Dissecting the CD8+ T cell responses to pre-erythrocytic malaria antigens

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I, Matthew Paul Gibbins, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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ABSTRACT

The pre-erythrocytic stages of malaria are the preliminary steps to a disease of massive global health importance. Following transmission of sporozoites by mosquito bite to a human host, a clinically silent period of parasite development in the liver preludes the harmful blood stages that characterise malarial infection. Creating a vaccine that targets these stages is a very attractive notion as it would prevent this burden of disease. Protection against the pre-erythrocytic stages has been shown to be dependent on CD8+ T cells. However, very few antigens that induce anti-*Plasmodium* CD8+ T cell responses have been identified, especially those expressed by the parasite when developing inside hepatocytes. Using mouse models, experimental genetics and bioinformatics tools, I present work that has progressed our understanding of CD8+ T cells induced in the pre-erythrocytic stages of malaria and assessed the ability of antigen-specific CD8+ T cells to protect against subsequent challenge following vaccination.

I have investigated and compared the differences in CD8+ T cell responses to a sporozoite (Circumsporozoite Protein) and a vacuolar membrane liver stage protein (Upregulated in Infectious Sporozoites gene 4) and showed that despite a divergence in immunogenicity when immunising with radiation attenuated sporozoites, both types of antigen are equally protective when mice are vaccinated with viral vectors to induce large antigen-specific CD8+ T cell populations. The natural immunogenicity of the liver stage antigen does not improve when liver stage development is extended by using drug prophylaxis. Additionally, I have compared the protection induced by liver stage antigens expressed constitutively after hepatocyte invasion and those expressed only after at least 12 hours post invasion. I have shown that some protection can be induced by the mid-late expressed Liver Specific Proteins 1 and 2, suggesting that these antigens are effectively presented and recognised by CD8+ T cells. This highlights the potential for the incorporation of liver stage antigens into next-generation malaria vaccines.

Additionally, I have investigated the role of the immunodominant CD8+ T cell epitope of Circumsporozoite Protein and showed that a significant level of protection is mediated by CD8+ T cells specific for this epitope. Nonetheless, following multiple immunisations with a parasite lacking this epitope, sterile protection can still be achieved, suggesting other antigens are important for parasite-induced protection. Thus, finally I go on to identify a number of novel CD8+ T cell epitopes from antigens expressed in the sporozoite and liver stage parasite, to further broaden our view of the CD8+ T cell responses induced during the pre-erythrocytic stages of malaria.

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STATEMENT OF CONTRIBUTIONS

The work presented in this thesis was conducted primarily at the London School of Hygiene and Tropical Medicine in the UK, but also at the Max Planck Institute for Infection Biology, in Berlin, Germany and at the Centre d'Immunologie et des Maladies Infectieuses at Sorbonne Université in Paris, France and the Instituto de Investigaciones Biotecnológicas at Universidad Nacional de San Martín in Buenos Aires, Argentina in concert with our collaborators.

Below I detail my contribution to all of the chapters I present here and the contributions of the collaborators listed as authors in the manuscripts that formulate these chapters.

Julius Hafalla (JH, my supervisor) and our collaborators (as indicated) conceived the studies presented in this thesis. Eleanor Riley (my co-supervisor) acted as a mentor during my PhD, providing stimulating and critical discussion during laboratory meetings.

CHAPTER 1

This chapter summarises the current knowledge about host-parasite interactions and the immune responses induced during the pre-erythrocytic stages of malaria. I also outline the current understanding of pre-erythrocytic stage targeted vaccines currently under investigation by the malaria and vaccine community.

CHAPTER 2

Contrasting immunogenicities of malaria pre-erythrocytic stage antigens are overcome by vaccination

The original study was conceived by JH with Olivier Silvie (OS) in the laboratory of Kai Matuschewski (KMat). OS generated the transgenic parasites and Katja Müller (KMü) performed the characterisation of the parasites. JH, KMü and I performed the immunological experiments and I performed the corresponding statistical analyses. Arturo Reyes-Sandoval (AR-S), Adrian Hill (AH) and Simon Draper (SD) provided the adenovirus AdOVA used in the vaccination experiments. JH and KMü performed the

vaccination experiments. I wrote the first draft of the paper. JH and I wrote the paper presented here with contributions from all authors.

CHAPTER 3

Extending expression of *Plasmodium* liver stage antigens does not improve cognate CD8+ T cell responses

The original study was conceived by JH with additional discussion from OS and KMat. KMü and I performed the immunological experiments and I performed the corresponding statistical analyses. I wrote the paper as presented here.

CHAPTER 4

Late liver stage antigens confer partial protection against the pre-erythrocytic stages of malaria

The original study was conceived by JH and OS. I generated the transgenic parasites with Sylvie Briquet (SB) and OS in Paris. I characterised the parasites, performed immunology and vaccination experiments in London with Niculò Barandun (NB) and Liya Mathew (LM) as part of their ETH Zürich and LSHTM MSc theses respectively. AR-S, AH and SD provided the adenovirus AdOVA used in the vaccination experiments. I performed the statistical analyses presented in the paper. I wrote the paper as presented here.

CHAPTER 5

The importance of the immunodominant CD8+ T cell epitope of *Plasmodium* circumsporozoite protein in parasite- and vaccine-induced protection

The original study was conceived by JH. Maya Glover (MG) and Jasmine Liu (JL) performed the initial experiments as part of their LSHTM MSc theses with KMü in the laboratory of KMat. I validated the immunological experiments and confirmed the screen for non-CSP epitopes presented as an addendum. I performed the statistical analyses. Karolis Bauza and A.R.-S. generated the CSP-expressing viruses Ad*Pb*CSP and

MVA*Pb*CSP. I wrote the first draft of the paper. JH and I wrote the paper as presented here.

CHAPTER 6

Identification of novel CD8+ T cell epitopes from the pre-erythrocytic stages of malaria

The original study was conceived by JH with guidance from Morten Nielsen (MN). I performed the bioinformatics analysis with Emilio Fenoy (EF) and Massimo Andreatta (MA). I performed the immunological epitope screens. The feature analysis was performed by EF. I wrote the paper as presented here with contributions from all authors.

CHAPTER 7

This chapter is a discussion of the findings presented, the implications that they have and potential future experiments that could be performed.

ADDITIONAL PUBLICATIONS

I also contributed to other projects that were not part of my PhD work and have not been presented in this thesis accordingly:

Müller K, Gibbins MP, Matuschewski K, Hafalla JCR. Evidence of cross-stage CD8+ T cell epitopes in malaria pre-erythrocytic and blood stage infections. Parasite Immunol. 2017 Jul;39(7).

Brugman VA, Kristan M, Gibbins MP, Angrisano F, Sala KA, Dessens JT, Blagborough AM, Walker T. Detection of malaria sporozoites expelled during mosquito sugar feeding. Sci Rep. 2018 May 15;8(1):7545.

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ABBREVIATIONS

Ad	adenovirus
APC	antigen presenting cell
AZ	azithromycin
CD	cluster of differentiation
CFSE	carboxyfluorescein succinimidyl ester
CHMI	controlled human malaria infection
CSP	circumsporozoite protein
DAPI	4′,6-diamidino-2-phenylindole
DC	dendritic cell
DHFR/TS	dihydrofolate reductase/thymidylate synthase
dLN	draining lymph node
EEF	exo-erythrocytic form
ETRAMP	early transcribed membrane protein
FCS	foetal calf serum
GAP	genetically attenuated parasite
GAPDH	glyceraldehyde-3-phosphate dehyrogenase
GFP	green fluorescent protein
HSP	heat shock protein
HSPG	heparan sulphate proteoglycan
IFN	interferon
KC	Kupffer cell
KO	knock out
LISP1	liver-specific protein 1
LISP2	liver-specific protein 2
LSEC	liver sinusoid endothelial cell
MHC	major histocompatibility complex
MHC-I	major histocompatibility complex class I
MVA	modified vaccinia virus Ankara

NK	natural killer cell
Pb	*Plasmodium berghei*
PEXEL	*Plasmodium* export element
Py	*Plasmodium yoelii*
PPM	parasite plasma membrane
PV	parasitophorous vacuole
PVM	parasitophorous vacuole membrane
RAS	radiation attenuated sporozoites
S20	sporozoite-specific gene 20
SEM	standard error of the mean
TCR	T cell receptor
Tg	*Toxoplasma gondii*
TNF-α	tumour necrosis factor α
TRAP	thrombospondin related adhesive protein
T_{RM}	tissue resident memory T cell
UIS4	upregulated in sporozoites gene 4
WT	wild type

CHAPTER 1

Introduction

INTRODUCTION

Malaria is a major global health challenge and remains a deadly disease with 3 billion people in the population at risk of infection[1]. Malaria is endemic to 91 countries in the tropical and subtropical regions of the globe[1]. There were around 216 million cases reported in 2016, resulting in around 445,000 deaths, with 80% of global cases occurring in 14 countries in sub-Saharan Africa and India[1].

Malaria sits on the world stage as one of the most high profile diseases and is one of the most heavily funded organisms destined for eradication although the resources are not always spread evenly between endemic countries[2, 3]. Reports indicate a general decline in malaria over the last decades however there are recent cases of countries rebounding from the brink of eradication as has been seen previously[4], including an increase of 5 million cases worldwide from that seen a year before in 2015[1]. The global reduction in malaria prevalence and related deaths is likely through an increased use of insecticide treated bed nets, indoor residual spraying and improved access to and availability of effective anti-malarials. Nonetheless, to achieve the World Health Organisation (WHO) vision of “A world free of malaria” a highly efficacious malaria vaccine is warranted.

Malaria in humans is caused by five species of the Apicomplexan protozoan *Plasmodium*. *Plasmodium falciparum* is the most prevalent human malaria parasite in Africa[5]. *P. vivax* is the most widespread human malaria parasite, constituting the majority of malarial cases in Asia, Central and South America, though it is often wrongly deemed to be of lower importance in terms of mortality and morbidity compared to *P. falciparum*[6]. Less common species include *P. malariae* and *P. ovale* which cause milder forms of malaria[7]. With importance to morbidity, *P. vivax* and *P. ovale* can form hypnozoites within the liver; metabolically active parasites waiting for reactivation by some as yet unknown trigger[8]. Relapses can often occur years later, if only the intermittent blood stage infections, and not the liver resident hypnozoites, are treated[9]. Finally and relatively

recently, zoonotic *P. knowlesi*, first surveyed in Sarawak (Malaysian Borneo)[10], was identified as the fifth species of *Plasmodium* that infects humans, though the natural hosts are macaque monkeys. Prior to this many malaria cases in Southeast Asia were microscopically misdiagnosed as *P. malariae*. However it has been shown that the severity and lethality of *P. knowlesi* infection is much greater than that of *P. malariae* infection[11].

The *Plasmodium* parasite is transmitted by female *Anopheles* mosquitoes and seeks to continuously cycle between the mosquito and vertebrate host. Many vertebrates are susceptible to malaria. *Plasmodia* species are known to infect sauropsids (birds and lizards), primates, bats and rodents[12]. Transmission can occur anywhere where the *Plasmodium* species, preferred vector and preferred host reside. The life cycle of malaria (discussed below) can be divided into three broad phases: vector stages, pre-erythrocytic stages and erythrocytic stages. Symptoms of malarial infection in humans are only exhibited in the erythrocytic or blood stage which can include cycles of fever, headaches and nausea as the parasite asexually reproduces. Severe malaria can result in anaemia, organ failure and cerebral malaria in complicated cases and- in non-immune individuals- will often result in death if left untreated as acute parasite infection is non-limiting.

THE LIFE CYCLE OF *PLASMODIUM*

The initiation of the life cycle of *Plasmodium* in the vertebrate host (pre-erythrocytic stages) starts with the injection of infectious sporozoites from an infected female *Anopheles* mosquito. The mosquito probes the skin before taking a blood meal which involves injecting saliva, which contains factors that help locate a blood vessel through vasodilation amongst other mechanisms[13]. This probing and salivation release the parasite from the mosquito salivary glands. The parasite in its elongated sporozoite form then glides in the skin, trickling out the dermis until it finds a capillary blood vessel[14]. Finding a dermal capillary, the parasite traverses the endothelium and passes into the

bloodstream[15]. From here, the parasite will travel to the liver to seek a hepatocyte to invade and develop into its liver stage form, an exo-erythrocytic form (EEF).

The sporozoite must cross the liver sinusoid, made up of fenestrated liver sinusoid endothelial cells (LSECs) and Kupffer cells (KCs), resident macrophages in the liver, in order to reach the parenchyma and hepatocytes below[16]. Sporozoites traverse several hepatocytes before invading properly[17] and initiating EEF development. Sporozoites traverse in a transient vacuole[18], whereas genuine invasion results in parasitophorous vacuole (PV) formation that protects the parasite from the cytoplasm of the hepatocyte. The PV is enclosed by a membrane, the parasitophorous vacuole membrane (PVM), composed of host cell origin which is then rapidly modified with parasite-derived proteins[19]. The EEF grows and undergoes mitotic division of the genome without cytokinesis (schizogony) within the PV to form thousands of merozoites inside the hepatocyte[20]. Membrane encompassed merozoites (merosomes) bud into the sinusoid of the liver and release merozoites into the bloodstream, where upon they can invade erythrocytes. This is the initiation of the erythrocytic stages and up until this point, an infected individual is unaware of the parasites in their body with the erythrocytic stages of malaria being the only stage responsible for the pathogenesis associated with malaria.

The merozoites replicate asexually, with invasion, development and rupture of erythrocytes occurring every 24, 48 or 72 hours, depending on the species of *Plasmodium*[21]. Merozoites invade erythrocytes through a variety of redundant mechanisms, utilising different erythrocyte and parasite membrane proteins. Erythrocyte proteins acting as receptors include glycophorins[22-24], complement receptor 1[25], basigin[26] and duffy antigen receptor for chemokines (DARC) in *P. vivax* invasion[27] with the parasite interacting via different erythrocyte binding ligand (EBLs), reticulocyte binding protein (RBPs) and Duffy-binding proteins (DBPs), released from the micronemes and rhoptries of the parasite, though many interactions have still to be resolved[28]. Once inside the erythrocyte, the merozoite replicates to form a schizont of 16-32 merozoites. Parasites

rupture their host erythrocyte synchronously before subsequently invading new nearby erythrocytes[21]. These processes induce a milieu of inflammatory cytokines and molecules from the immune system which induces the bouts of fever which are famously associated with malarial disease[29]. If left untreated this replication will continue, which in non-immune individuals can result in death by anaemia, organ failure and/or cerebral malaria as parasites sequester in different locations in the body. Cerebral malaria has been proposed to occur by several different mechanisms and the pathogenesis of severe malaria has also been found to vary depending on endemic location and age of host[30].

At some stage, although it is not fully determined what the trigger(s) are, some asexual blood stage parasites change and commit to development into gametocytes, the sexual stage of the parasite. This switch requires the gene *ap2-g* to be epigenetically derepressed[31] and through a positive feedback loop[32] produce increasingly levels of AP2-G, an AP2 domain DNA-binding protein transcription factor. AP2-G is the master regulator of sexual commitment[32, 33] and is a transcriptional activator for early gametocytogenesis genes[34]. Once fully mature, gametocytes are believed to remain dormant in G_0 phase of the cell cycle inside erythrocytes until uptake by a mosquito[35]. Both male and female gametocytes are required to be taken up by a mosquito to generate the next parasite progeny in the vector stages.

Following uptake of male and female gametocytes into a female mosquito, by means of blood meal, the mature gametocytes escape from their erythrocytes and form gametes. Inside the midgut of the mosquito, both gametocytes round up with the male gametocyte undergoing three rounds of rapid replication to form 8 motile microgametes which leave the erythrocyte and adhere to neighbouring erythrocytes in an observable process called exflagellation[36]. Differentiation into gametes is caused by two environmental triggers: a drop in temperature[37] and mosquito derived xanthurenic acid[38, 39]. Microgametes go in search for a female macrogamete, to initiate fertilisation, with fusion of plasma membranes and nuclear fusion to form a tetraploid, or diploid zygote[40]. The zygote

transforms into a motile ookinete, which traverses the midgut epithelium and embeds itself between the basement epithelium and basal lamina to form an oocyst[41]. Following successful embedding, mitotic division occurs[40] and sporozoites are formed within syncytial lobes of the oocyst called sporoblasts[42] in a process called sporogony. Rupture of the oocysts release sporozoites into the haemocoel of the mosquito either through sporozoites actively escaping the oocyst membrane[43] or the action of a protease[44]. Once in the haemocoel, the sporozoites travel via haemolymph circulation and attach and invade salivary glands to further mature[45]. The host mosquito now harbours infectious sporozoites ready to be released during probing and salivation, prior to blood feeding, thus restarting the *Plasmodium* life cycle.

THE PRE-ERYTHROCYTIC STAGES OF MALARIA

The development of an efficacious vaccine against malaria is still a long way away, though major advances in technology and techniques have allowed more information to be derived from the host-parasite interactions of the parasite at its different life stages and the immune responses that are induced. Alongside advances in vaccine development, there is now increased understanding of how to best induce appropriate immune responses to provide protection against parasite development in these stages. Focusing on the pre-erythrocytic stages, I will review the biological and immunological processes that occur at these stages, derived from murine and human studies and highlight recent advances in these fields. Creating a vaccine against the pre-erythrocytic stage is attractive because elimination of the parasite at an early time point following sporozoite injection by mosquito and prior to fulminant blood stage infection would prevent all malarial pathology associated with the erythrocytic stage and also prevent further transmission of the parasite.

Previously, the pre-erythrocytic stages of *Plasmodium* infection were considered a singular process. However, given the changes in parasite form and migration through multiple locations in the vertebrate host, we, amongst others, have seen it pertinent to

reconsider these stages in terms of these differences. While sporozoites and EEFs are perpetually interlinked, classical immunology would suggest that sporozoite and EEFs would be dealt with quite differently. Considerations of the environment in which the parasite is found will also likely have an impact on the immune responses that are induced.

HOST-PARASITE INTERACTIONS

Entry and exit from the skin

Sporozoites are injected intradermally into the skin of the host, by mosquito bite, where they search for a blood vessel in order to travel to the liver. Experimentally, only around 100 sporozoites are injected into the skin by a single infected mosquito, though inoculum dose and ability of infected mosquitoes to inject sporozoites varies[46], with around 1-2.5 sporozoites per second released from the proboscis[47, 48]. There is recent evidence from human and mouse studies that mosquitoes with high numbers of sporozoites in the salivary gland post feeding are more likely to have transmitted the parasite[49]. New advances with the engineering of enamel coated glass pipettes may allow more physiological injection of sporozoites intradermally for intravital imaging rather than that previously executed using metal needles[50].

Sporozoites move in the skin and liver by gliding on the extracellular substrate using the surface Thrombospondin Related Anonymous Protein (TRAP) that connects to a submembrane actin-myosin motor, which propels the parasite forward[51]. TRAP originates in the microneme organelles at the apical end of the sporozoite[52], which also release other proteins important for adhesion and motility[53]. In humans, the αv-subunit of integrins, with a preference for $\alpha v\beta 3$ integrins, are the direct host receptors for *P. falciparum* TRAP[54]. Sporozoites glide at an average speed of 1-2μm/s[55] but show an increasingly constrained motility at the inoculation site. The peak of sporozoite motile and dispersal activity occurs in the first 15 minutes, with more sporozoites exhibiting a

circling rather than meandering behaviour as time progresses[15]. Sporozoites face a tripartite fate once deposited in the skin[14, 55] with ~60% parasites remaining in the skin (sporozoites start to be killed off after 3 hours in the dermis[14]) and the other sporozoites either successfully enter the bloodstream via a blood vessel (25%) or enter a lymphatic vessel (15%)[55]. The sporozoite uses gliding motility and cell traversal to move between different cells including fibroblasts and leukocytes to reach a vessel[56] with traversal through endothelial cells to finally reach the lumen of the vessel. Several proteins that have important roles in gliding and cell traversal have been investigated for role in sporozoite exit from the skin. TRAP is essential for gliding[57, 58]; the proteins Sporozoite microneme Protein Essential for Cell Traversal (SPECT) and Perforin-Like Protein 1 (PLP1) are important for cell traversal of phagocytes, to avoid clearance and prevent infection of cells in the skin which the parasite is not destined for[59]. Also, TRAP-Like Protein (TLP) and Phospholipase (PL) both may have a role in sporozoite traversal in the dermis as parasite liver loads are significantly lower in mice receiving intradermal injections of murine infective *P. berghei PbTLP*$^{-}$ [60] or PbPL knock-out[61] (KO) parasites compared to wild-type (WT) parasites. Invasion of blood vessels seems to be aided by some structural tropism, with a preference for blood vessels with a similar curvature to the sporozoite[60] that strengthens the argument that sporozoites are guided to blood vessels more by physical characteristics than a reliance of chemotactic signals[61].

Entering the lymphatics is considered a dead end for the parasite[62] in terms of development as sporozoites become trapped in the proximal draining lymph node where they mostly associate or are taken up by CD11c+ dendritic cells (DCs) and while EEFs do form in endothelial cells, they do not reach full development akin to intra-hepatocyte development[55]. Interestingly, EEF development has also been shown to occur at the inoculation site in the skin[63, 64]. Up to 10% of sporozoite remaining in the skin can develop into EEFs, which are comparable to those seen in the hepatocytes, but with a reduced susceptibility to primaquine[63, 64]. However, the ability to induce a blood stage infection *in vivo* could not be demonstrated[63, 64]. Only one study could induce parasitaemia, by

intravenous injection of skin derived *P. berghei* merozoites, in naïve mice[63]. In fact, *P. berghei* has also been found growing in immunoprivileged hair follicles[63] although the possibility that they could represent a dormant reservoir has not been further investigated and it is not known how this may translate to human *Plasmodia*. However, sporozoites that lack the N-terminus of Circumsporozoite Protein (CSP), that masks the C-terminal Type I Thrombospondin Repeat (TSR) motif to prevent invasion of hepatocytes until the right time, cannot leave the skin but subsequently develop and induce a blood stage infection *in situ*, indicating a key role for CSP conformation in maintaining the sporozoite in a migratory state[65]. CSP is the major surface protein of the sporozoite, attached to the parasite plasma membrane (PPM) by a C-terminal glycosylphosphatidylinositol anchor[66]. It is a conserved protein across *Plasmodia* species with conserved N-terminal and C-terminal regions flanking a central species-specific repeat section[67]. CSP is necessary for sporozoite development and hepatocyte targeting[68] and is constantly shed from the sporozoite during gliding motility[69].

