.0005 and ****: P=<0.0001



Supplementary Figure 3. Nucleus count of HC-04.j7 cells. A. Nucleus count of uninfected (dark grey), SGE stimulated (light grey) and Pf infected hepatocytes (white with blue) after co-culture of YLNKIQNSL-specific CD8$^+$ T-cells. CSP epitope YLNKIQNSL added as positive control (striped) or no CD8$^+$ T-cells added (white with black), n= 2-6, total of 9 automatically selected fields per well, average of 2-3 wells. B. Brightfield images of SGE stimulated, Pf infected and CSP epitope YLNKIQNSL stimulated HC-04.j7 cells after overnight co-culture with YLNKIQNSL-specific CD8$^+$ T-cells prior fixation and staining. Clustering of YLNKIQNSL-specific CD8$^+$ T-cells shown within the black circle.