Seeking a hepatocyte

Once sporozoites have traversed the endothelia of dermal blood vessels and entered the bloodstream, the destination they seek is the hepatocytes of the liver. Development in hepatocytes in mammals is an obligatory step in the *Plasmodia* life cycle and sporozoites are passively transported to the liver sinusoid in a matter of minutes[70]. The liver sinusoid is the open pore capillary network of the liver which perfuses the parenchyma plates of hepatocytes made up of Liver Sinusoid Endothelial Cells (LSECs), whose fenestrations allows passage of small molecules across their cytoplasm into the perisinusoidal space of Disse which separates the vessel from the hepatocytes[71]. The sinusoid also contains Kupffer cells (KCs), vascular resident macrophages, found in the lumen, and stellate cells, in the space of Disse, which secrete Heparan Sulphated Proteoglycans (HSPGs). Sporozoites passing through the liver in the blood are essentially stalled in the sinusoid and associate with the lumen by interaction between the parasite surface CSP with HSPGs[72] that protrude through the LSEC fenestrations,

like a signal indicating that the sporozoite has reached the liver[73]. Sporozoites need to traverse the sinusoid to arrive at the hepatocytes. Sporozoites can cross the endothelium in a number of ways. They can traverse KCs[16, 74, 75], LSECs[16] or migrate between LSECs[16] with the vast majority of entry events being traversal related through KCs[16]. Cell traversal seems to act two-fold, as the quickest way to get across the sinusoid to the hepatocytes and as a mechanism to avoid phagocytosis by KCs[16]. Traversal of KCs has been proposed to occur by interaction of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) on the sporozoite surface with CD68, a transmembrane glycoprotein, expressed on cells of the macrophage lineage, with interference of this interaction conferring protection from sporozoite challenge[76].

The CSP-HSPG interaction as well as acting as the pathway marker is also believed to be the driver of shifting sporozoites into an invasive phenotype[77]. The sporozoites exhibit cell traversal as a normal phenotype, however when they come into contact with the uniquely high level of sulphation on HSPGs as seen in the liver, parasite calcium dependent protein kinase 6 (CDPK6) is activated[77] that leads to N-terminal CSP cleavage by a cysteine protease[78], which in turn exposes the TSR C-terminal fragment[65]. This exposure of the TSR is the indicator of an invasive phenotype. However, it is unclear when sporozoites switch to invasive phenotype, which could occur in the sinusoid lumen, later in the space of Disse or in the parenchyma. Switching to invasion appears not to be a binary process, with several signals likely required to execute a fully invasive phenotype[79] and thus sporozoites are seen to continue to exhibit cell traversal, passing through several hepatocytes before 'choosing' a hepatocyte to invade and develop in[17]. It has been shown that sporozoites traverse hepatocytes using transient vacuoles, which they can escape from before leaving the cell through the action of PLP1[18], though whether this is the case in other cell types it is not known. SPECT and PLP1 have been shown to be essential for human hepatocyte traversal of *P. falciparum* and that traversal is important for invasion for human hepatocytes in humanised mice *in vivo*[80]. A further traversal related protein, LIMP (referring to the sporozoite phenotype when the protein

is epitope tagged), has been characterised with KO parasites Δ*limp* showing a severe reduction in gliding and adhesion to hepatocytes, a complete inability to traverse hepatocytes and a vastly reduced ability to invade hepatocytes[81].

The trigger for the unmasking of the TSR of CSP and the exact interactions that the TSR has with the hepatocyte to initiate invasion are not known. Using experimental genetics, several proteins have been found to have critical and major roles in invasion, but the actual series of events that occur during invasion have not been completely realised, in contrast to the extensive knowledge of events occurring during erythrocyte invasion of merozoites[28]. Sporozoite invasion of hepatocytes has been shown to be influenced by cGMP and Ca^{2+} signalling with an essential and important role for cGMP-dependent protein kinase (PKG) and calcium-dependent protein kinase 4 (CDPK4) respectively, with invasion affected by the enzymes limiting sporozoite motility[82]. TRAP binds to HSPGs through its extracellular domain and binds the actin-myosin motor through its cytoplasmic domain[83] using aldolase as a bridge[84, 85]. P52 and P36 are members of the 6-cys protein family and are likely interlinked in their invasive roles. 6-cys proteins contain one or multiple s48/45 domains, a conserved protein fold containing 6-cysteines which can form disulphide bridges[86]. Proteins containing these domains have been found on the surface of gametes, sporozoites and merozoites with many having adhesion related functions[86]. Deletions or disruptions of P52 and/or P36 genes in *P. berghei*[87, 88], *P. yoelii*[89] and *P. falciparum*[88, 90] have been shown to result in inabilities to invade or subsequent disruption in PVM and EEF development. CD81 and SR-BI are hepatocyte membrane proteins required for invasion but in *P. berghei*, *P. yoelii* and *P. falciparum*, the different species have differing usage of the receptors to mediate invasion with P36 mediating these interactions[91]. Invasion results with the movement of the parasite through a moving junction involving actin remodelling[92] and invagination of the hepatocyte. A number of rhoptry proteins have been shown to be important in the formation of the moving junction[93, 94], though the roles of rhoptry proteins in sporozoite invasion have been less well understood in comparison to their role in merozoite invasion[28]. During invasion,

TRAP also provides the traction for the parasite to move against and as such is translocated to the posterior end of the parasite where it is then cleaved to release the bond with HSPG[58].

Thus, the parasite has successfully invaded and now resides in a parasitophorous vacuole (PV) encapsulated by a parasitophorous vacuole membrane (PVM) of host cell origin, essentially evading detection inside the cell and destruction through the endosomal-lysosomal pathway. The next stage begins with the parasite changing from an invasive phenotype, by clearing its micronemes[95], to a replicative and metabolically active phenotype. Upon invasion, the sporozoite localises proximal to the hepatocyte nucleus and endoplasmic reticulum[19]. The sporozoite undergoes cytoskeleton remodelling starting with a hump proximal to its nucleus. The inner membrane complex (IMC) breaks down, the micronemes and contents are released into the PV and the parasite rounds up, containing only the organelles it requires for biosynthesis[96].

Development inside a hepatocyte

In the hepatocyte, the parasite remodels the PVM with parasite-derived proteins to allow it to complete EEF development[97]. Many proteins associate with the PVM providing different functions to ensure survival. Although the parasite is metabolically capable, the acquisition of host factors and metabolites surely aids its development. Only two sets of molecules have been formally shown to have host-PVM interactions and these are with respect to host factor acquisition. Both Exported Protein 1 (EXP1) and Upregulated In Sporozoites gene 3 (UIS3) protein are expressed early on in EEF development[45, 98] and have been shown to recruit Apolipoprotein H (ApoH)[99] and Liver Fatty Acid Binding Protein 1 (FABP1)[100] respectively which are proposed to be crucial for EEF development. UIS3 is essential for development[101], and while an EXP1 knock-out could not be generated, deletion of the C-terminal fragment of EXP1 that interacts with ApoH significantly reduced liver parasite burden[99]. Acquisition of other metabolites from the host including lipids[102-104], glucose[105], arginine[106], biotin[107] and metal ions[108-110] have also

been shown to promote EEF development. Of the parasite expressed membrane transporters[109-111] described, with two being described as required for liver stage infection[110, 111], all have been identified in the PPM and until recently none have been characterised at the PVM. Aquaporin-3 has been shown to locate to the PVM and its absence significantly reduces EEF development but whether a disruption in transport of water and glycerol in *Plasmodium* causes this defect has yet to be determined[112]. It has been noted that molecules up to 855Da can pass through the PVM[19] but how larger molecules make it through remains a mystery. Several publications have pointed to methods relating to vesicular transport involving the endocytic[113, 114] and autophagy[115] pathways as additional methods for acquisition of nutrients from the host, possibly through the use of the tubovesicular network of the parasite[116]. Parasite-derived subversion of the autophagy machinery has also been described[117], perhaps ensuring the parasite can benefit fully from the nutrition autophagy could supply.

Other parasite-derived proteins have also been shown to have an association with the PVM at various stages in EEF development, however their functions remain a mystery despite absences in these proteins leading to impaired or completely ablated EEF development[97]. The early expressed PVM protein encoded by Upregulated in Infectious Sporozoites gene 4 (UIS4) [45] is crucial for EEF development[118] and like UIS3, is highly transcribed in the sporozoite[119] presumably to allow quick remodelling of the PVM, with translational repression preventing UIS4 protein expression until the sporozoite formatively invades a hepatocyte[120]. Liver-specific proteins 1 and 2 (LISP1 and LISP2) have peaks of expression later in EEF development[121, 122]. LISP1 KO parasites, *Lisp1*Δ, have an impaired ability to rupture the PVM[121] and LISP2 have also been shown to translocate into the hepatocyte cytosol and nucleus where they are proposed to modify the host environment for the benefit of the parasite[122]. Recently, Sporozoite surface Protein Essential for Liver stage Development (SPELD), as the name suggests, is a protein expressed in sporozoites and early stage EEFs and is required for early EEF development[123]. SPELD localises at the PVM at 17 hours but not later[123]. *pbspeld* KO

parasites arrest with a very significant impairment in development and no merozoites are formed when mice receive infectious mosquito bites[123]. Regarding the disposal of waste products from the EEF, there is no clear evidence for how the parasite manages this while remaining hidden from the host with no description of a food vacuole as described in blood stage parasites[124]. Nonetheless, a potential iron detoxification mechanism has been proposed, as *Plasmodium* express an orthologue of a plant vacuolar iron transporter (VIT) which transports Fe^{2+} ions. VIT has been shown to be important for parasite EEF and blood stage (BS) growth[125].

The process of DNA replication and division of the parasite begins around 20 hours after invasion of the hepatocyte[126]. It involves schizogony, an obscure variation of mitosis to that exhibited normally in eukaryotes. The nucleus undergoes division and replication 13-14 times to generate a syncytium, a multinucleated schizont containing tens of thousands of nuclei[127]. At the same time, the apicoplast and mitochondria form intertwined branched structures, appearing to remain singular organelles and do not associate with the nuclei[128]. Entering the cytomere stage after nuclear division has been completed; the PPM invaginates and leads to the partitioning of the cytoplasm, nuclei associate with the plasma membrane with close proximity to the apicoplast, and the mitochondria clump in the centre. In a synchronous fashion, the apicoplast divides, followed by the mitochondria and the invagination of the PPM ultimately results in cytokinesis and daughter merozoite formation. The RNA-binding protein PlasMei2 has been shown to be a critical factor in late schizogony[129]. *P. yoelii plasmei2*$^{-}$ KO parasites exhibit incorrect DNA segregation and organelle maturation with no cytomere formation and a failure in merozoites formation[129]. Autophagy (ATG)-related protein 8, ATG8, an ubiquitin-like protein associated with autophagosome formation[130], has also been implicated in apicoplast maintenance, in addition to microneme dissolution after invasion and merozoite differentiation[95]. With relevance to hypnozoite forming *Plasmodia*, the hypnozoite EEFs of *P. vivax* in human liver-chimeric mice do not undergo schizogony

but stay as dormant trophozoites[8], though the processes involved in preventing schizogony or later re-activation of hypnozoites are not known.

Egress of merozoites out of the hepatocyte is the next step in the journey. The release of merozoites and the death of the host hepatocyte is very much interlinked, to ensure the parasite evades destruction by hepatocyte-driven and immune system-driven responses for the greatest possible time and reach the blood with the minimum of fuss. Egress again involves the parasite using the host cell for its own benefit, before killing it and leaving little trace for the immune system[131]. First the PVM is broken down inside the hepatocyte which is mediated by phospholipase (PL)[132]. LISP1 is also important for PVM rupture[121], however it has no functional protease domain and its mechanism of action has yet to be formally shown: it may act as a receptor or upstream molecule for PL or other protease action. The release of merozoites into the hepatocyte cytoplasm is closely followed by the disintegration of the host mitochondria essentially preventing the cell from producing ATP[131].Upon PVM rupture, the actin cytoskeleton of the hepatocyte dissociates from the plasma membrane with a concomitant modulation of membrane content possibly caused by a disruption in protein biosynthesis[20]. This actin-membrane disruption leads to detachment of the hepatocyte from the parenchyma. The dissociated hepatocyte, with the parasites inside, passes through the sinusoid, squeezing through the gaps between LSECs, whereby it comes into contact with the shear forces in the blood vessel which cause merosomes (pockets of membrane containing merozoites) to bud off from the hepatocyte[20]. These merosomes have then been shown, in rodent models, to release the merozoites in the pulmonary capillaries of the lung[133], presumably to enhance erythrocyte infection. Over time, the membrane of what remains of the hepatocyte, after most of the parasites have been released, loses phosphatidylserine asymmetry and membrane integrity[131] which signals to phagocytes to engulf the remains. The wrapping of the merozoites inside a hepatocyte derived membrane also mediates extra protection against KCs, who would recognise merozoites as foreign, and the removal of major histocompatibility complex (MHC) class I molecules from the merosome

prevents recognition by T cells[131]. All in all, the parasite has a very elegant and co-ordinated approach to entry, development and egress out of hepatocytes to ensure full development and survival.

IMMUNE RESPONSES TO THE PRE-ERYTHROCYTIC STAGES

There is limited data for naturally acquired immunity against the pre-erythrocytic stages of *Plasmodium* infection, with contributions from antibodies and T-cells[134] but ultimately never the acquisition of sterile protection. A number of factors contribute to this lack of protective immunity including the size and site of sporozoite inoculum, the tolerogenicity of the liver, protection of the EEF by PVM and immunosuppressive nature of the blood stages[135]. Thus, it is imperative to understand the immune responses that are induced by the parasite in order to be able to enhance them through vaccination and induce, develop and sustain sterile protection. While clinically these stages may appear silent, immune responses are most definitely being induced, with the parasite simultaneously trying to avoid them. Through experimentation, mainly using mouse models, the immune responses occurring during the pre-erythrocytic stages, particularly those that can induce protection, are being established. With this knowledge, the next generations of malaria vaccines can be developed to enhance immune responses and subvert parasite immune evasion.

Innate immune response evasion and exploitation

The first induction of host immunological responses occurs as soon as the mosquito probes the skin looking for a blood vessel to feed from. The saliva of the mosquito has immunomodulatory properties[136] and probing contributes to Damage-Associated Molecular Patterns (DAMPs) and presumably Pathogen-Associated Molecular Patterns (PAMPs), although no sporozoite PAMPs have been discovered yet[137]. Mast cells have also been shown to be recruited upon probing by mosquitos[138]. Mast cell degranulation and release of histamine increases extravasation of fluid in blood vessels[139], which the sporozoites may exploit in their search for a dermal blood vessel. This increase in

vascular permeability also leads to influx of leukocytes[138], including neutrophils and resident myeloid cells first, followed by the recruitment of monocytes at the inoculation site and proximal draining lymph node[140]. The skin stage is possibly the most overlooked part of the pre-erythrocytic stages of malaria. The longest time that the parasite is exposed and extracellular in the vertebrate host is when sporozoites are deposited in the skin, so this would be a great avenue for targeting[70].

Upon entering the liver, *Plasmodium* elicits further responses from the innate immune system with the secretion of Type I and II interferons (IFNs) by hepatocytes. Also produced in response to *Plasmodium* blood stages[141], *in vivo* type I IFN release by hepatocytes infected with *P. berghei*[142] or *P. yoelii*[143] results in recruitment of leukocytes to the liver by signalling through the cytosolic receptor melanoma differentiation-associated protein 5 (MDA5). This suggests host sensing of parasite RNA with further signalling through mitochondrial antiviral signalling protein (MAVS) and transcription factors Interferon-Regulatory Factors 3 (IRF3) and IRF7[142, 143]. While this release of Type I IFN does not peak until the final stages of EEF development in mouse models and thus subsequently does not affect EEF development upon primary infection, their release has been shown to recruit Natural Killer (NK) T cells which have been shown to be crucial in combating subsequent infections through IFN-γ production[143]. The role of Type I IFNs following sensing of hepatic parasites in human infection and the subsequent downstream responses remain to be determined.

Parasite antigen presentation and T cell priming

Early on, CD8+ T cells were shown in mouse models to be the critical leukocyte for pre-erythrocytic driven protection following vaccination with radiation attenuated sporozoites (RAS)[144] with a correlation between CD8+ T cells and protection later observed in humans vaccine studies[145, 146]. The role of CD4+ T cells in pre-erythrocytic immunity is less clear. In mouse models their role seems to be dependent on mouse strain, vaccine strategy and parasite used[147]. In humans, different vaccination strategies indicate

conflicting correlations between CD4+ T cells and protection[145, 146, 148]. The process of pre-erythrocytic parasite antigen processing and presentation and T cell priming is poorly understood. Vertebrate hosts are exposed to sporozoite antigens as sporozoites migrate and traverse cells or through cross-presentation following sporozoite degradation by phagocytosis. Exposure to liver stage antigens occurs following arrested development of the EEF or following phagocytosis of the dead infected hepatocyte after the merosomes have been released[149].

Sporozoites antigens are presented by cells in the liver as well as in the draining lymph nodes (dLNs). Despite entering the lymphatics and reaching a developmental dead end, the sporozoite plays a crucial role in priming the immune system against sporozoite antigens. In the skin dLN, migratory sporozoites have been shown to prime protective *P. yoelii* CSP-specific CD8+ T cells[150], with *P. berghei* CSP-specific CD8+ T cells being primed by dLN–resident CD8α+ DCs[151]. While data suggests that the skin dLN is the major site for priming of CD8+ T cells specific for sporozoite antigens following natural intradermal inoculation of sporozoites[150], dermal inoculation of live attenuated sporozoites induces weaker CD8+ T cell responses than intravenous inoculation[152]. Previously this was thought to be a result of a reduced number of parasites reaching the liver but it now seems that inoculation and prolonged exposure in the skin induces more regulatory adaptive immune responses with a development preference for IL-10 producing B and T cells, though the reason for this and the interactions in the skin that induce this dampening of immune responses in liver and skin dLNs is not known. In addition to the dLN, intravenous injection of RAS leads to priming of CD8+ T cells by CD8α+ DCs in the spleen[153].

Antigen presentation and CD8+ T cell priming in the liver is equally poorly understood. LSECs, KCs, DCs and hepatocytes in the liver can all present antigen with differing degrees of efficiency which most often lead to tolerance in the liver, however immune

responses can be induced. The liver is inherently a tolerogenic organ given the large amount of blood and lymph that flows through it, containing food breakdown products and microbial antigens from the gastrointestinal tract and systemic blood circulation. A continuous production of IL-10 in the liver maintains this tolerogenic environment[154], though in the case of infection, the switch can be made. IFN production can lead to an upregulation of major histocompatibility complex (MHC) class I and II presentation although how tolerogenic signals are overcome is not known with the likely involvement of many factors making the process very complex[155].

LSECs are likely heavily exposed to sporozoite surface antigens including CSP, which can translocate into the cytosol following cell contact[156], or antigens can be cross-presented by LSECs acting as antigen presenting cells (APCs) to activate naïve CD8+[157] and CD4+ T cells[158]. However, most entry events by sporozoites seeking the hepatic parenchyma occur with an involvement with KCs[16]. Traversal would lead to release of sporozoite antigens in the KC cytoplasm, which has been shown for CSP *in vitro*[74]. However, it is unknown what the role of KCs is in relation to immunity and antigen presentation. CSP has shown to have a ribotoxic effect on macrophages[159] and there is *in vitro* evidence that KCs do not survive sporozoite traversal and undergo apoptosis[160]. This would limit the capacity of KCs as APCs. However, *in vivo* responses of KCs following challenge of naïve and RAS-immunised mice are vastly different. Upon challenge, immunised mice induced an upregulation of antigen presentation with an increase in MHC class I (MHC-I) molecules, costimulatory molecules and IL-12 expression on KCs[161]. The mechanism of RAS inducing this activation of KCs is not known, but it seems to mediate the transition of the liver from a tolerogenic to a more inflammatory environment. Inflammation in other systems has also been shown to abrogate tolerance induction by KCs[162]. This suggests that KCs do not have a definitive role as APCs in the context of malaria, but activation of KCs (by an unknown mechanism) leads to increased capacity for phagocytosis by KCs in immunised individuals. Other data suggests a similar notion that sporozoites use their cell traversal capabilities to be

able to avoid KCs to successfully infect the liver, otherwise they would be rapidly phagocytosed[16]. Further research is required to fully determine the roles of LSECs and KCs in parasite antigen presentation in the liver and their capacity to prime and recall effector T cell responses, rather than induce tolerance[163, 164].

Hepatic DCs are immature at resting state[154]. The exact mechanisms of recruitment and/or priming of DCs associated with *Plasmodium* liver infection is poorly understood. Hepatic DCs are located in the periportal and pericentral regions[154] and draining lymph nodes[165]. Protective immunity induced in a *P. berghei* model presented an association with CD8α+ DC accumulation in the liver after RAS immunisation[166, 167]. This recruitment and movement of DCs to the liver takes time. There are several options that might occur to activate CD8+ T cells in the liver. These DCs may travel back to the portal regions and draining lymph nodes to present antigen and activate CD8+ T cells, or directly present and activate CD8+ T cells in the liver sinusoid. The fact that large numbers of CD8+ T cells are required for protection[168] indicates that the elimination of infected hepatocytes is an inefficient process which likely requires large numbers of DCs for optimal antigen presentation and CD8+ T cell activation[149]. More work is required to determine where and how hepatic DCs sample and present parasite antigens and the mechanism by which CD8+ T cells are activated and act upon infected hepatocytes. Given the priming seen in the skin dLNs, it also been proposed that liver dLNs could be most likely site for priming of CD8+ T cells against liver stage and blood stage antigens[149]. Hepatic DCs have also been shown to be able to present antigens after phagocytosing dead infected hepatocytes (following merosome release or EEF arrest)[169] but whether these migrate to the dLNs is unknown[170].

Parasite development in hepatocytes (in addition to EEF development in the skin) is the only time EEF antigens are expressed so hepatocytes are a crucial cell type for presentation of these antigens as well as signalling their infected status. While antigen presentation on hepatocytes usually results in tolerisation[170], hepatocytes have been

shown to successfully prime naïve CSP-specific CD8+ T cells[171] and convert systemically primed effector CD8+ T cells to become liver-resident memory cells[172]. Parasite-derived peptides have been shown to reach the cytosol of the hepatocyte, with loading of MHC-I molecules occurring in an endosomal-independent TAP-dependent manner[173, 174]. Using bone chimeras, it has also been determined that the elimination of hepatocytes occurs in an antigen dependent manner[150, 173, 175]. While the repertoire of proteins expressed by the EEF is vast, only a few proteins have been shown to induce antigen-specific CD8+ T cell driven responses that protect against sporozoite infection. That being said, these include sporozoite surface proteins[176, 177], proteins involved in traversal[178], EEF proteins[179] and PVM associated proteins[180]. This illustrates the range of proteins that can potentially be presented by hepatocytes and induce protective responses. Protective CD8+ T cell responses can also be induced using a parasite that expresses SIINFEKL from ovalbumin in the context of heat shock protein 70 (HSP70), indicating cytosolic parasite antigen can also be presented on hepatocytes[173].

Effector functions of CD8+ T cells

As mentioned before, a key role for CD8+ T cells in protective immunity was determined early on[144] with CD8+ T cell depletion abrogating protection[144, 181]. However now it has also been shown that for long lasting complete protection, a threshold of parasite-specific memory CD8+ T cells are required which is postulated to be 100-1000 times greater than that needed for protection against viral or bacterial pathogens[182]. The reason for this is not fully understood but it is generally accepted that very few sporozoites reach the liver, which is a massive organ and given the short duration of EEF development in mice (2 days), this means that a very large CD8+ T cell compartment would be required to find and kill all the infected hepatocytes. It has also been found, however, that fewer parasites also lead to reduced CD8+ T cell responses, presumably because it is harder to initiate proliferation and a large response if the target is so small[183]. Thus a fine balance connects the number of inoculated sporozoites and memory CD8+ T cells to drive an optimum response which will result in the killing of all infected hepatocytes and sterile

protection[183]. Naïve CD8+ T cells take several days to become fully activated, proliferate and gain effector function. Proliferation of adoptively transferred naïve CSP-specific transgenic CD8+ T cells could only be detected in the spleen after 2 days following immunisation with *P. yoelii* RAS[184]. While induction of IFN-γ production in transferred naïve CD8+ T cells was rapidly observable after 24 hours following antigen exposure with RAS, only mice harbouring activated effector CD8+ T cells at the time of challenge could kill infected hepatocytes[184]. Mice harbouring naïve CD8+ T cells at challenge did not induce significant levels of killing presumably because all EEFs had developed and merozoites had been released before the CD8+ T cells could be activated, proliferate and exercise their effector functions[184]. This stands in contrast to memory CD8+ T cells which can produce IFN-γ within 4 hours after reactivation[150]. In humans, the role of naïve CD8+ T cells may be different given the extended development of the EEF (~7 days depending on the species of *Plasmodium*). Immunisation with viral vectors against *P. falciparum* TRAP[185] indicated that fewer IFN-γ producing CD8+ T cells were required for protection in humans compared to that required in mice. Still CD8+ T cells are estimated to be looking for one infected hepatocyte out of 10^6 in mice and one out of 10^9 in humans which is quite the race against time with a single infected hepatocyte able to propagate a blood stage infection[186].

Following clearance of infections, a subset of effector CD8+ T cells differentiate into memory T cells: either central memory (T_{CM}) or effector memory T cells (T_{EM})[187]. Central memory T cells have shown to have a limited role in providing protection against sporozoite infection[188] with the majority of parasite-specific T cells in the liver following RAS immunisation exhibiting a T_{EM} phenotype[189]. Recently tissue resident memory (T_{RM}) CD8+ T cells have also been described as being crucial for protection of RAS immunised mice from sporozoite challenge[172]. Tissue resident T cells are a non-circulating population of memory T cells found in all non-lymphoid tissues with a distinct phenotype to T_{CM} and T_{EM}, expressing CD69[190] and low levels of KLRG1[172] and are retained in the

liver by their expression of CXCR6[191]. They have been shown to patrol the sinusoid[192] and targeting CD8+ T cells to the liver using a systemic prime and liver trap immunisation protects immunised mice from sporozoite challenge[172]. This immunisation strategy could be a great tool for the generation of new malaria vaccines to improve killing of EEFs in the liver.

As previously mentioned, infected hepatocytes are required to express and present parasite specific peptides on MHC molecules to be eliminated by the cognate CD8+ T cell[150, 173, 175]. It has been shown that antigen-specific[173, 193] and non-specific CD8+ T cells[193] cluster around EEFs. Through using antigenically different parasites it has also been shown that there is a lack of bystander effect in EEF elimination by CD8+ T cells[194]. The mechanism for elimination of EEFs by CD8+ T cells has been heavily researched; however, the exact mechanism is still to be determined. It is clear from mouse studies that depending on the strain of mouse and species of *Plasmodium* used, that the effector CD8+ T cell mechanisms differ[147, 195]. Effector molecules investigated include cytokines, cytotoxic proteins and death receptors. However, there is contention over whether cytotoxic granules can be released onto hepatocytes by CD8+ T cells given that significant contact between CD8+ T cell and target is normally required.

It has been suggested that contact-dependent killing can occur *in vitro* with the release of perforin[196] however; there is little *in vivo* evidence of contact dependent mechanisms for EEF elimination. Intravital imaging in mice has demonstrated that antigen-specific CD8+ T cells cluster around EEFs but contact between lymphocyte and hepatocyte was not conclusively demonstrated[173, 193]. Another study showed that parasite-specific CD8+ T cells are immobilised in the liver but no contact is made with EEFs[192]. Adoptively transferred CD8+ T cells have been shown to exhibit slow velocity[192, 193] and immobilisation in the liver for at least 3 days following transfer[192], which may be due to differing anatomical locations and microenvironmental changes altering local T cell differentiation[149] or a slowing to survey presenting hepatocytes in an antigen-specific

manner[197]. Thus, the lack of contact with EEFs but an observed reduction in EEFs following adoptive transfer of parasite-specific CD8+ T cells[192, 193] heavily suggests a role for soluble factors and contact independent mechanisms i.e. not the release of cytotoxic granules. In fact, using perforin$^{-/-}$ or granzyme B$^{-/-}$ KO mice or mice homozygous for Fas ligand (FasL) mutations indicated that these factors were dispensable for protecting RAS or viral vector immunised mice challenged with sporozoites[147, 198, 199].In addition, where clustering was seen, multiple EEF death phenotypes were observed[193] and protection could be maintained by adoptively transferring CD8+ T cells lacking the ability to produce IFN-γ and/or perforin[173] which indicates that multiple mechanisms are involved in protection. It has been found that T cells can monitor hepatocytes using trans-endothelial hepatocyte-lymphocyte interactions (TEHLIs) that stretch through the fenestrations of the LSECs avoiding the need for extravasation across the sinusoid[200]. However, given the division between lymphocyte and hepatocyte and the lack of evidence for extravasation *in vivo*, it is unlikely that sufficient contact is made by TEHLI to form a functional immunological synapse and allow cytotoxic granule release[201]. Thus, it has been proposed that CD8+ T cells in the liver use TEHLIs to survey hepatocytes, and when a cognate interaction has been made, the minimal contact of the TEHLI forms a stimulatory synapse that is sufficient to allow cytokines to be released and act on the infected hepatocytes[149, 202].

IFN-γ is generally considered the central mediator of protection against EEFs[203]. Recombinant IFN-γ was first shown to inhibit murine and human *Plasmodia* EEF development *in vitro*[204-206]. Then *in vivo*, systemic blockage of IFN-γ was shown to inhibit EEF development in immunised mice that were normally protected against sporozoite challenge[144]. The first CD8+ T cell response that definitively demonstrated that antigen-specific protection based on IFN-γ secretion was identified from a CD8+ T cell clone specific for an epitope from CSP with cross-specificity for *P. berghei* and *P. yoelii*[207]. IFN-γ from CD8+ T cells has been shown to function by activating the L-arginine-dependent

inducible production of nitric oxide synthase pathway, which was shown to be crucial for protection in RAS immunised mice[208]. IFN-γ production induces increased production of inducible nitric oxide synthase (iNOS), the enzyme that converts L-arginine to L-citrulline and nitric oxide. Nitric oxide then acts on several metabolic pathways by reaction with iron centres, formation of reactive oxygen species and nitrosation of nucleophilic centres[209] which are toxic to the EEF[208]. iNOS can act to inhibit EEF development[192] or kill the parasite in the hepatocyte completely[208]. This will likely depend on the concentration of CD8+ T cells and IFN-γ in the local area surrounding the EEF[149]. Long lasting protection, in mice immunised with *P. berghei* RAS or infectious sporozoites under drug prophylaxis, correlates with sustained IFN-γ responses from hepatic memory CD8+ T cells[210].

However, as mentioned before, multiple soluble effector molecules may act on infected hepatocytes as IFN-γ independent protection has been reported[147, 195]. It is becoming clear in mouse models that levels of protection are dependent on murine host strain and *Plasmodium* species. Comparing two commonly used mouse strains, C57BL/6 mice require more immunisations to induce a greater antigen-specific memory CD8+ T cell response and afford protection than in BALB/c and protection against *P. berghei* is more easily achieved than against *P. yoelii*[168]. Using mice that express the same MHC alleles, differences in protection have shown to be due to the murine genetic background[211]. In terms of effector molecules, blockage of IFN-γ in *P. yoelii* challenged RAS immunised B10.D2 mice had no effect on protection and only a partial effect in CD-1 mice[147]. *P. yoelii* or *P. berghei* challenge of IFN-$\gamma^{-/-}$ BALB/c mice, immunised by prime-boost (DCs and recombinant *Listeria monocytogenes*) to induce CSP-specific CD8+ T cell responses, showed a 35% and 50% reduction in protection respectively in the absence of IFN-γ[195]. This incomplete abolition of protection indicates that other effector molecules can mediate protection. In the same study, neutralisation of TNF-α reduced protection against *P. berghei* challenge by 40% and *P. yoelii* challenge by 85%[195]. Depletion of

perforin had no effect on protecting mice from *P. berghei* infection but reduced protection by 50% when mice were challenged with *P. yoelii*[195]. The role of perforin has not been fully determined as some models show correlation with protection while others indicate a dispensability for protection. It is particularly interesting that there is a correlation in RAS vaccinated human volunteers between perforin producing CD8+ T cells and protection[145]. Further work will be required to determine the role of perforin given that immunological synapses between T cells and hepatocytes have yet to be described *in vivo*. But this shows that, in addition to IFN-γ, other cytokines can have a role in protecting against sporozoite infection, indicating that CD8+ T cell mediated protection occurs in a multi-faceted manner. Consequently, in addition to a requirement for CD8+ T cells above a certain threshold, the ability of these cells to produce IFN-γ and other cytokines, alternatively or in concert, seems to define whether a host can be robustly protected from sporozoite infection.

Antibodies against *Plasmodium* sporozoites

Anti-sporozoite antibodies are induced by individuals living in malaria endemic areas, however antibody titres against sporozoite antigens are lower than those against blood stage antigens[212]. This is probably due to the much shorter time that the sporozoite is visible to the immune system, compared to merozoites. Passive transfer of IgG from immune adults was shown to reduce parasitaemia in children[213], and more recently administering monoclonal antibodies from individuals naturally exposed to *P. falciparum* has been shown to reduce parasite liver development in liver-chimeric humanised mice following *P. falciparum* challenge[214]. Using RAS, the first sporozoite-specific antibodies determined were those against CSP[215], the major sporozoite surface protein, with transfer of monoclonal antibodies shown to protect mice from sporozoite challenge[216]. This finding inspired the development of a CSP-based vaccine[217] which would then lead to the development of RTS,S/AS01, the most advanced malaria vaccine to date. There is an association between titres of anti-CSP antibodies and vaccine efficacy in RTS,S

trials[218]. Thus while antibodies have been shown to immobilise sporozoites in the skin[219], it is likely that a very high titre of anti-sporozoite antibodies would be required to prevent all sporozoites from leaving the skin and prevent sporozoites infecting hepatocytes if they manage to exit the dermis unscathed. Antibodies against other parasite antigens have also been identified with titres for TRAP and LSA-1 correlating with reduced incidence of clinical malaria in *P. falciparum* naturally exposed Kenyan children[220]. While antibodies clearly play a role in reducing *Plasmodium* sporozoite infections (and particularly acting against blood stage infection), complete protection from sporozoite challenge can be achieved in B-cell depleted mice[221], indicating that they are not the major player in providing protection against pre-erythrocytic immunity.

PRE-ERYTHROCYTIC STAGE VACCINES

Over the last 50 years, malaria vaccines have been gathering momentum. Despite the reduction in malaria incidence seen over the last couple of decades, elimination of malaria is highly unlikely without the advent, introduction and deployment of an efficacious vaccine. There have been major breakthroughs and movements in malaria vaccine generation however, it has become apparent that the complexity of *Plasmodium* compared to other pathogens such as viruses is slowing down vaccine development in terms of time from inception to successful, deployed vaccine.

The initial inklings that a malaria vaccine was possible, came from two major findings. Firstly, in endemic settings, individuals that are constantly exposed to infectious mosquito bites and malaria infection develop immunity against the disease over time[222]. While it is extremely rare, and thus probably undocumented, that individuals develop sterile immunity against parasites, asymptomatic malaria infections are very common. This development of immunity has been associated with age though the immunological basis has yet to be fully unravelled[222]. The other finding resulting in actual sterile protection, which spearheaded the malaria vaccine movement was the use of radiation treated sporozoites as an experimental vaccine. Multiple immunisations with sporozoites

attenuated by radiation, leading to random DNA damage, were first shown to protect mice[223] and then humans[224, 225] and non-human primates[226] from infectious non-irradiated sporozoite challenge in a stage[227] and species[228] specific manner. These findings show that the immune system develops following exposure to parasite antigens and upon reinfection these responses can be recalled to slow parasite replication in the blood or impact parasite development in the liver. In this way, a vaccine could be developed to prime and enhance the immune system to fight *Plasmodium* parasites. Here I focus on the development of pre-erythrocytic vaccines.

With the discovery that radiation-attenuated sporozoite (RAS) vaccination induces sterile protection, this should have been the end of malaria. However, the technicalities behind production of a RAS vaccine stymied its introduction. Challenges include dissection of salivary gland sporozoites from mosquitoes by hand (to date no automated machine exists) followed by purification, the exposure of sporozoites to a standardised level of radiation (too much will make sporozoites nonviable and non-immunogenic, too little and breakthrough blood stage infections could occur) and a suitable method of cryopreservation and transport to site, followed by appropriate storage.

While RAS was established as the 'gold standard' vaccine against pre-erythrocytic malaria, research became more focused on generating a subunit vaccine, akin to more traditional vaccines, which would probably be less onerous to manufacture under regulations.

Pre-erythrocytic subunit vaccines

Subunit vaccines to date targeting the pre-erythrocytic stages have been designed to induce antibody responses against the sporozoite or induce T cell responses against the EEFs. RTS,S/AS01 is the most advanced *P. falciparum* malaria vaccine which is currently seeking approval for licensure. RTS,S is based on the central repeat region and C-terminal region of *P. falciparum* CSP, which contains B cell and T cell epitopes respectively, conjugated to hepatitis B virus surface antigen (HBsAg). The latest

longitudinal results come from a large multi-site Phase III trial in Africa, conducted with children (5-17 months) and young infants (6-12 weeks) who received three doses of RTS,S (or control rabies or meningococcal serogroup C conjugate vaccines - C3C), one month apart, with an RTS,S booster (R3R) or a control boost (R3C - control boost was meningococcal serogroup C conjugate vaccine) at month 20[229]. Assessing the number of clinical malaria cases compared to control groups, vaccine efficacy of RTS,S/AS01 to prevent episodes of clinical malaria in children over 48 months was 36.3% in the R3R group and 28.3% in the R3C group. In young infants, over 38 months, vaccine efficacy was 25.9% in the R3R group and 18.3% in the R3C group[229]. The trial detailed a gradual waning of vaccine efficacy as has been previous noted[230, 231]. The booster dose in children provided incremental efficacy of 25.6% in the first 12 months following booster administration which drops to 16.2% by the study end 27 months later. In infants, incremental efficacy of the booster dose was 22.3% after 12 months and 17.5% after 18 months[229]. The efficacy of RTS,S/AS01 against severe malaria was much less pronounced than that preventing clinical episodes. In vaccinated children, RTS,S/AS01 induced anti-CSP antibody titres and anti-CSP CD4+ T cell responses, which have been proposed to correlate with protection[232, 233]. Thus RTS,S/AS01 induces modest vaccine efficacy with value gained from an additional booster dose. However there is concern over cases of meningitis and febrile convulsions following administration of RTS,S/AS01 which has yet to be explained[229]. In addition, it has been noted that children who received RTS,S/AS01 experienced more malaria episodes, than those in the control group, several years after vaccination possibly because while the vaccine provided immunity against sporozoites, the children had a delay in developing blood stage immunity[234]. It is unclear whether additional boosters every year or so will benefit. This notion, as well as safety, are being considered as RTS,S/AS01 is further analysed before it can be approved to be rolled out. With antibody titres likely to be the mode of action of RTS,S/AS01, additional steps are being taken in earlier stage clinical trials to improve antibody titres and thus protection by altering the dose regimen[235] or *P. falciparum* CSP composition in the vaccine[236, 237].

Subunit vaccines against the liver stage of *Plasmodium* infection have tended towards the need to induce high numbers of parasite-specific CD8+ T cells which target infected hepatocytes. As previously mentioned, a numerical threshold of CD8+ T cells is required to protect mice[182] from subsequent sporozoite challenge and therefore this requires the use of different vaccine platforms compared to those that are designed to induce high antibody titres. This has included the use of viral vectors to administer malaria antigens into the host[238]. While it is well accepted that boosting with subsequent immunisations improves a vaccine's efficacy, it seems that using heterologous viral vectors encoding the same antigen improves efficacy better than homologous boosting[239]. So far, the most effective regimen for inducing high levels of human malaria antigen-specific CD8+ T cell is using AdCh63 prime followed by MVA boost encoding *Pf*TRAP linked to a multi-epitope string of other malaria epitopes (ME-TRAP)[185, 238]. This vaccine induces IFN-γ producing CD8+ T cells observable in peripheral blood which correlates with protection from sporozoite challenge in humans[185]. Research is ongoing to determine other antigens that are presented on hepatocytes, including antigen combinations, which could produce large viral vectored vaccine-induced CD8+ T cell responses.

Whole sporozoite vaccines

With the concept of using multiple antigens in a vaccine and the less than satisfactory vaccine efficacy imparted particularly by RTS,S/AS01, research has returned to whole sporozoite vaccines (WSVs). As mentioned before, WSVs in animals and humans have been shown to induce protective responses[223-225 226]. WSV benefit from the exposure of the host to many different parasite antigens, not just the one or two encoded in the subunit vaccine. There are four WSV strategies currently under investigation in humans and animal models[240].

Published in 2002, vaccination with RAS was shown to promote long lasting sterile immunity against challenge, when individuals were immunised with a thousands of bites from *P. falciparum* infected mosquitoes[241]. Extensive research into using RAS as a

vaccine outside of experimental medicine followed this result with the development of Sanaria Inc., a company that is able to produce, purify, attenuate and cryopreserve a metabolically active, non-replicating *P. falciparum* sporozoite vaccine (PfSPZ) in a standardised manner than meets all the regulatory requirements of the United States Food and Drug Administration[242]. With the intention of delivering a radiation attenuated whole sporozoite vaccine, they produced a vaccine that could induce complete sterile protection, through multiple high dose intravenous immunisations[145]. While intravenous injection may not seem the best method of administration, it has been shown that intradermal or subcutaneous injection of PfSPZ induced weaker immune responses and failed to protect from infectious mosquito bite challenge[243]. However, recently it has been shown that intradermal vaccination using a needle and a laser to locally damage blood vessels near the injection site is a better proxy for mosquito infection than traditional needle-administered intradermal inoculation as it mimics the damage induced by the mosquito proboscis[244]. While induction of immune responses with this method was vastly improved compared to traditional intradermal inoculation, CD8+ T cell responses did not completely match those induced by intravenous inoculation. Nonetheless, immunisation with purified *P. yoelii* RAS by this method was able to completely protect against sporozoite challenge equivocal to intravenous immunisation[244]. The authors suggest that technical developments in vaccine administration could permit PfSPZ to be administered intradermally, which is less effort than intravenous injection and would be more cost-effective[244], though whether the same results are replicated in humans has yet to be determined.

Other whole sporozoite vaccines have entered clinical trials following on from advances in genetic manipulation of *Plasmodia*. Genetically attenuated parasites (GAPs) administered as sporozoites, were first developed in order to achieve parasites that arrest at a more precise time than occurring with irradiated sporozoites[101]. GAPs involve the knock-out (KO) of particular gene(s) that are crucial for liver stage development but do not affect other stages of the *Plasmodia* life cycle[240]. GAPs need to be safe attenuated

parasites (abrogating blood stage infection following sporozoite infection) but the parasite also needs to induce potent sterilising protective responses[240]. The first GAP to enter clinical testing in humans[146] was a double KO of two proteins of the 6-cys family (P52 and P36) which are vital for EEF development[87, 88, 91, 245]. But, upon high bite exposure from infected mosquitoes, a breakthrough infection with *p52⁻/p36⁻* occurred[146]. However, further removing the *sap1 (slarp)* gene from the parasite lead to a fully attenuated GAP following mosquito bite[246]. These two GAPs target the early stages of EEF development, however a GAP lacking *fabb/f*[247], a gene that encodes a protein involved in fatty acid synthesis, led to parasites that arrested later than early arresting RAS[248]. *fabb/f⁻* GAP gave long lasting sterile immunity against sporozoites challenge[248] as well as blood-stage challenge highlighting stage-transcending immunity[248, 249]. However, FabB/F is essential for *P. falciparum* to produce sporozoites and thus is not an option for human GAPs[250]. Nonetheless the generation of the GAP *lisp2⁻/plasmei2⁻*, a double KO in *P. yoelii*, showed very late liver arrest with no breakthrough infections and long lasting sterile immunity against infectious sporozoites with induction of liver resident memory CD8+ T cells[251]. With high conservation of these genes in human *Plasmodia*, the generation of a long lasting sterilely protecting human GAPs could be soon on the horizon.

A further WSV strategy that has shown promise is the administration of infectious sporozoites concomitantly with anti-malarial drugs (ChemoProphylaxis and Sporozoites – CPS). Similar to GAPs, drug prophylaxis would ensure full EEF development and thus increased antigen exposure to the host immune system, with the premise of inducing broader action immune responses. Immunisation of sporozoites by mosquito bite[252, 253] or intravenous injection[148] with chloroquine cover provided complete sterile protection. While efficacy against heterologous challenge was shown to be limited[254], continual administration of chloroquine not appearing to be the most appropriate regimen considering chloroquine resistance in endemic settings and the potential regulatory issues of generating a live unattenuated parasite, the amount of sporozoites required for

CPS immunisation to achieve protection is 10-100-fold less than that required for RAS immunisations[255]. While subjects are exposed to transient low level parasitaemia during vaccination, immunity is targeted mainly against pre-erythrocytic antigens[256], with the contribution of chloroquine to induce cross-stage protective immunity, as seen in animals studies[257, 258], impossible to study in human vaccine studies where monitoring parasitaemia over time is not possible.

Other drugs have been tested as alternatives to chloroquine for CPS. Mefloquine was shown in humans to give equivalent results to chloroquine following human trails receiving infectious *P. falciparum* mosquito bites[259] in humans. In mice, primaquine[260], pyrimethamine[261], piperaquine[262], artesunate[263], azithromycin[260, 264] and clindamycin[260, 264] have been tested and compared, with azithromycin inducing the best immunity and protection[260]. This suggests that full EEF development without the release of viable merozoites is most beneficial for protection, which corroborates data suggesting blood stage infections negatively impacts pre-erythrocytic immunity[265].

The least investigated WSV strategy has been using chemically attenuated parasites, where sporozoites[266, 267] or blood stage merozoites[268] are incubated with centamycin or Tafuramycin-A, *Seco*-cyclopropyl pyrrolo indole analogues, that are thought to irreversibly alkylate polyA rich DNA regions. In both stages of the life cycle, complete attenuation of growth occurs, and sterile immunity induced. While there are concerns over complete attenuation and the possibility of reversion and drug toxicity to hosts, safety and immunogenicity trials in humans using *P. falciparum* infected erythrocytes treated with Tafuramycin-A is underway to assess the viability of this strategy for vaccination[269] which may then lead on to vaccination with sporozoites.

Nonetheless, while many still deem the use of whole sporozoite vaccines, especially in malaria endemic areas, as unlikely, the use of RAS, GAPs and CPS has allowed many immunological determinants induced by these vaccines to be identified. Correlates of

protection have been achieved and the contribution of different arms of the immune system induced by these vaccines have been investigated, first in mice and then tested and translated into human vaccine studies. Despite few clinical studies, of which many were conducted in malaria-naïve cohorts, some assertions have been made to the responses induced by WSVs.

CD8+ T cells are considered the major effector immune response eliciting sterile protection following WSV, with varying contributions from CD4+ T cells and antibodies[270]. In humans, studying the contribution of T cells is difficult as depletion studies and determining the presence of liver resident memory T cells is not possible. However, vaccination with RAS[145] or *p52⁻/p36⁻* GAP[146] induced parasite-specific IFN-γ producing CD8+ T cells detectable in peripheral blood in most subjects, with a dose dependent response[145]. In a CPS study using chloroquine, only 2/9 protected individuals had observable parasite-specific IFN-γ CD8+ T cells in peripheral blood[148]. This may be the result of the protective CD8+ T cells remaining trapped in the liver as has been shown in mouse models using a late arresting GAP[251] and RAS immunised non-human primates[243], though why RAS and GAP generally induce this peripheral blood correlate but not CPS is not known. Another CPS study found an association between Granzyme B expression on peripheral blood CD8+ T cells, following stimulation with *P. falciparum* infected erythrocytes, and protection, which may indicate the induction of protective responses to late-liver stage antigens[271].

The protective role of CD4+ T cells in pre-erythrocytic immunity after vaccination is less clear. Mouse models suggest the role is dependent on mouse strain, vaccine strategy and parasite used[147]. It has also been proposed that the role of CD4+ T cells in protection is dependent on timing of challenge in RAS[270] and possibly other WSVs. In humans, intravenous vaccination with RAS induces CD4+ T cell response whose magnitude correlates with vaccine dose[145]. In addition, immunisation with *p52⁻/p36⁻* GAP[146] and

sporozoites administered under chloroquine prophylaxis[148], induced polyfunctional cytokine-producing memory CD4+ T cell responses. This correlated with pre-erythrocytic and erythrocytic protection in the CPS study[148] but correlations to protection could not be determined when using a GAP[146]. A further association in a CPS study was found between protection and CD107a expression on peripheral blood CD4+ T cells, following stimulation with *P. falciparum* infected erythrocytes, which may again indicate responses to late-liver stage antigens[271].

The contribution of antibodies to protection is the least clear adaptive immune response. As mentioned before, anti-sporozoite antibody responses can be induced in mice using WSVs[215, 216] however protection is also achievable upon depletion of B cells[221]. In human studies, vaccination with RAS induced high anti-CSP antibody titres, which correlated with immunisation dose, and higher titres were observed in protected individuals compared to non-protected individuals[145]. Results from CPS studies show that antibodies against CSP[148, 252, 256, 272] and other proteins expressed in sporozoites and EEFs[148, 272] were induced, but anti-CSP antibodies were deemed to be short-lived[253] and no correlation was found between protection and antibody responses to any protein[148]. Immunisation with *p52⁻/p36⁻*[146] and *p52⁻/p36⁻/sap1⁻*[246] GAPs also elicited antibodies against CSP[146, 246] and other sporozoite and EEF proteins[246]. It is clear that while antibodies induced by WSVs have significant functional activity in the ability to block sporozoite invasion *in vitro*[145, 146, 148, 243, 273] and in chimeric mice[274], they are probably not the main effector of the immune system targeting *Plasmodium* in the liver. The role of blood-stage reactive antibodies to protect against blood stage parasites following sporozoite vaccination has also not been fully determined. It is not determined in humans what level of exposure to blood stage parasites is required to confer protection, whether human late arresting GAPs could induce stage-transcending immunity[249, 251] and the full impact of blood stage parasites on immunoregulatory mechanisms which may negatively impact other immune responses[265].

Further insights into WSV-induced immunity will be achieved with the increasing number of clinical trials using RAS, GAPs and CPS. Through this, the differences in the strategies and (protective) immune responses induced can be delineated. It is also imperative to have more data from clinical trials in malaria endemic settings to determine correlates of protection.

Overall, whole sporozoite vaccines have been very useful in informing how the immune system responds to malaria parasites and also how best to modulate immune responses to achieve protection against the pre-erythrocytic stages. Given the complexity of the parasite and natural immune responses, fast-forwarding natural acquired immunity is probably not the most appropriate method as sterile protection is never induced[275]. Instead, vaccines against pre-erythrocytic immunity should be more focused towards developing unnaturally high immune responses to induce sterile protection[240].

To improve existing vaccines, it is this unnatural immunity that needs to be maintained to sustain protection. In terms of targeting sporozoites, strong and durable titres of broad acting polyclonal antibodies are required to maximise inhibition of sporozoite traversal and invasion. A strong, broad acting antibody response would likely also favour a reduction in merozoites in the blood, however, the acquisition of sterile protection against blood stage parasites, and the role of blood stage parasites in dampening pre-erythrocytic responses, has yet to be fully unravelled despite stage-transcending immunity being possible in mouse models with late arresting GAPs[249, 251]. The identification of new targets is also a high priority using the plethora of new genetic, transcriptomic and proteomic data sets and tools.

To improve current CD8+ T cell inducing strategies, while it may be difficult to determine, assessing the ability of vaccines to induce *Plasmodium* specific-liver resident CD8+ T cells will be vital as a correlate for protection, as these cells have been shown to play a major role in protection and can be induced by vaccination[172, 251, 276-278] in mice and non-

human primates. Developing a vaccine to induce large numbers of potent liver resident CD8+ T cells as well as developing an assay to determine this induction will be crucial next stages in developing and testing efficacious pre-erythrocytic targeting malaria vaccines. Further identification of the targets of these protective responses will also aid the development of next generation T-cell targeting vaccines, particularly late expressed EEF antigens which have been shown to give superior immunity to earlier expressed EEF antigens[248].

Vaccines targeting the other stages of the *Plasmodium* life cycle also exist, based on inducing strong antibody responses against merozoites and sexual stages, to protect against blood stage infection and prevent transmission of the parasite to the mosquito[255]. The advent of an efficacious subunit vaccine could involve combining antigenic elements from all of the *Plasmodium* life cycle stages to achieve long-lasting, cross-stage immunity against malaria.

AIMS AND RESEARCH OBJECTIVES

The importance of CD8+ T cells in conferring protection to the pre-erythrocytic stages of malaria is apparent, however few antigens have been described as being responsible for these immunological responses. More work has been carried out looking at the responses to sporozoite antigens, particularly CSP, with EEF antigens being very much a mystery in terms of induced CD8+ T cell responses and protective capability. Sporozoites are extracellular and thus antigens would be accessible to the immune system for presentation prior to hepatocyte invasion. Conversely, EEFs are hidden inside a hepatocyte and surrounded by a parasitophorous vacuole membrane, blocking access to the cytosol and antigen processing machinery. Given this, my working hypothesis was that sporozoite antigens are more immunogenic than EEF antigens, as they are more likely to be presented to CD8+ T cells.

The overall aim of this PhD project was to determine the CD8+ T cell responses and subsequent protection induced by different antigens expressed in the pre-erythrocytic stages of *Plasmodium* infection with a focus on EEF antigens.

My research objectives were to:

- Compare a sporozoite surface and an EEF vacuolar membrane antigen and investigate the effect of spatial and temporal differences of pre-erythrocytic antigen expression on CD8+ T cell responses and their roles in vaccine-induced protection (Chapters 2 and 3).
- Compare early and later expressed EEF antigens to investigate the effect of temporal expression of antigens expressed by EEFs on CD8+ T cell responses and vaccine-induced protection (Chapter 4).
- Investigate CD8+ T cell responses and protection induced by the immunodominant CD8+ T cell epitope of sporozoite surface circumsporozoite protein (Chapter 5).

- Identify novel CD8+ T cell epitopes from pre-erythrocytic antigens, especially those expressed in the EEF, using bioinformatics tools and laboratory screening (Chapter 6).

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SECTION A – Student Details

Student	Matthew Paul Gibbins
Principal Supervisor	Julius Clemence R. Hafalla
Thesis Title	Dissecting the CD8+ T cell responses to pre-erythrocytic malaria antigens

If the Research Paper has previously been published please complete Section B, if not please move to Section C

SECTION B – Paper already published

Where was the work published?			
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SECTION C – Prepared for publication, but not yet published

Where is the work intended to be published?	Nature Medicine
Please list the paper's authors in the intended authorship order:	Katja Müller*, Matthew P. Gibbins*, Arturo Reyes-Sandoval, Adrian V. S. Hill, Simon J. Draper, Kai Matuschewski, Olivier Silvie & Julius Clemence R. Hafalla *contributed equally
Stage of publication	**Not yet submitted**

SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	I performed immunological experiments, analysed data and performed the statistical analyses. I wrote the first draft of the paper.

Student Signature: [redacted] Date: 17/09/18

Supervisor Signature: [redacted] Date: 17/09/18

CHAPTER 2

Contrasting immunogenicities of malaria pre-erythrocytic stage antigens are overcome by vaccination

Contrasting immunogenicities of malaria pre-erythrocytic stage antigens are overcome by vaccination

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ABSTRACT

Vaccine discovery and development critically depends on predictive assays, which prioritise protective antigens. Immunogenicity is considered one important criterion for progression of candidate vaccines to further clinical evaluation, including phase I/ II trials. Here, we tested this assumption in an infection and vaccination model for malaria pre-erythrocytic stages. We engineered *Plasmodium berghei* parasites that harbour a well-characterised H-2-K^b epitope for stimulation of CD8+ T cells either as an antigen in the circumsporozoite protein (CSP), the surface coat protein of extracellular sporozoites or in the upregulated in sporozoites gene 4 (UIS4), a major protein associated with the parasitophorous vacuole membrane (PVM) that surrounds the intracellular exo-erythrocytic forms (EEF). We show that the antigen origin results in profound differences in immunogenicity with a sporozoite antigen eliciting robust and superior antigen-specific CD8+ T cell responses, whilst an EEF antigen evokes poor responses. Despite their contrasting immunogenic properties, both sporozoite and EEF antigens gain access to antigen presentation pathways in hepatocytes. Recognition and targeting by vaccine-induced, antigen-specific effector CD8+ T cells results in high levels of protection when targeting both antigens. Our study is the first demonstration that poor immunogenicity of EEF antigens does not preclude their susceptibility to antigen-specific CD8+ T cell killing. Our findings that antigen immunogenicity is an inadequate predictor of vaccine susceptibility have wide-ranging implications on antigen prioritisation for the design and testing of next-generation malaria vaccines.

INTRODUCTION

Malaria, caused by the apicomplexan parasites *Plasmodium*, is responsible for more than 200 million clinical cases and over 440,000 deaths annually worldwide[1]. Whilst current malaria control strategies have led to marked reduction in incidence rate, cases, and mortality for the past 16 years, a highly efficacious vaccine is likely essential to approach the ambitious World Health Organisation's (WHO) vision of "a world free of malaria". Targeting the malaria pre-erythrocytic stages, an obligatory and clinically silent phase of the parasite's life cycle, is considered an ideal and attractive strategy for vaccination; inhibiting parasite infection of and development in hepatocytes results in preclusion of both disease-causing blood stages and transmissible sexual stages. Yet, despite intensive research for over 25 years, a highly efficacious pre-erythrocytic stage vaccine remains elusive[2]. An in-depth characterisation of how the complex biology of pre-erythrocytic stages influences the generation of protective immune responses is warranted to inform the design of future malaria vaccines.

CD8+ T cells are crucial mediators of protective immunity to malaria pre-erythrocytic stages[3]. Whilst often considered as a single phase of the parasite's life cycle, the malaria pre-erythrocytic stage is comprised of two different parasite forms: (i) sporozoites, which are motile extracellular parasites that are delivered by infected mosquitoes to the mammalian host, and (ii) exo-erythrocytic forms (EEF; also known as liver stages), which are intracellular parasites resulting from the differentiation and growth of sporozoites inside a parasitophorous vacuole (PV) within hepatocytes[4]. How these two spatially different parasite forms and the ensuing temporal expression of parasite-derived antigens impact the magnitudes, kinetics and phenotypes of CD8+ T cell responses elicited following infection is poorly understood. Furthermore, the complexity within the pre-erythrocytic stages has fuelled a long-standing debate focused on the contributions of distinct sporozoite and EEF antigens in parasite-induced responses, and whether sporozoite or EEF proteins are better targets of vaccines.

Our current understanding of CD8+ T cell responses to malaria pre-erythrocytic stages has been largely based on measuring responses to the H-2-K^d-restricted epitopes

of *P. yoelii* (*Py*)[5] and *P. berghei* (*Pb*)[6] circumsporozoite proteins (CSP), the major surface antigen of sporozoites. Many of these fundamental studies have focused on using infections with irradiated sporozoites, the gold-standard vaccine model for malaria. Infection with *Py* sporozoites elicits an expected T cell response typified by early activation and induction of effector CSP-specific CD8+ T cells followed by contraction and establishment of quantifiable memory populations[7]. CSP-specific CD8+ T cells are primed by dendritic cells that cross-present sporozoite antigens via the endosome-to-cytosol pathway[8]. Yet, CSP is a unique antigen because it is expressed in both sporozoites and EEFs[9]. Whilst the expression of CSP mRNA ceases after sporozoite invasion, the protein on the parasite surface is stable and endures in EEFs during development in hepatocytes[10]. *In vitro* data indicate that primary hepatocytes process and present *Pb*CSP-derived peptides to CD8+ T cells in a proteasome-dependent manner, involving export of antigen to the cytosol[8]. Taken together, these data imply that sporozoite antigens induce quantifiable CD8+ T cell responses after infection. Antigens that have similar expression to CSP, persisting to EEFs and with epitope determinants expressed on hepatocytes, are excellent targets of CD8+ T cell-based vaccines.

The paucity of EEF-specific epitopes has hindered not only our ability to understand the immune responses that are evoked whilst the parasite is in the liver, but also their utility as targets of vaccination. Accordingly, the contribution of EEF-infected hepatocytes in the *in vivo* induction of CD8+ T cell responses is poorly understood. The liver is an organ where the primary activation of CD8+ T cells is generally biased towards the induction of tolerance[11,12]. Yet, studies in other model systems have demonstrated antigen-specific primary activation within the liver[13]. Another confounding issue with EEFs is their development in PVs with constrained access to the hepatocyte's cytosol[4]. Nonetheless, if CD8+ T cells specific for EEF antigens are primed, do they expand and contract with distinct kinetics? Moreover, are EEF-specific epitopes efficiently generated for recognition and targeting by vaccine-induced CD8+ T cells? Answers to these questions will be key for antigen selection and design of future malaria vaccines.

In this study, we compared the initiation and development of CD8+ T cell

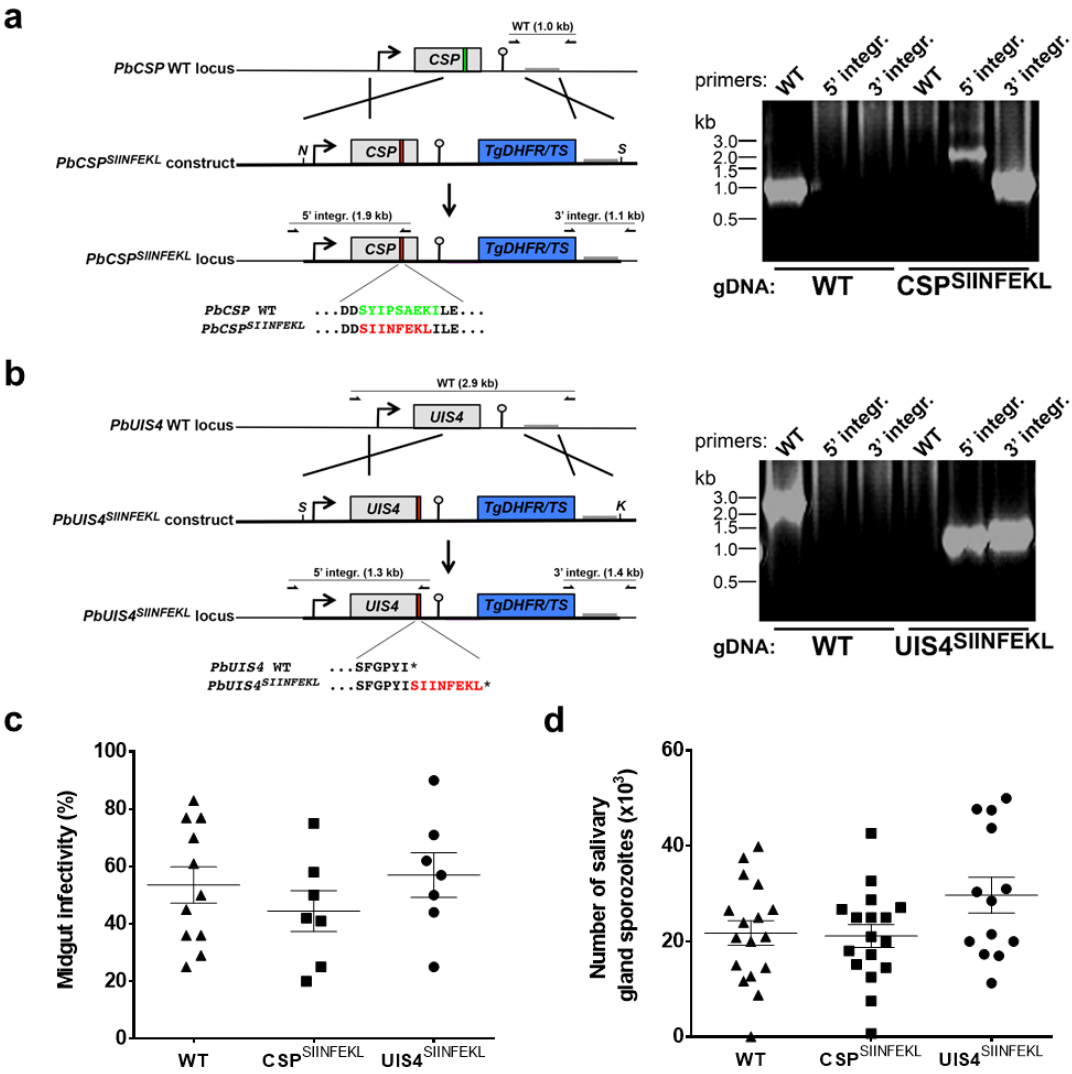
responses – elicited following parasite infection – to CSP, a sporozoite antigen, and to upregulated in infective sporozoites gene 4 (UIS4), an EEF-specific vacuolar protein[14]. UIS4, a member of the early transcribed membrane protein (ETRAMP) family, is abundantly expressed in EEFs and associates with the PVM[14]. Whilst UIS4 mRNA expression is present in sporozoites, translation is repressed until when EEFs develop[10]. To control for epitope specificity, we generated *Pb* transgenic parasites that incorporate the H-2-K^b epitope SIINFEKL, from ovalbumin, in either CSP or UIS4. We assessed the immunogenic properties of SIINFEKL expressed in the context of CSP and UIS4, defining immunogenicity as the ability of CSP and UIS4 to induce SIINFEKL-specific CD8+ T cell responses following immunisation with irradiated *Pb* transgenic sporozoites. We followed the kinetics of the CD8+ T cell response to each antigen and effector functions induced. Furthermore, we evaluated the capacity of vaccine-induced CD8+ T cells to target these parasites in a mouse challenge model. Our data shows disparate immunogenic properties between a sporozoite and an EEF vacuolar membrane antigen but equivalent susceptibility to vaccine-induced CD8+ T cells.

RESULTS

Transgenic CSP^{SIINFKEL} and UIS4^{SIINFEKL} parasites display normal sporozoite motility and liver invasion

We generated, by double homologous recombination, transgenic *Pb* parasites expressing the immunodominant H-2-K^{b}-restricted CD8+ T cell epitope of ovalbumin (SIINFEKL) in the context of the sporozoite surface antigen CSP or the EEF vacuolar membrane antigen UIS4 **(Figure 1a, b)**. Constructs included the *TgDHFR/TS* positive selection cassette and incorporated SIINFEKL in the context of the gene open reading frame. For CSP^{SIINFEKL}, SIINFEKL replaced SYIPSAEKI, the immunodominant H-2-K^{d}-restricted CD8+ T cell epitope of CSP, which allowed for recognition in H-2-K^{b}-carrying C57BL/6 mice. For UIS4^{SIINFEKL}, the SIINFEKL epitope was added to the immediate C-terminus of the UIS4 protein. Appending the C-terminus was chosen because it had been shown in *Toxoplasma gondii* that the potency of the immunodominant epitope of GRA6 was associated with its C-terminal location, which may have enhanced the presentation by parasite-infected cells[15]. Whilst undefined for UIS4 itself, it has been shown for several other ETRAMPs that the C-terminus faces the host cell cytoplasm[16], which might enhance exposure to the MHC I machinery.

The resulting parasites showed a phenotype comparable to WT parasites, with comparable midgut infectivity **(Figure 1c),** number of salivary gland sporozoites **(Figure 1d)**, functional sporozoite motility **(Figure 1e)** and normal invasive capacity and development inside hepatocytes **(Figure 1f)**. Thus, the introduced mutations to generate CSP^{SIINFEKL} and UIS4^{SIINFEKL} parasites did not interfere with the completion of the life cycle, in either mosquito vector or mouse. All C57BL/6 mice that received 800 sporozoites of either CSP^{SIINFKEL} or UIS4^{SIINFEKL} intravenously developed a patent blood stage infection by day 4, comparable to infection with WT sporozoites (data not shown).


a
WT (1.0 kb)
PbCSP WT locus
CSP
PbCSPSIINFEKL construct
N
CSP
TgDHFR/TS
S
5' integr. (1.9 kb)
3' integr. (1.1 kb)
PbCSPSIINFEKL locus
PbCSP WT ...DDSYIPSAEKILE...
PbCSPSIINFEKL ...DDSIINFEKLILE...
primers: WT 5' integr. 3' integr. WT 5' integr. 3' integr.
kb
3.0
2.0
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1.0
0.5
gDNA: WT CSPSIINFEKL
b
WT (2.9 kb)
PbUIS4 WT locus
UIS4
PbUIS4SIINFEKL construct
S
UIS4
TgDHFR/TS
K
5' integr. (1.3 kb)
3' integr. (1.4 kb)
PbUIS4SIINFEKL locus
PbUIS4 WT ...SFGPYI*
PbUIS4SIINFEKL ...SFGPYISIINFEKL*
primers: WT 5' integr. 3' integr. WT 5' integr. 3' integr.
kb
3.0
2.0
1.5
1.0
0.5
gDNA: WT UIS4SIINFEKL
c
Midgut infectivity (%)
100
80
60
40
20
0
WT
CSPSIINFEKL
UIS4SIINFEKL
d
Number of salivary gland sporozoites (x10³)
60
40
20
0
WT
CSPSIINFEKL
UIS4SIINFEKL

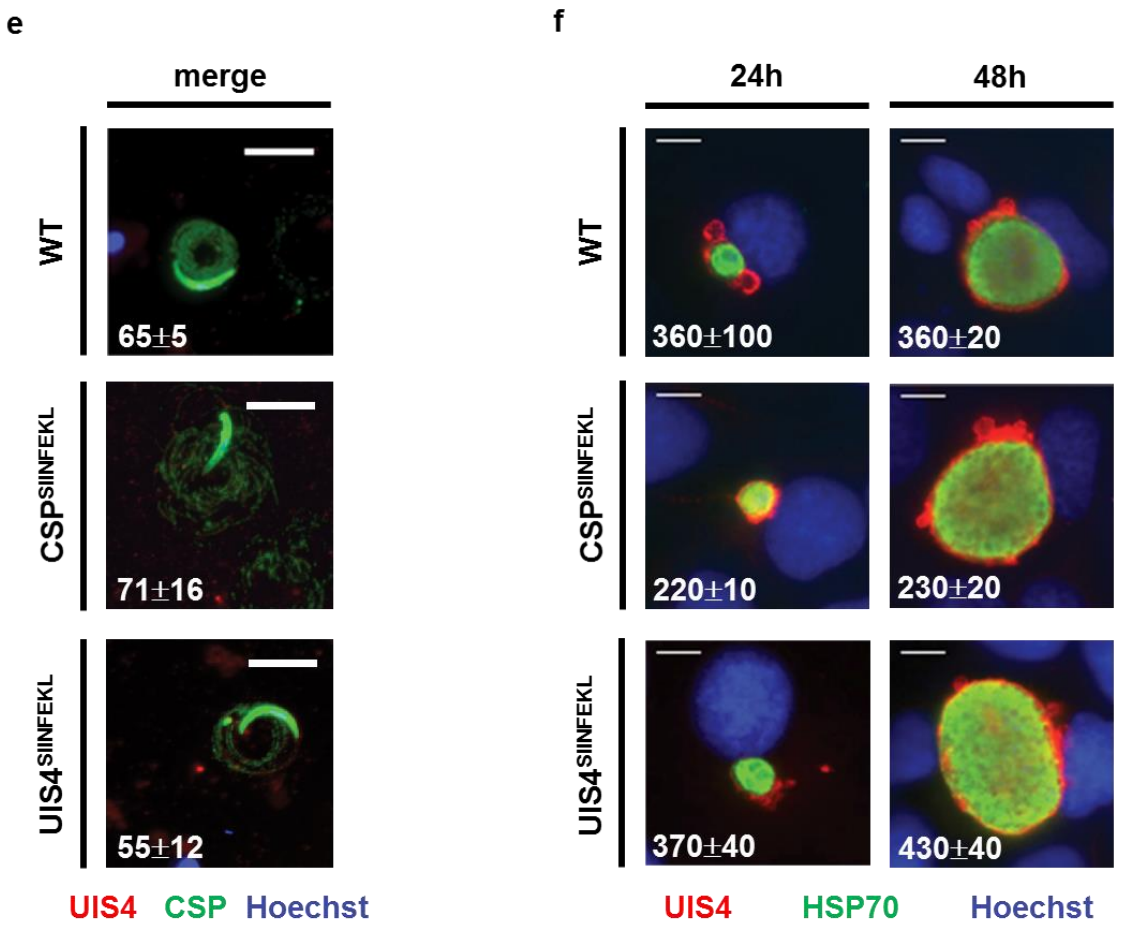


Figure 1: Generation and characterisation of transgenic CSP^{SIINFEKL} and UIS4^{SIINFEKL} *P. berghei* lines

Plasmodium berghei parasites expressing the CD8+ T cell epitope of ovalbumin, SIINFEKL, in the context of CSP or UIS4 were generated using double homologous recombination, combining drug-resistance selection (through incorporation of the *dhfr/ts* gene from *Toxoplasma gondii*) and cloning by limiting dilution to select for correctly recombined parasites. **(a,b)** Diagrams illustrate the reverse genetics strategy. **(a)** In CSP^{SIINFEKL} SIINFEKL replaces the immunodominant CD8+ T cell epitope SYIPSAEK(I) of CSP. **(b)** In UIS4^{SIINFEKL} SIINFEKL is adjoined to the carboxyl-terminus of the UIS4 protein. Purified schizonts of WT *P. berghei* ANKA were transfected with linearized plasmid by electroporation as described[17], and immediately injected intravenously in the tail vein of a mouse. The day after transfection, pyrimethamine (70 mg/l) was orally administered in the drinking water for selection of transgenic parasites. Transgenic

clones were generated in mice by *in vivo* cloning by limiting dilution. Correct integration of the constructs and purity of the transgenic lines was verified by diagnostic PCR using primer combinations specific for the unmodified *CSP* or *UIS4* locus, and for the 5' and 3' recombination events as indicated by lines, arrows and expected fragment sizes. **(c)** Oocyst midgut infectivity of mosquitoes infected with WT, $CSP^{SIINFEKL}$ or $UIS4^{SIINFEKL}$. The mean (±SD) of infected midguts was enumerated 10-14 days after infection (n= at least 7 infections). **(d)** Salivary glands were isolated from WT, $CSP^{SIINFEKL}$ or $UIS4^{SIINFEKL}$ infected mosquitoes and mean sporozoite numbers (±SD) were enumerated between 18-23 days after infection (n= at least 13 infections). **(e)** Sporozoite immunofluorescent antibody staining of WT, $CSP^{SIINFEKL}$ or $UIS4^{SIINFEKL}$ sporozoites after gliding on BSA-coated glass slides. Shown are microscopic images of the respective sporozoites that were stained with anti-CSP (green), anti-UIS4 (red) and nuclear stain Hoechst 33342 (blue). Scale bars, 10μm. The numbers show mean percentage (±SD) of sporozoites with trails (n≥220 sporozoites viewed from two independent experiments). **(f)** Fluorescent-microscopic images of EEF-infected Huh7 hepatoma cells. 24 and 48 hours after infection with WT, $CSP^{SIINFEKL}$ or $UIS4^{SIINFEKL}$ sporozoites, the cells were fixed and stained with anti-UIS4 (red), anti-HSP70 (green) and the nuclear stain Hoechst (blue). Scale bars: 10μm. The numbers show mean numbers of intracellular parasites (±SD; n≥ 200 EEFs viewed, from three independent experiments, and for WT from two independent experiments).

Peripheral blood CD8+ T cell responses and early proliferative capacity of splenic CD8+ T cells are superior if elicited by a sporozoite surface protein in contrast to a vacuolar membrane protein in the infected liver

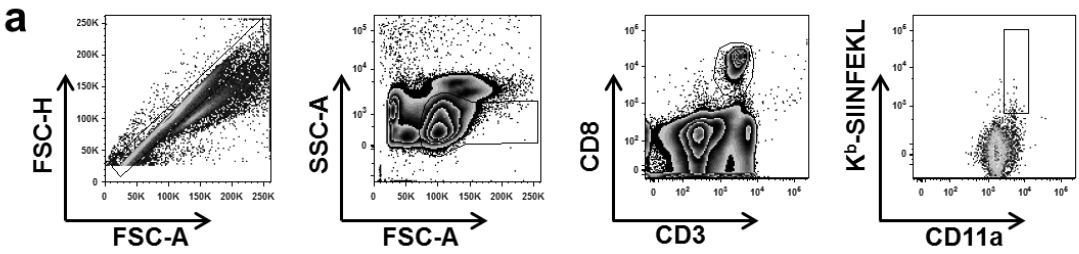
We first wanted to determine whether the generated transgenic parasites allow antigen-specific responses to be tracked using SIINFEKL as a surrogate CD8+ T cell epitope for sporozoite surface and EEF vacuolar membrane antigens. To this end, we assessed the kinetics of the CD8+ T cell response following intravenous immunisation with $CSP^{SIINFEKL}$ or $UIS4^{SIINFEKL}$ sporozoites. To augment the CD8+ T cell response, mice were adoptively transferred with 2 x 10^6 OT-I cells expressing a SIINFEKL-specific TCR[8], prior to receiving 10,000 γ-radiation attenuated WT, $CSP^{SIINFEKL}$ or $UIS4^{SIINFEKL}$ sporozoites. Prior work showed that γ-radiation attenuation of *P. berghei* sporozoites does not impact host cell invasion and UIS4 expression[18].

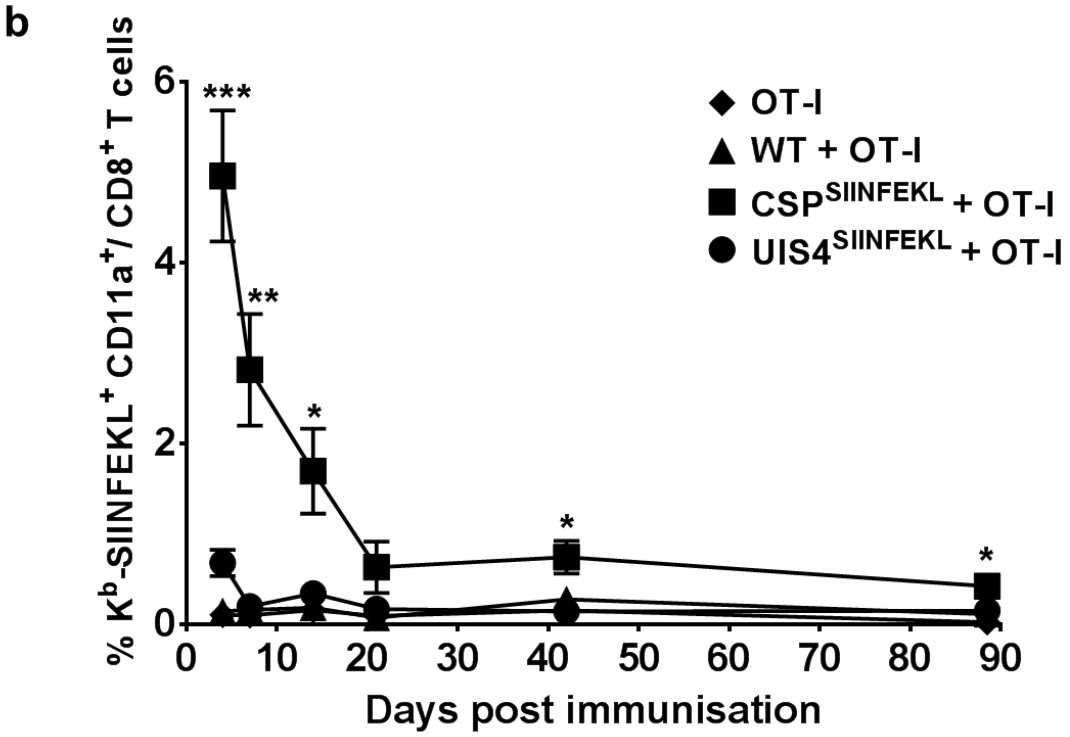
Peripheral blood was taken at days 4, 7, 14, 21, 42 and 88 after immunisation and CD8+ T cell responses were analysed after staining with H-2-K^b-SIINFEKL pentamers and for CD11a, a marker for antigen-experienced T cells[19,20] (**Figure 2a)**. A substantial proportion of K^b-SIINFEKL+ CD11a+ CD8+ T cells were observed in mice immunised with $CSP^{SIINFEKL}$; the response was highest on day 4, reaching 5% of all antigen-experienced CD8+ T cells, and declined steadily until day 21, when the response stabilised and remained unchanged for several weeks **(Figure 2b)**. In marked contrast, $UIS4^{SIINFEKL}$ immunisation induced a poor CD8+ T cell response; the proportion of K^b-SIINFEKL+ CD11a+ CD8+ T cells was only higher than the control groups at day 4 after immunisation, and the response remained within background levels for the duration of the experiment. Control groups included mice receiving OT-I cells only or in addition to WT sporozoites, which lack SIINFEKL sequences.

The poor CD8+ T cell response induced by $UIS4^{SIINFEKL}$ sporozoites, as compared to $CSP^{SIINFEKL}$, led us to characterise the early events in the proliferation and differentiation of these cells. Mice were adoptively transferred with CFSE-labelled OT-I cells and immunised with γ-radiation attenuated WT, $CSP^{SIINFEKL}$ or $UIS4^{SIINFEKL}$

sporozoites. 5 days later, as shown by gating on CD8+ T cells **(Figure 2c, g)**, immunisation with $CSP^{SIINFEKL}$ sporozoites recruited K^b-SIINFEKL+ CD8+ T cells to undergo massive proliferative activity, which was 6x larger than that observed with $UIS4^{SIINFEKL}$ sporozoites, in good agreement with the peripheral blood data described above **(Figure 2b)**. Consistent with the activation of these cells, the proliferation of antigen-specific CD8+ T cells by both parasites was associated with the development of effector and effector-memory phenotypes as evidenced by upregulation of CD11a and CD49d, and downregulation of CD62L, respectively **(Figure 2d-f)**.

Taken together, these findings establish that immunisations with $CSP^{SIINFEKL}$ and $UIS4^{SIINFEKL}$ sporozoites permit antigen-specific responses to be tracked longitudinally in the peripheral blood. Importantly, we demonstrate that a sporozoite surface protein evokes a CD8+ T cell response of superior magnitude than an EEF vacuolar membrane protein following immunisation with malaria sporozoites.


a
FSC-H
FSC-A
SSC-A
FSC-A
CD8
CD3
K^b-SIINFEKL
CD11a


b
% K^b-SIINFEKL^+ CD11a^+/ CD8^+ T cells
Days post immunisation
OT-I
WT + OT-I
CSP^SIINFEKL + OT-I
UIS4^SIINFEKL + OT-I
***
**
*
*
*

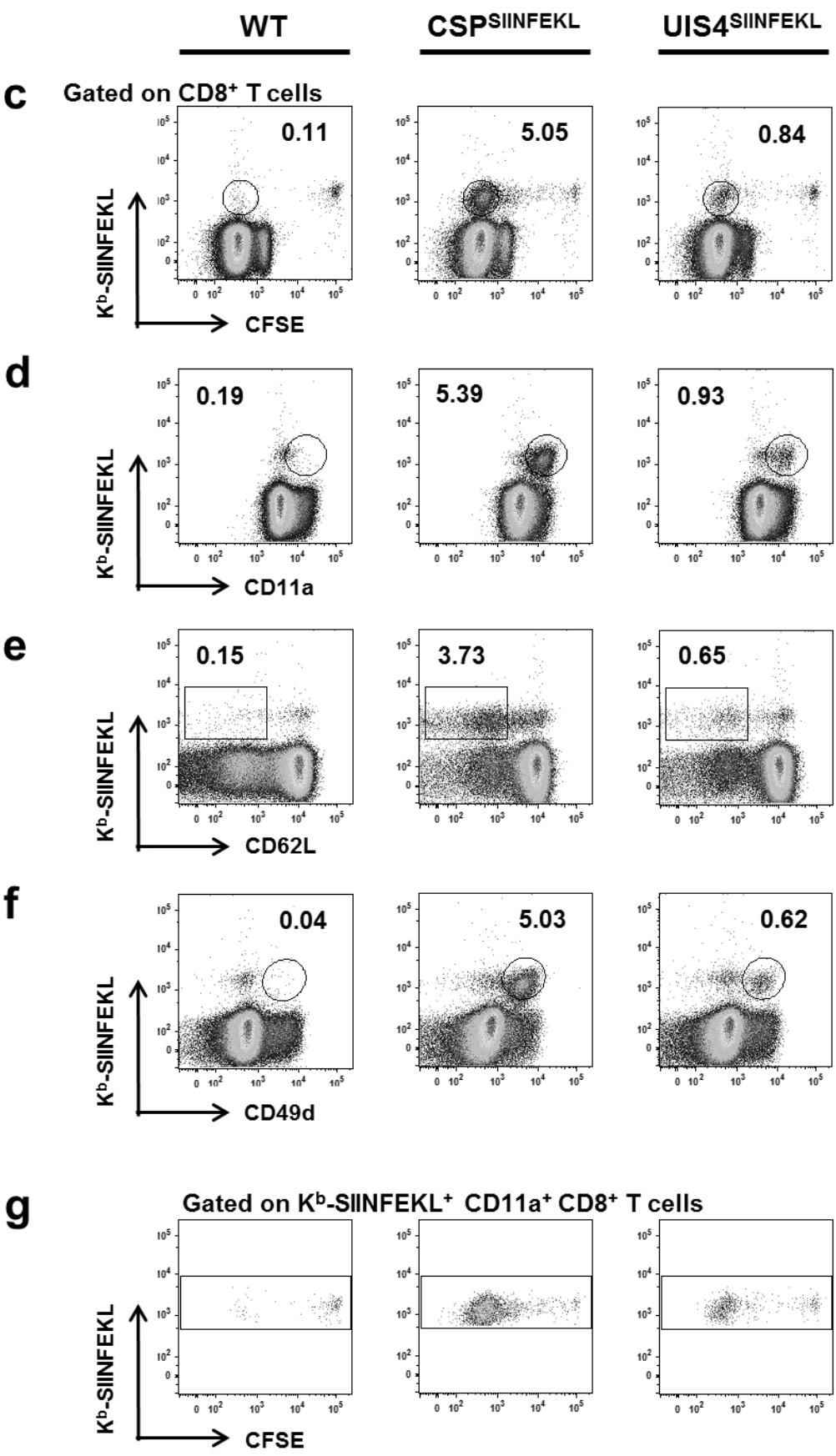

WT
CSP^SIINFEKL
UIS4^SIINFEKL
c
Gated on CD8+ T cells
0.11
5.05
0.84
Kb-SIINFEKL
CFSE
d
0.19
5.39
0.93
Kb-SIINFEKL
CD11a
e
0.15
3.73
0.65
Kb-SIINFEKL
CD62L
f
0.04
5.03
0.62
Kb-SIINFEKL
CD49d
g
Gated on Kb-SIINFEKL+ CD11a+ CD8+ T cells
Kb-SIINFEKL
CFSE

Figure 2: Kinetics of CD8+ T cell responses induced by transgenic parasites.

(a-b) C57BL/6 mice (n=3-5 per group) received $2x10^6$ OT-I cells alone (diamonds) or were additionally immunised with 10,000 γ-radiation attenuated WT (triangles), $CSP^{SIINFEKL}$ (squares) or $UIS4^{SIINFEKL}$ (circles) sporozoites intravenously. **(a)** Flow cytometry plots show the gating strategy for identifying K^b-SIINFEKL+ CD11a+ CD8+ T cells. **(b)** Peripheral blood was obtained on days 4, 7, 14, 21, 42 and 88 after immunisation and stained for K^b-SIINFEKL+ CD11a+ CD8+ T cells. Line graph shows data pooled from two experiments (mean values ± SEM; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; Welch's t-test comparing $CSP^{SIINFEKL}$ and $UIS4^{SIINFEKL}$). **(c-g)** C57BL/6 mice (n=4 per group), which received $2x10^6$ CFSE-labelled OT-I splenocytes, were immunised with 10,000 γ-radiation attenuated WT, $CSP^{SIINFEKL}$ or $UIS4^{SIINFEKL}$ sporozoites intravenously. 5 days later, mice were sacrificed, spleens harvested and splenocytes assessed for **(c)** CFSE dilution and stained *ex vivo* **(d-f)** for effector CD8+ T cell surface markers. Shown are flow cytometry plots of K^b-SIINFEKL co-staining with markers of effector phenotypes: **(d)** $CD11a^{hi}$, **(e)** $CD62L^{lo}$, **(f)** $CD49d^{hi}$ and **(g)** the proliferation of CFSE-labelled antigen experienced K^b-SIINFEKL+ CD11a+ CD8+ T cells.

High magnitude splenic and hepatic CD8+ T cell responses to a sporozoite antigen

Previous research has shown that CD8+ T cells are primed primarily in the spleen following intravenous immunisation with malaria sporozoites[21] and that liver lymphocytes form a front-line defence against developing EEFs in hepatocytes[22,23]. Thus, we further analysed the development of CD8+ T cell responses in the spleens and livers of mice adoptively transferred with OT-I cells and intravenously immunised with WT, $CSP^{SIINFEKL}$ or $UIS4^{SIINFEKL}$ sporozoites. Consistent with our aforementioned results, surface staining of splenic and liver lymphocytes showed higher proportion and absolute numbers of K^b-SIINFEKL+ CD11a+ CD8+ T cells at day 14 and day 42 following immunisation with $CSP^{SIINFEKL}$ compared to $UIS4^{SIINFEKL}$ sporozoites **(Figure 3a-c)**. In addition to CD11a upregulation, the splenic and liver CD8+ T cells, elicited by both $CSP^{SIINFEKL}$ or $UIS4^{SIINFEKL}$ sporozoites, had effector and effector memory cell phenotypes (CD62L-, CD49d+ and CD44+) **(Supplementary Figure 1).** Although low, the numbers of antigen-specific CD8+ T cells induced by $UIS4^{SIINFEKL}$ sporozoites were within the detection limits of the assay.

To assess for effector functions, splenic and liver lymphocytes were stimulated *ex vivo* with the SIINFEKL peptide. Generally, higher numbers (proportion and absolute numbers) of IFN-γ-secreting CD8+ T cells were observed at day 14 and day 42 following immunization with $CSP^{SIINFEKL}$ compared to $UIS4^{SIINFEKL}$ sporozoites **(Figure 3d-f)**. In addition, these CD8+ T cells also expressed TNF and IL-2, suggesting some potential polyfunctionality **(Supplementary Figure 2)**.

Altogether, even though effector and effector memory CD8+ T cell responses can be detected against both sporozoite surface protein and EEF vacuolar membrane protein antigens following immunisation with malaria sporozoites, the two antigens show a striking difference in the magnitude of CD8+ T cell responses they induce.

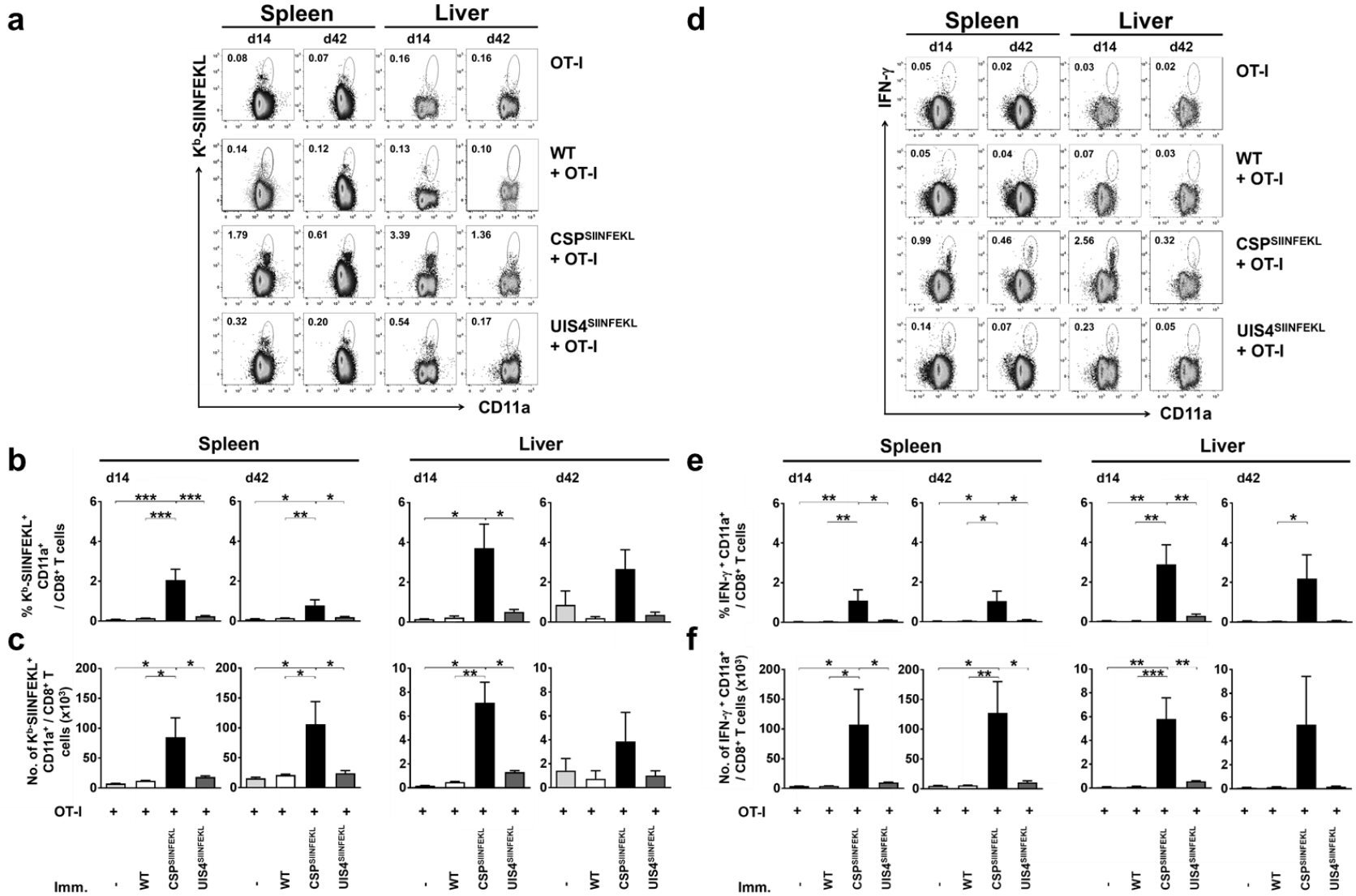

a
Spleen
Liver
d14
d42
d14
d42
Kb-SIINFEKL
CD11a
0.08
0.07
0.16
0.16
OT-I
0.14
0.12
0.13
0.10
WT + OT-I
1.79
0.61
3.39
1.36
CSPSIINFEKL + OT-I
0.32
0.20
0.54
0.17
UIS4SIINFEKL + OT-I
d
IFN-γ
CD11a
0.05
0.02
0.03
0.02
OT-I
0.05
0.04
0.07
0.03
WT + OT-I
0.99
0.46
2.56
0.32
CSPSIINFEKL + OT-I
0.14
0.07
0.23
0.05
UIS4SIINFEKL + OT-I
b
% Kb-SIINFEKL+ CD11a+ / CD8+ T cells
e
% IFN-γ+ CD11a+ / CD8+ T cells
c
No. of Kb-SIINFEKL+ CD11a+ / CD8+ T cells (x10³)
f
No. of IFN-γ+ CD11a+ / CD8+ T cells (x10³)
OT-I
Imm.
-
WT
CSPSIINFEKL
UIS4SIINFEKL

Figure 3: Sporozoite surface antigen induces a higher CD8+ T cell response than EEF vacuolar membrane antigen in the spleen and liver.

C57BL/6 mice (n=3-5 per group) received $2x10^6$ OT-I cells alone or were additionally immunised with 10,000 γ-radiation attenuated WT, $CSP^{SIINFEKL}$ or $UIS4^{SIINFEKL}$ sporozoites intravenously. Spleens and livers were harvested either at day 14 or day 42. Proportions and numbers of **(a-c)** K^b-SIINFEKL+ CD8+ T cells were enumerated or **(d-f)** IFN-γ-secreting CD8+ T cells following restimulation *ex vivo* with SIINFEKL peptide were quantified. Flow cytometry plots show representative percentages of CD8+ T cells co-stained with CD11a and **(a)** K^b-SIINFEKL or **(d)** IFN-γ. The upper panel of bar charts **(b, e)** show the percentage of co-stained CD8+ T cells and the lower panel **(c, f)** the absolute cell counts. Bar charts show mean values (±SEM) from one representative experiment of two experiments performed (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; one-way ANOVA with Tukey's multiple comparison test).

Quantification of endogenously produced antigen-specific CD8+ T cells following intravenous or intradermal parasite immunisation

Previous work tracking responses to SIINFEKL-tagged proteins has used adoptively transferred cells from OT-I mice, with all T cells from these mice expressing T cell receptors specific to SIINFEKL[8,24]. We employed this robust approach by adoptively transferring a fixed amount of OT-I splenocytes in order to augment the response and allow visualisation **(Figures 2 and 3)**. Next, we wanted to explore whether we can capture the endogenous K^b-SIINFEKL+ CD11a+ CD8+ T cell population, which is elicited by immunising with parasites without OT-I cell transfer. We performed *ex vivo* restimulation of lymphocytes with SIINFEKL peptide followed by flow cytometry and were able to clearly identify the endogenous population with a trend complementary to our earlier results **(Figure 4a-c)**. Immunisation with $CSP^{SIINFEKL}$ sporozoites elicited a superior splenic and liver CD8+ T cell response than with $UIS4^{SIINFEKL}$ sporozoites. As expected, the proportion and absolute cell numbers were considerably lower than with adoptive transfer of OT-I cells, but this did not preclude the ability to visualise IFN-γ-secreting CD8+ T cells and capture the differences between the two groups.

Under normal conditions of transmission, sporozoites are delivered into the host skin by mosquito bite. All preceding immunisation experiments were performed with parasites injected intravenously. As a proxy for the natural route of infection, whilst ensuring consistent quantities of parasites were inoculated, $CSP^{SIINFEKL}$ and $UIS4^{SIINFEKL}$ sporozoites were injected via the intradermal route into the ear pinnae. Under these conditions, CSP still induced a greater number of IFN-γ-secreting SIINFEKL-specific CD8+ T cells following restimulation with SIINFEKL compared to UIS4, with a comparable magnitude as after intravenous injection **(Figure 4d-f)**. Thus, these biologically and immunologically more appropriate data entirely recapitulate the strong immunogenicity of a sporozoite surface antigen compared to an EEF vacuolar membrane protein.

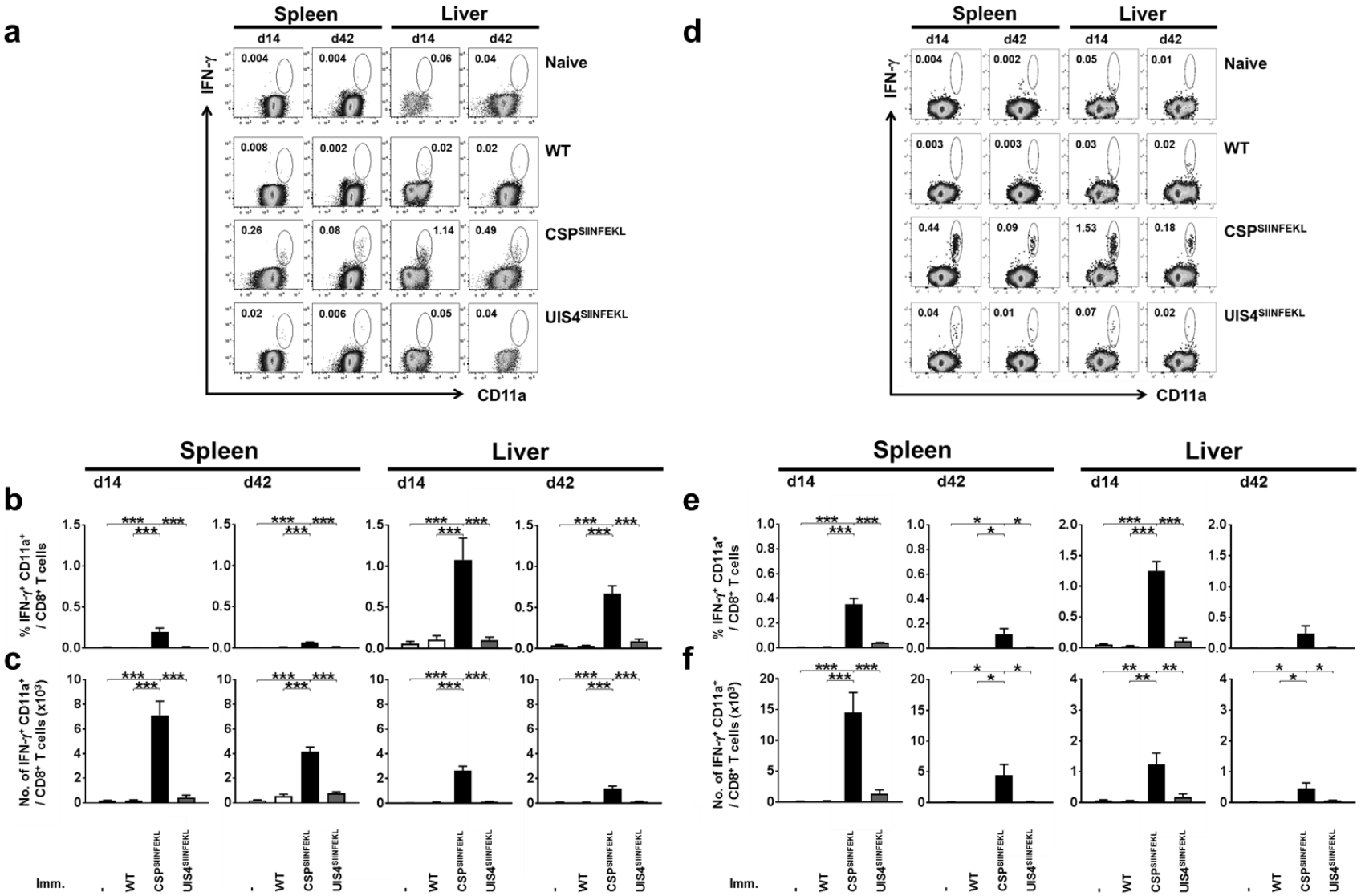

a
Spleen
Liver
d14
d42
d14
d42
IFN-γ
0.004
0.004
0.06
0.04
Naive
0.008
0.002
0.02
0.02
WT
0.26
0.08
1.14
0.49
CSP^SIINFEKL
0.02
0.006
0.05
0.04
UIS4^SIINFEKL
CD11a
d
Spleen
Liver
d14
d42
d14
d42
IFN-γ
0.004
0.002
0.05
0.01
Naive
0.003
0.003
0.03
0.02
WT
0.44
0.09
1.53
0.18
CSP^SIINFEKL
0.04
0.01
0.07
0.02
UIS4^SIINFEKL
CD11a
b
Spleen
Liver
d14
d42
d14
d42
% IFN-γ+ CD11a+ / CD8+ T cells
c
No. of IFN-γ+ CD11a+ / CD8+ T cells (x10³)
Imm.
-
WT
CSP^SIINFEKL
UIS4^SIINFEKL
e
Spleen
Liver
d14
d42
d14
d42
% IFN-γ+ CD11a+ / CD8+ T cells
f
No. of IFN-γ+ CD11a+ / CD8+ T cells (x10³)
Imm.
-
WT
CSP^SIINFEKL
UIS4^SIINFEKL

Figure 4: OT-I cells are not required to detect SIINFEKL-specific CD8+ T cell responses.

C57BL/6 mice (n=3-6 per group) received 10,000 γ-radiation attenuated WT, $CSP^{SIINFEKL}$ or $UIS4^{SIINFEKL}$ sporozoites, either **(a-c)** intravenously or **(d-f)** intradermally. Additional control mice did not receive sporozoites. Spleens and livers were harvested either at day 14 or day 42, and IFN-γ-secreting lymphocytes following restimulation *ex vivo* with SIINFEKL peptide were quantified. Flow cytometry plots show representative percentages of CD8+ T cells co-stained with IFN-γ and CD11a **(a, d)**. The upper panel of bar charts **(b, e)** show the percentage of CD11a+ IFN-γ+ CD8+ T cells and the lower panel **(c, f)** the absolute cell counts. **(b-c)** Bar charts show mean values (±SEM) from one representative experiment of two experiments performed (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; one-way ANOVA with Tukey's multiple comparison test). **(e-f)** Bar charts show mean values (±SEM) from one experiment performed (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; one-way ANOVA with Tukey's multiple comparison test).

Increasing the amount of EEF vacuolar membrane antigen does not impact on CD8+ T cell responses

Both CSP and UIS4 are essential proteins expressed by the sporozoite and EEF respectively, and both proteins are important for survival and succession into the subsequent life stage and parasite form[10,14,25]. Previous studies have shown that the magnitude of the CD8+ T cell response to a sporozoite surface antigen depended on the amount of parasites used for immunisation[26]. Hence, poor immunogenicity of an EEF vacuolar membrane protein could be a result of the lower level of protein expression during parasite infection. It is possible to enhance CD8+ T cell responses by increasing the number of parasites used for immunisation[26]. Therefore, we immunised groups of mice with 8,000 $CSP^{SIINFEKL}$, 8,000 $UIS4^{SIINFEKL}$ or 64,000 $UIS4^{SIINFEKL}$ sporozoites and compared the magnitude of the elicited antigen-specific responses. Strikingly, the CD8+ T cell response following 8x sporozoite immunisation dose with $UIS4^{SIINFEKL}$ did not increase proportionally and was not significantly higher than immunisation with a 1x dose **(Figure 5a, b)**. This result suggests that, in the context of attenuated sporozoite immunisation, EEF vacuolar membrane antigens induce poor CD8+ cell responses and increasing antigen fails to substantially improve the magnitude of these CD8+ T cell responses.

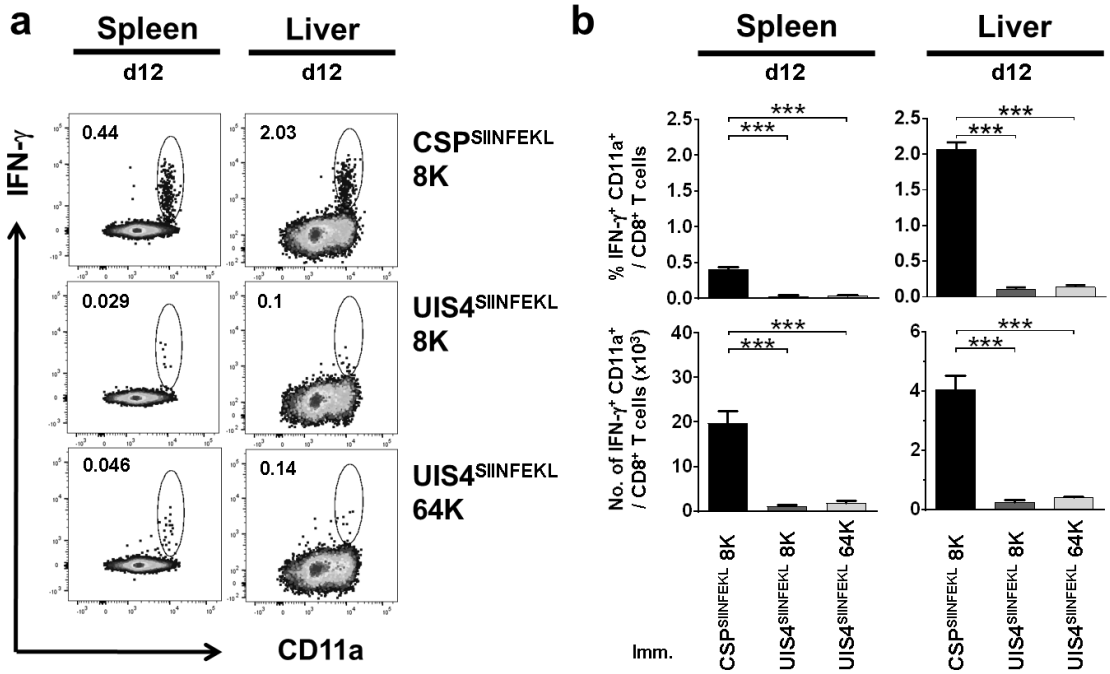


Figure 5: Increasing antigen dose does not improve antigen-specific CD8+ T cell responses to an EEF vacuolar membrane protein.

C57BL/6 mice (n=4 per group) received an intravenous dose of 8,000 γ-radiation attenuated CSP^{SIINFEKL} or UIS4^{SIINFEKL} sporozoites or 64,000 γ-radiation attenuated UIS4^{SIINFEKL} sporozoites. Spleens and livers were harvested at day 12 and IFN-γ-secreting lymphocytes following restimulation *ex vivo* with SIINFEKL peptide were quantified. **(a)** Flow cytometry plots show representative CD8+ T cells co-stained with IFN-γ and CD11a. **(b)** The upper panel of bar charts show the percentage of CD11a+ IFN-γ+ CD8+ T cells and the lower panel the absolute cell counts. Bar charts show mean values (±SEM) from one representative experiment of two experiments performed (***, $p<0.001$; one-way ANOVA with Tukey's multiple comparison test).

Immunogenicity of parasite antigens does not predict effector responses following vaccination

Our findings thus far showed that sporozoite surface proteins appear more immunogenic than EEF vacuolar membrane proteins and raised an intriguing and important question; does immunogenicity predict susceptibility to vaccine-induced effector responses? To address this, we vaccinated mice, which had received OT-I cells, with a recombinant adenovirus expressing full-length ovalbumin[27]. This vaccination protocol resulted in frequencies of ~7.5% SIINFEKL-specific CD8+ T cells in peripheral blood **(Figure 6a, b)**. Vaccinated mice were then challenged with $CSP^{SIINFEKL}$ or $UIS4^{SIINFEKL}$ sporozoites, and protection was assessed by two complementary assays; (i) determination of the reduction of parasite load in the liver **(Figure 6c)**, and (ii) induction of sterile protection **(Figure 6d)**. Vaccinated mice challenged with $CSP^{SIINFEKL}$ or $UIS4^{SIINFEKL}$ sporozoites showed a dramatic reduction in parasite load in the liver **(Figure 6c)** as compared to vaccinated mice challenged with WT parasites. Strikingly, there was no statistical difference in the protection observed when vaccinated mice were challenged with either $CSP^{SIINFEKL}$ or $UIS4^{SIINFEKL}$ sporozoites. Consistent with these findings, both groups of vaccinated mice challenged with either $CSP^{SIINFEKL}$ or $UIS4^{SIINFEKL}$ sporozoites exhibited sterile protection of comparable levels **(Figure 6d)**. These findings indicate that spatial and temporal aspects of antigen expression may affect protein immunogenicity in the context of parasitic infection but not necessarily the same target's susceptibility to antigen-specific CD8+ T cell killing.

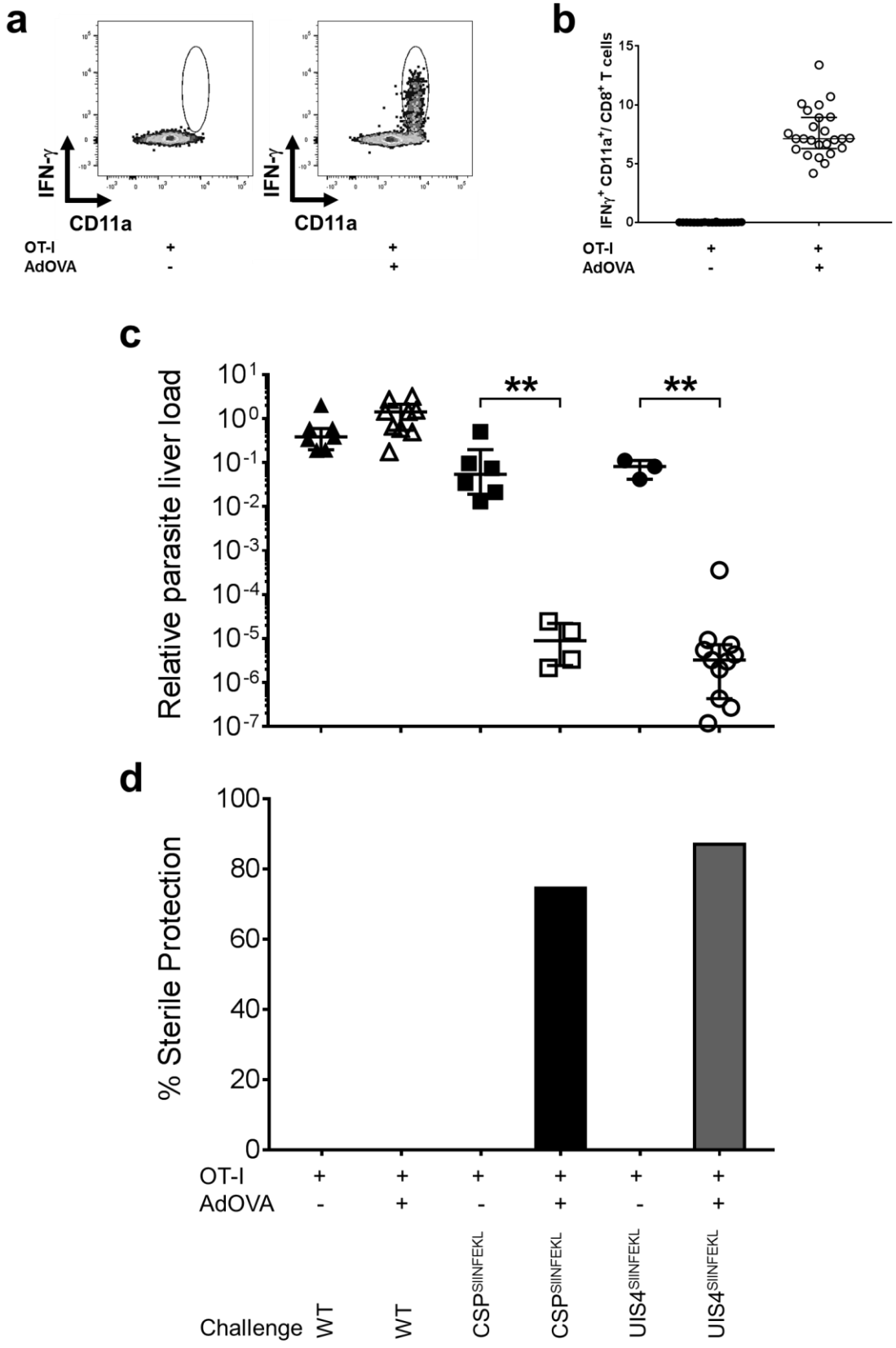

a
IFN-γ
CD11a
IFN-γ
CD11a
OT-I + +
AdOVA - +
b
IFNγ+ CD11a+/ CD8+ T cells
15
10
5
0
OT-I + +
AdOVA - +
c
Relative parasite liver load
10^1
10^0
10^-1
10^-2
10^-3
10^-4
10^-5
10^-6
10^-7
**
**
d
% Sterile Protection
100
80
60
40
20
0
OT-I + + + + + +
AdOVA - + - + - +
Challenge WT WT CSPSIINFEKL CSPSIINFEKL UIS4SIINFEKL UIS4SIINFEKL

Figure 6: Sporozoite surface and EEF vacuolar membrane antigens are presented to vaccine-induced CD8+ T cells for killing, leading to sterile protection.

Mice received $1x10^8$ ifu recombinant AdHu5 expressing whole ovalbumin (AdOVA) and/or $2x10^6$ OT-I splenocytes. **(a)** Flow cytometry and **(b)** scatter plots represent CD8+ T cells derived from peripheral blood co-stained with IFN-γ and CD11a, following *ex vivo* restimulation with SIINFEKL. **(c)** Protective efficacy as measured by quantitative real-time PCR. Groups of mice (n=3-11 per group) were vaccinated as described and challenged 19 days later with 10,000 WT, $CSP^{SIINFEKL}$ or $UIS4^{SIINFEKL}$ sporozoites. 42 hours later livers were removed and parasite load was assessed by qPCR. Plots show the relative parasite load of mice in each condition (**, $p<0.01$; Mann-Whitney U test). **(d)** Proportion of sterile protection after immunization. Mice (n=8 per group) were vaccinated as described and were challenged with 1,000 WT, $CSP^{SIINFEKL}$ or $UIS4^{SIINFEKL}$ sporozoites. Data for **a-d** are from one representative experiment of two experiments performed with scatter plots showing median values + interquartile range.

DISCUSSION

The malaria pre-erythrocytic stages have been a prime target for the development of a *Pf* vaccine for more than 35 years. Indeed, RTS,S/AS01, the most advanced malaria sub-unit vaccine candidate to date is based on CSP, the major surface protein of sporozoites[28]. Yet, final results of the Phase III trial showed that RTS,S/AS01 offers only modest efficacy, which rapidly wanes over time[29]. Thus, there is an imperative need not only to widen the pursuit for new sub-unit vaccine candidates, but also to radically improve the antigen selection process. Antigens are generally prioritised based on a range of criteria, including their immunogenicity in the context of parasitic infection. We examined this notion in an infection and vaccination model for malaria pre-erythrocytic stages.

The malaria pre-erythrocytic stages consist of two spatially-different parasite forms: extracellular sporozoites and intracellular EEFs. The transformation of sporozoites into EEFs involves regulation at both transcriptional[30] and translational[31,32] levels, resulting in both spatial and temporal expression of many antigens that are distinct for each parasite form[33]. Whilst our current understanding of immune responses to malaria pre-erythrocytic stages has focused on CSP, the lack of well-defined epitopes that are expressed only by EEFs has restrained fundamental studies investigating the contributions of EEF antigens in parasite-induced CD8+ T cell responses and their value as target of vaccines.

In this study, we contrasted the development of CD8+ T cell responses induced by CSP and UIS4, two major proteins expressed by sporozoites and EEFs, respectively. We generated transgenic *Pb* parasites where SIINFEKL is expressed as part of either CSP or UIS4, allowing the presentation of the epitope at the same space and time as the respective protein. This approach is in contrast to a more common strategy of inserting the whole ORF of ovalbumin to be expressed as part of an *Pb* endogenous gene and then tracking the immune response elicited by this extraneous molecule[24,34]. Since CSP is expressed in both sporozoites and EEFs, the processing and presentation

of the SIINFEKL in CSP^{SIINFEKL} occurs as soon as sporozoites are inoculated and are able to interact with dendritic cells, which present antigens via an endosome-to-cytosol pathway[8]. CSP also has direct access to the hepatocyte's cytosol for processing and presentation of the CSP-derived epitope[8]. However, since UIS4 is expressed only in the PVM of EEFs, processing and presentation of the epitope in UIS4^{SIINFEKL} is restricted to just hepatocytes.

Our results establish that following sporozoite-immunisation, a sporozoite surface protein induces superior CD8+ T cell responses – as measured both by pentamer staining and by IFN-γ secretion following peptide stimulation – than an EEF vacuolar membrane protein. Detailed kinetic and phenotypic analysis of the development of antigen-specific CD8+ T cells to both CSP and UIS4 revealed that the responses only differ in magnitude but not in durability, demonstrating the ability of both antigens to elicit effector and effector memory responses. There was no difference in our results whether sporozoites are delivered using the commonly used intravenous immunisation or the more physiological intradermal delivery. We also showed that increasing the number of UIS4^{SIINFEKL} parasites used for immunisation did not augment CD8+ T cell responses, signifying that the poor immunogenicity of an EEF vacuolar membrane protein is not a due to the level of UIS4 expression during parasite infection. Our findings support the idea that EEF antigens have minimal contributions to the magnitude of immune responses following whole sporozoite immunisation, which corroborates with prior data showing that that hepatocytes are poor at priming T cell responses[11,12].

Regardless of their differing CD8+ T cell immunogenicities in the context of parasitic infection, we further demonstrated that both sporozoite and EEF antigens are effectively targeted by antigen-specific effector CD8+ T cells, which were generated by vaccination using priming and boosting with recombinant viruses expressing the epitope. Importantly, mice harbouring vaccine-induced, antigen-specific CD8+ T cells were comparably protected when challenged with either CSP^{SIINFEKL} or UIS4^{SIINFEKL}. These findings imply that both sporozoite and EEF antigens comparably access the antigen presentation pathways in hepatocytes leading to recognition of defined epitopes.

Our study is the first demonstration that poor natural immunogenicity, in this case of an EEF antigen, does not preclude antigen-specific CD8+ T cell killing. Our findings that antigen immunogenicity in this context is an inadequate predictor of vaccine efficacy have wide-ranging implications on antigen prioritisation for the design and testing of next generation malaria vaccines. To broaden the repertoire of liver-stage malaria vaccines, antigens secreted into the hepatocytes of either infected or traversed cells must be tested as these constitute promising targets of anti-malaria vaccines. Additionally, combining EEF antigens with CSP would be a favourable concept.

While CSP and UIS4 only represent one antigen expressed in the sporozoite or EEF, they act as good surrogates for assessing CD8+ T cell responses to sporozoite and EEF antigens. Both antigens are highly abundant in their respective life stages, so should represent antigens that are likely to have significant access to the antigen presentation machinery compared to lesser expressed proteins. These antigens have also offered an insight into the effect of temporal and spatial factors of antigen expression on CD8+ T cell responses. Temporally, CSP protein is expressed early in the pre-erythrocytic stages like other sporozoite antigens, while UIS4 protein is expressed later, following hepatocyte invasion, like all EEF-specific antigens. Spatially, CSP is expressed on the sporozoite surface as it travels through the skin and bloodstream in search of the liver and so other sporozoite antigens have similar niches, contacting many cells and increasing their propensity to be presented. UIS4 protein is expressed solely by the parasite in the hepatocyte and exported to the PVM, much like other EEF proteins. The close proximity of the PVM to the hepatocyte cytosol may also increase the likelihood of the protein being processed and presented, compared to a protein remaining inside the parasite plasma membrane. This is not to say that CSP or UIS4 are completely akin to all sporozoite or EEF antigens respectively. To fully resolve the spatiality effects of antigen expression on CD8+ T cell responses in the pre-erythrocytic stages of malaria, further antigens would need to be investigated. For instance, the differences between intracellular and extracellular sporozoite antigens, and intracellular EEF antigens and those exported to the PVM should be compared in their capability to induce CD8+ T cell

responses and be susceptible to vaccine-induced CD8+ T cells. The temporal effects of EEF antigen expression on CD8+ T cell responses have also been started to be probed to see if later expressed EEF antigens induce similar CD8+ T cell responses compared to those expressed earlier like UIS4 (Chapter 4: Gibbins et al., paper in preparation).

A key direction for future research will be identifying the mechanisms by which EEF antigens elicit protection and finding new assays to easily distinguish good vaccine targets, namely those antigens that can protect (via susceptibility to vaccine-induced CD8+ T cells) rather than those that naturally induce strongly immunogenic responses. Ultimately, the molecular mechanisms of presentation of EEF antigens, those expressed in the PVM and within the parasite itself, onto the surface of infected hepatocytes remains to be fully understood. Determination of the processes involved in parasite antigen presentation in the pre-erythrocytic stages of malaria may elucidate links to protection and the identification of further antigens that could drive the development of an efficacious protective malaria vaccine.

METHODS

Ethics and animal experimentation

Animal procedures were performed in accordance with the German 'Tierschutzgesetz in der Fassung vom 18. Mai 2006 (BGBl. I S. 1207)' which implements the directive 2010/63/EU from the European Union. Animal experiments at London School of Hygiene and Tropical Medicine were conducted under license from the United Kingdom Home Office under the Animals (Scientific Procedures) Act 1986. NMRI, CD-1, C57BL/6 and OT-I laboratory mouse strains were bred in house at LSHTM or purchased from Charles River Laboratories (Margate, UK or Sulzfeld, Germany). Female mice were used for experiments at the age of 6-8 weeks.

Generation of transgenic parasites

Transgenic *P. berghei* ANKA mutants CSP^{SIINFEKL} and UIS4^{SIINFEKL} were developed using double homologous recombination. In the CSP^{SIINFEKL} mutant, the CSP gene is altered so the epitope SYIPSAEKI (residues 252-260) is replaced with the H-2^{b} restricted *Gallus gallus* ovalbumin epitope SIINFEKL. In the UIS4^{SIINFEKL} mutant, the SIINFEKL epitope is appended to the C-terminal end of the UIS4 protein. Clonal parasite lines were generated by limiting dilution. Details of plasmid design, including the primers used and the cloning of parasites can be found in **Supplementary Experimental Procedures** and **Supplementary Table 1**.

Plasmodium berghei ANKA immunisation

P. berghei wild type (WT; strain ANKA clone c15cy1 or clone 507) parasites and CSP^{SIINFEKL} and UIS4^{SIINFEKL} (clone c15cy1) parasites were maintained by continuous cycling between murine hosts (NMRI or CD-1) and *Anopheles stephensi* mosquitoes. Infected mosquitoes were kept in incubators (Panasonic and Mytron) at 80% humidity and 20°C. Sporozoites were isolated from salivary glands and γ-irradiated at 1.2 x 10^4 cGy. Mice were immunised intravenously in the lateral tail vein or intradermally in the ear pinnae with 10,000 sporozoites, unless otherwise stated, and challenged with either

1,000 or 10,000 sporozoites injected intravenously.

Indirect fluorescent antibody staining (IFA) of sporozoites

Epoxy-covered 8-well glass slides were coated with 3% BSA-RPMI. 10,000 sporozoites were added per well in 3% BSA-RPMI and incubated for 45 minutes during which the shed surface proteins are deposited in the gliding motility process. Sporozoites and their trails were stained with a mouse anti-CSP[35] primary antibody and a rabbit polyclonal anti-*Pb*UIS4[31] primary antibody and the respective fluorescently labelled secondary antibodies. Nuclei were stained with Hoechst 33342 and slides mounted with 'Fluoromount-G' (Southern Biotech). Sporozoites and trails were analysed by fluorescent microscopy (Zeiss Axio Observer).

***In vitro* infection of hepatoma cells and fluorescent staining**

In vitro EEF development was analysed in infected Huh7 hepatoma cells for 24 and 48 hours. Triplicate Labtek (Permanox plastic - Nunc) wells were infected with 10,000 transgenic CSP^{SIINFEKL} or UIS4^{SIINFEKL} parasites and duplicate wells were infected with 10,000 WT parasites. Infected cells were analysed by fluorescence microscopy using a mouse anti-*Pb*HSP70[36] and a rabbit polyclonal anti-*Pb*UIS4[31] primary antibody, the respective fluorescently labelled secondary antibodies and nuclear staining with Hoechst 33342. Staining were analysed by fluorescent microscopy (Zeiss Axio Observer).

Quantification of SIINFEKL-specific CD8+ T cell responses

Spleens and livers were harvested from immunised or naïve mice and perfused with PBS. Lymphocytes were derived from spleens by passing through 40 or 70μm cell strainers (Corning) and from livers by passing through 70μm cell strainers (Corning). Red blood cells were lysed with PharmLyse (BD), and lymphocytes were resuspended in complete RPMI (cRPMI- RPMI + 10% FCS + 2% Penicillin-Streptomycin + 1% L-glutamine (Gibco)). For cell counting, lymphocytes were diluted 40x with Trypan Blue

(ThermoFisher Scientific) and enumerated using a Neubauer 'Improved' haemocytometer (Biochrom). Alternatively, lymphocytes were counted using a MACSQuant flow cytometer (Miltenyi Biotec), using propidium iodide (PI) (Sigma Aldrich) or, in the case of hepatic lymphocytes, using CD45.2-Alexa647 (Biolegend) to distinguish between hepatocytes and lymphocytes, prior to PI administration and counting. Peripheral blood was acquired by tail vein puncture collected in Na^+ heparin capillary tubes (Brand) and assayed in 96-well flat bottom plates (Corning). For CD8+ T cell stimulations, 2-3x10^6 splenocytes or 1-2x10^5 liver cells were incubated with SIINFEKL peptide (Peptides and Elephants, Henningsdorf) at a final concentration of 10μg/ml in the presence of Brefeldin A (eBioScience). Cells were incubated at 37°C, 5% CO_2 for 5-6 hours, before incubation at 4°C overnight. For staining of cell surface markers and intracellular cytokines, cells were incubated for 1 hour at 4°C. Cells derived from the spleen or liver were fixed with 4% paraformaldehyde, and cells from peripheral blood were fixed with 1% paraformaldehyde between the extra- and intracellular staining steps. Data was acquired by flow cytometry using an LSRII or LSRFortessa (BD). Antibodies used for staining were as follows; BD: CD3 (500A2); eBioScience: CD8 (53-6.7), CD11a (M17/4), CD49d (R1-2), CD62L (MEL-14), CD44 (IM7), IFN-γ (XMG1.2), TNF-α (MP6-XT22) and IL-2 (JES6-5H4); ProImmune: H-2-K^b-SIINFEKL pentamers.

CFSE labelling of OT-I cells

Spleens from OT-I mice were lysed and cells washed twice in PBS without serum. Splenocytes resuspended at a density of 5x10^6 cells/ml in PBS had 1:5,000 CFSE (ThermoFisher Scientific) added and were incubated in the dark at room temperature, with gentle inversion for 4 minutes. The labelling reaction was quenched with cRPMI and cells washed twice in cRPMI. Cells were recounted and 2x10^6 cells were injected per mouse.

Vaccination with OVA expressing recombinant adenovirus

To assess parasite liver load after vaccination with virus-expressed OVA, groups of C57BL/6 mice were immunised with recombinant human adenovirus serotype 5 (AdHu5) expressing full-length chicken ovalbumin (AdOVA)[27]. Each mouse received $1x10^8$ infective units (ifu) in a volume of 100µl administered intramuscularly (50µl into each thigh). At the same time vaccinated and control mice received OT-I splenocytes intravenously ($2x10^6$ cells/mouse). 19 days after vaccination, mice were challenged with 10,000 WT, $CSP^{SIINFEKL}$ or $UIS4^{SIINFEKL}$ sporozoites administered intravenously. 42 hours after the challenge the livers were harvested and homogenised in TRIzol (ThermoFisher Scientific) for total RNA isolation. Afterwards, cDNA was generated using the RETROScript Kit (Ambion). Quantitative real-time PCR was performed using the StepOnePlus Real-Time PCR System and Power SYBR Green PCR Master Mix (Applied Biosystems). Relative liver parasite levels were quantified using the ΔΔCt method comparing levels of *P. berghei 18S* rRNA normalised to mouse *GAPDH* mRNA[31]. To assess sterile protection, AdHu5 OVA-vaccinated and control mice received $2x10^6$ OT-I splenocytes one day prior to vaccination. 14 days after vaccination, all mice were challenged with 1,000 WT, $CSP^{SIINFEKL}$ or $UIS4^{SIINFEKL}$ sporozoites. Blood smears were taken from day 3-14 after challenge to determine the presence of blood stage parasites.

Statistics

Data were analysed using FlowJo version 9.5.3 (Tree Star Inc., Oregon, USA), Microsoft Excel and GraphPad Prism v7 (GraphPad Software Inc., CA, USA). We used Mann-Whitney U test for analysing data from two groups that were not normally distributed, and Welch's t-test or one-way ANOVA with Tukey's multiple comparison test for normally distributed data.

AUTHOR CONTRIBUTIONS

O.S. and J.C.R.H. designed the experiments in the laboratory of K.Matuschewski; O.S. generated the transgenic parasites $CSP^{SIINFEKL}$ and $UIS4^{SIINFEKL}$; K.Müller, M.P.G., O.S. and J.C.R.H. performed experiments and analysed data; A.R.-S., A.V.S.H. and S.J.D. provided the adenovirus AdOVA; M.P.G. and J.C.R.H. wrote the paper; all authors commented and revised the manuscript.

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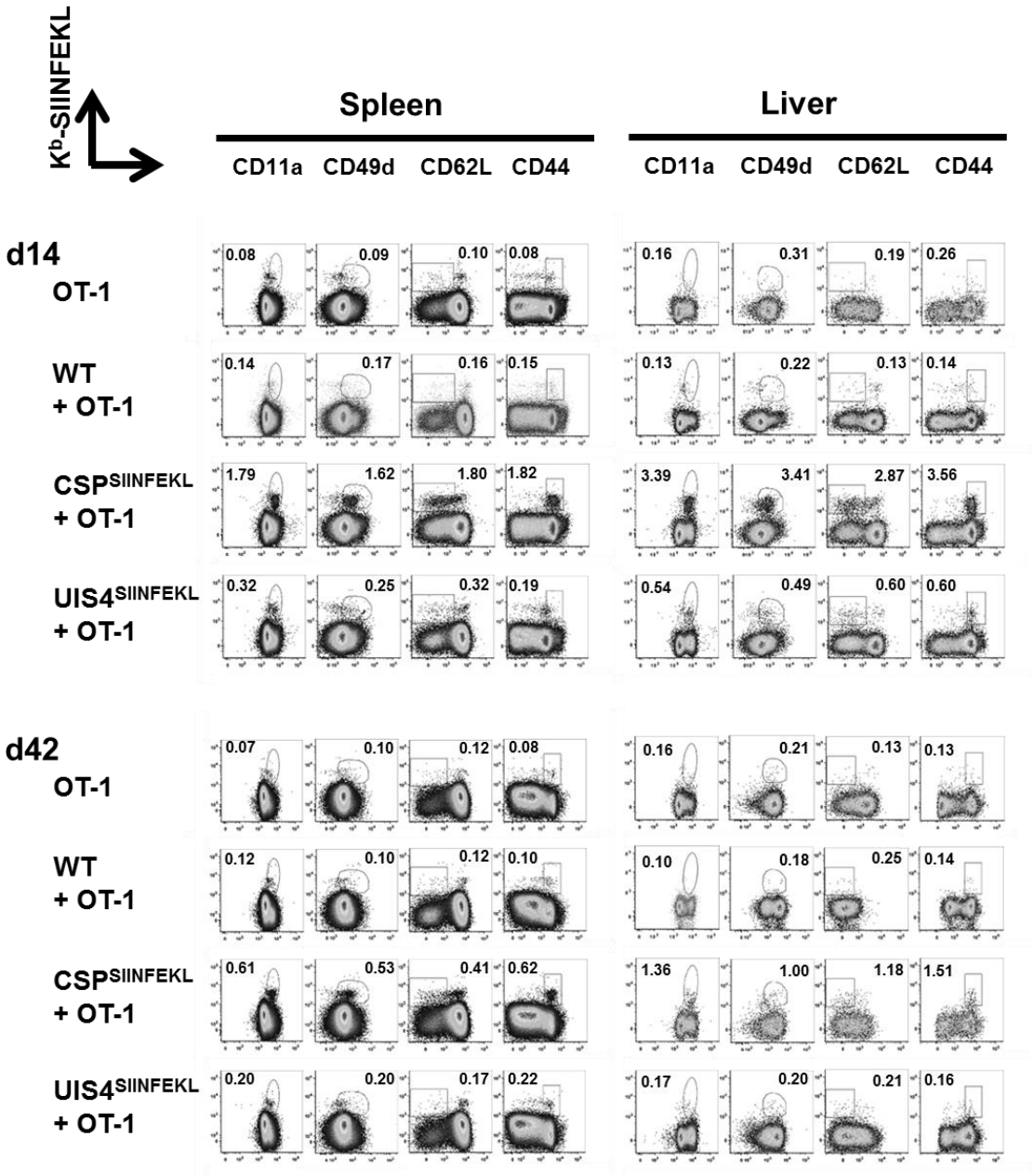
COMPETING INTERESTS

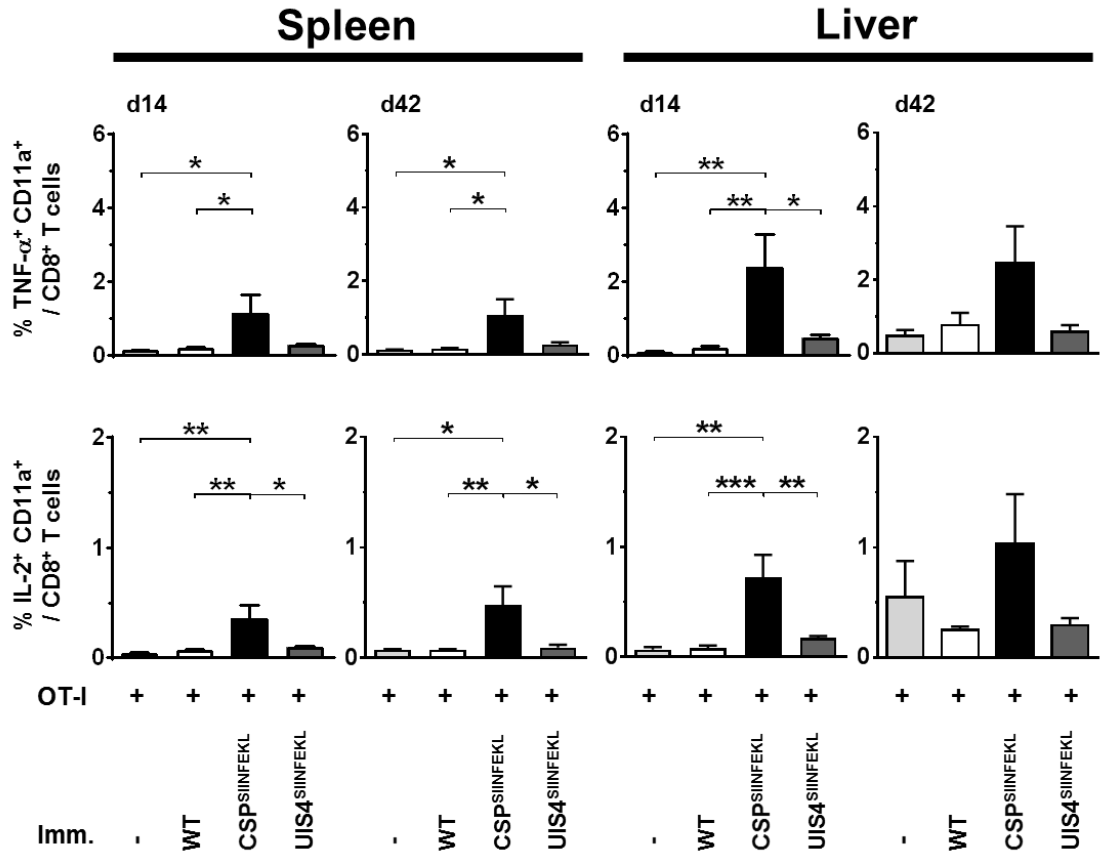
S.J.D. is a named inventor on patent applications relating to malaria vaccines, adenovirus vaccines and immunisation regimens.

SUPPLEMENTARY FIGURES



Supplementary Figure 1: Sporozoite surface antigen induces a greater effector CD8+ T cell phenotype than EEF vacuolar membrane antigen.

C57BL/6 mice (n=3-5 per group) received $2x10^6$ OT-I cells alone or were additionally immunised with 10,000 γ-radiation attenuated WT, $CSP^{SIINFEKL}$ or $UIS4^{SIINFEKL}$ sporozoites intravenously. Spleens and livers were harvested either 14 or 42 days later, and proportions of CD8+ T cells expressing effector surface markers were quantified. Flow cytometry plots show representative percentages of CD8+ T cells co-staining K^b-SIINFEKL and markers of effector phenotype ($CD11a^{hi}$, $CD49d^{hi}$, $CD62L^{lo}$, $CD44^{hi}$).



Supplementary Figure 2: Antigen experienced SIINFEKL-specific CD8+ T cells also produce TNF-α and IL-2.

C57BL/6 mice (n=3-5 per group) received 2x10^6 OT-I cells alone or were additionally immunised with 10,000 γ-radiation attenuated WT, CSP^{SIINFEKL} or UIS4^{SIINFEKL} sporozoites intravenously. Spleens and livers were harvested either 14 or 42 days after immunisation and lymphocytes restimulated *ex vivo* with SIINFEKL peptide at 10μg/ml per well for 5-6 hours. The upper panel of bar charts show the percentage of CD11a+ TNF-α secreting CD8+ T cells, the bottom panel CD11a+ IL-2 secreting CD8+ T cells. This is a representation of one experiment from two experiments performed. Bar charts show mean values (±SEM) from representative experiments (*, $p<0.05$, **, $p<0.01$, ***, $p<0.001$; one-way ANOVA with Tukey's multiple comparison test).

SUPPLEMENTARY EXPERIMENTAL PROCEDURES

Generation of CSP^{SIINFEKL} and UIS4^{SIINFEKL} transgenic *P. berghei* parasite lines

B3D-CSP^{SIINFEKL} plasmid was assembled by successive cloning of three fragments, CSP-C, CSP-B and CSP-A, obtained by PCR amplification from *P. berghei* ANKA genomic DNA followed by restriction enzyme digestion. These fragments correspond respectively to a 3' homology region downstream of CSP (CSP-C, 0.7 kb), a fragment comprising the CSP ORF downstream of the SYIPSAEKI epitope followed by the CSP 3' UTR (CSP-B, 0.8 kb) and a fragment comprising a 5' promoter region followed by the CSP modified ORF where the SYIPSAEK coding sequence has been replaced by a SIINFEKL coding sequence (CSP-A, 1.8 kb). The resulting B3D-CSP^{SIINFEKL} plasmid, containing the *Toxoplasma gondii* dihydrofolate reductase/thymidylate synthase (*TgDHFR/TS*) pyrimethamine resistance cassette flanked by CSP-A and CSP-B on one side, and CSP-C on the other, was linearized with *Not*I and *Sac*II before transfection. Integration of the construct after double crossover homologous recombination results in replacement of the WT CSP gene by a modified copy containing the SIINFEKL coding sequence instead of the SYIPSAEKI coding sequence. The B3D-UIS4^{SIINFEKL} plasmid was assembled by successive cloning of three fragments, UIS4-A, UIS4-B and UIS4-C, obtained by PCR amplification from *P. berghei* ANKA genomic DNA followed by restriction enzyme digestion. These fragments correspond respectively to a fragment comprising a 5' upstream sequence followed by the UIS4 entire ORF fused in frame to the SIINFEKL coding sequence (UIS4-A, 1.2 kb), to the UIS4 3' UTR sequence (UIS4-B, 0.6 kb) and to a 3' homology region downstream of UIS4 (UIS4-C, 0.9 kb). The resulting B3D-UIS4^{SIINFEKL} plasmid, containing the *TgDHFR/TS* pyrimethamine resistance cassette flanked by UIS4-A and UIS4-B on one side, and UIS4-C on the other, was linearized with *Sac*II and *Kpn*I before transfection. Integration of the construct after double crossover homologous recombination results in replacement of the WT UIS4 gene by a modified copy containing the SIINFEKL coding sequence just upstream of a STOP codon. *P. berghei* CSP^{SIINFEKL} and UIS4^{SIINFEKL} parasites were generated by transfection of *P.*

berghei ANKA with linearized B3D-CSP^{SIINFEKL} and B3D-UIS4^{SIINFEKL} plasmids, respectively. Purified schizonts of WT *P. berghei* ANKA (clone c15cy1) were transfected with 5-10µg of linearized plasmid by electroporation using the AMAXA Nucleofector device (program U33), as described[17], and immediately injected intravenously in the tail vein of a mouse. The day after transfection, pyrimethamine (70 mg/l) was administrated in the mouse drinking water, for selection of transgenic parasites. Transgenic clones were isolated after limiting dilution and injection into mice. Correct integration of the constructs and purity of the transgenic lines was verified by analytical PCR using primer combinations specific for the unmodified CSP or UIS4 locus, and for the 5' and 3' recombination events. All primers used in this study are indicated in **Supplementary Table 1**.

	Oligonucleotide	Sequence 5´ → 3´
Production of B3D-CSP^{SIINFEKL} construct	CSP-A forward	ATAAGAATGCGGCCGCATGGTTATATTTTGTGCAATGCTAAAATGG
	CSP-A reverse	CGGAATTCTAGTATCAGTTTTTCAAAGTTGATTATACTATCGTCATTATTATTATTTTTGTTATTG
	CSP-B forward	GGACTAGTGAATTCGTTAAACAGATCAGGGATAGTATCACAGAGG
	CSP-B reverse	CCGCAATTGTACAAAAAATATTTTCGACAAAGGATAACG
	CSP-C forward	CCCAAGCTTTGGGAATCTATTTTACAATATTATTTAAGGG
	CSP-C reverse	CGGGGTACCCCGCGGTTATTGAAAAAGACACAAAATAGCTAG
Production of B3D-UIS4^{SIINFEKL} construct	UIS4-A forward	TCCCCGCGGATAGCTATATTTTATGGTTGATCCTTTCC
	UIS4-A reverse	GGACTAGTTTACAGTTTTTCAAAGTTGATTATACTTATGTATGGGCCGAATGATTTATTTTCC
	UIS4-B forward	GGACTAGTTTCATTATGAGTAGTGTAATTCAGAAAGAG
	UIS4-B reverse	CCGGAATTCTATGTAAAAAAGTTTGCATATACGGCTG
	UIS4-C forward	CCCAAGCTTAGTGAAATATAAATATGAATGGAAGCAGCC
	UIS4-C reverse	CGGGGTACCAGCAGCTAATGTCAATATATTTTATGCAC
Genotyping of transgenic parasites	TgDHFR forward	CGCATTATATGAGTTCATTTTACACAATCC
	OVA reverse	CTAGTTTACAGTTTTTCAAAGTTGATTATAC
	CSP WT forward	TGTGAACTTTTCCTTATTTATTACGATTATG
	CSP test forward	AATATGAGCACGCTTTTACTTTGTCCAGG
	CSP test reverse	ACGAATCGAAATAAGTTACTATTCGTGCC
	UIS4 test forward	TGGTTCTTAATATTATTTTGGATACATGC
	UIS4 test reverse	CTCGTGTCCTTTGTAGTAAAAATAAACC

Supplementary Table 1 – Primers used to generate and genotype CSP^{SIINFEKL} and UIS4^{SIINFEKL} transgenic parasites.

Restriction sites in the primer sequences are underlined.

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Student	Matthew Paul Gibbins
Principal Supervisor	Julius Clemence R. Hafalla
Thesis Title	Dissecting the CD8+ T cell responses to pre-erythrocytic malaria antigens

If the Research Paper has previously been published please complete Section B, if not please move to Section C

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SECTION D – Multi-authored work

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CHAPTER 3

Extending expression of *Plasmodium* liver stage antigens does not improve cognate CD8+ T cell responses

Extending expression of *Plasmodium* liver stage antigens does not improve cognate CD8+ T cell responses

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ABSTRACT

Sporozoite antigens are the basis of many vaccines currently being tested against the pre-erythrocytic stages of malaria. Research focusing on liver stage antigens is lacking and their contribution to pre-erythrocytic immunity is less well understood. Whole sporozoite vaccination studies have shown that immunisation with late liver stage arresting parasites lead to improved protection against infectious sporozoite challenge. Nonetheless, few liver stage antigens have been discovered that can induce protection against sporozoite challenge. Previously our group had shown that UIS4, a parasitophorous vacuolar membrane (PVM) protein, expressed constitutively during liver stage development of the parasite, induces poor CD8+ T cell responses following immunisation with radiation attenuated *P. berghei* sporozoites. Given that radiation attenuated *P. berghei* sporozoites arrest early in their host hepatocytes, we employed an alternative sporozoite immunisation strategy in an effort to improve endogenous CD8+ T cell responses to UIS4. Here we employed azithromycin prophylaxis to ensure full liver stage development. However, we found that increasing the duration of expression of UIS4 in the liver stage parasite does not improve its cognate CD8+ T cell responses. Therefore, it is now important to define if late-expressing EEF antigens can contribute to protective CD8+ T cell responses against the pre-erythrocytic stages of malaria.

INTRODUCTION

There is a far greater abundance of research delineating *Plasmodium* sporozoite antigens, the immune responses they induce and their potential for use in malaria pre-erythrocytic vaccines compared to antigens expressed in the liver stage or exo-erythrocytic forms (EEFs) of malaria infection. Early identification of pre-erythrocytic antigens focused on sporozoite antigens due to their high immunogenic capacity. Sporozoites are an extracellular form of the parasite, clearly visible to the immune system and have been found to induce strong antibody responses[1]. Experimentally, the generation of sporozoites from mosquitoes is also much easier than generating EEFs, which require a suitable hepatocyte tissue system[2]. However, to generate an efficacious pre-erythrocytic vaccine, it is imperative to investigate immune responses to the parasite as it resides in the liver. During this development in a hepatocyte, the parasite is stationary, thus fit for immune-mediated destruction, which would prevent the subsequent symptomatic and transmissible stages.

Vaccination induced protection against the pre-erythrocytic stages of malaria was first shown to be possible in animals and humans using radiation attenuation sporozoites (RAS)[3-5]. RAS became the gold standard that all future malaria vaccines were compared to. Sterile protection induced by RAS has been shown to be mediated primarily by CD8+ T cells[6, 7]. Efficient recall of CD8+ T cell responses following presentation of parasite antigens on hepatocytes is crucial due to the short liver stage (in mice infected with *Plasmodium berghei* this is around 48-52 hours[8]), in order to kill all the developing EEFs. Despite the protection RAS can mediate, around 24 hours post immunisation with *Plasmodium berghei* RAS, parasites arrest their development in the hepatocyte as they reach the early schizont stage[9, 10]. In contrast, genetically attenuated parasites (GAPs) that arrest later in EEF development have been generated, with immunisation offering greater levels of sterile protection against pre-erythrocytic infection compared to RAS or early-arresting GAPs[11, 12].

In addition, another whole sporozoite vaccine approach involving administration of antibiotics as anti-malarial chemoprophylaxis can protect mice from challenge[13]. Antibiotics target the apicoplast of the parasite, which are a relict plastid-like organelle obtained by endosymbiosis. The apicoplast has lost its photosynthetic properties however it is still an essential organelle for the parasite, with antibiotic treated asexual forms of *P. falciparum* unable to develop inside red blood cells (RBCs) following reinvasion, and perpetuate the infection[14], leading to a 'delayed death' phenotype. Apicoplasts in the progeny are unable to branch and segregate, unlike the nucleus[14]. Thus, where daughter merozoites do not contain a nucleus and an apicoplast, perpetuation of the infection is not possible. In the EEF, the action of antibiotics on the parasite is similar. EEF development appears to occur as normal, with clear schizogony of the nucleus. However, the antibiotics target the apicoplast, with a lack of branching occurring upon schizogony[15, 16]. The merosomes that are produced following *P. berghei* sporozoite infection of hepatoma cells treated with antibiotics *in vitro* contain non-viable merozoites, with these merozoites unable to infect mice when administered intravenously[16]. As in the RBC, antibiotics target the apicoplast of the EEFs in hepatocytes to prevent normal branching morphology during schizogony and daughter merozoites fail to be infectious and produce progeny because they fail to inherit an apicoplast[16].

Several antibiotics have been tested as prophylactic drugs concomitant with sporozoite immunisation and the greatest levels of sterile protection, following challenge, occurred when mice were immunised multiple times under azithromycin cover[13]. The level of protection was also greater compared to immunisation with chloroquine prophylaxis or RAS[13] and mice receiving three immunising doses of sporozoites with azithromycin prophylaxis survived a second re-challenge 6 months after the first challenge[16]. In these investigations, azithromycin was given as three doses of 160mg/kg intraperitoneally[13, 16], however in humans this can be given orally. Prevention of malaria by daily dosing with azithromycin has been shown to be effective in Kenya[17] and

Indonesia[18] indicating a generally safe, widely available antibiotic could be repurposed as a malaria prophylactic[19].

Molecular docking techniques have tried to identify the mode of action of azithromycin. There is evidence that point mutations in the apicoplast large subunit unit (LSU) rRNA gene and apicoplast-encoded ribosomal protein L4 (*rpl4*) gene confer azithromycin resistance in *P. falciparum*. With these mutations, azithromycin is unable to bind to L4, which complexes with L22 and the LSU rRNA at the nascent peptide exit tunnel[20]. This suggests that azithromycin blocks apicoplast development by inhibiting the apicoplast translation machinery, preventing new apicoplast-encoded polypeptides from being released from the ribosome. Using *in silico* docking software suggests this docking of azithromycin to apicoplast ribosomal proteins is organelle specific, as azithromycin does not dock in mitochondrial ribosomal proteins with the same affinity due to different side chains affecting the environment of the docking site[21].

As mentioned above, azithromycin allows full development of the EEF and merosome formation, which may be the reason for the increased sterile protection it induces. This increased protection may be achieved using the same mode of action as seen for late arresting GAPs[12]. It is hypothesised that upon extended EEF development, extended repertoires of antigens are expressed thus leading to a more diverse CD8+ T cell repertoire[12]. In addition to increased repertoires, the length of single antigen expression may also be increased. However, the CD8+ T cell responses induced by EEF proteins are currently not well defined.

Previous research from our group has shown that CD8+ T cell responses to an EEF vacuolar membrane protein, UIS4[22], were weaker compared to those against circumsporozoite protein (CSP) when mice were immunised with *P. berghei* RAS. Nonetheless, both sporozoite and EEF antigen could robustly protect when used in a viral vaccination regimen (Chapter 2: Müller and Gibbins, paper in preparation). Both

antigens are abundantly expressed, with CSP constitutively present at the sporozoite surface and UIS4 constitutively present at the PVM following sporozoite invasion of a hepatocyte[22, 23]. Here we wanted to determine if the magnitude of antigen-specific CD8+ T cells responses to this EEF antigen could be improved by altering the method of parasite attenuation. In a side-by-side study, we compared CD8+ T cell responses following different immunisation strategies of C57BL/6 mice with *P. berghei* transgenic parasites expressing the reporter CD8+ T cell epitope SIINFEKL in the context of CSP or UIS4 (Chapter 2: Müller & Gibbins, paper in preparation). Mice were immunised either with RAS or sporozoites and azithromycin prophylaxis. Here we hypothesised that prolonged PVM protein expression would increase CD8+ T cell responses against EEF vacuolar membrane proteins.

MATERIALS AND METHODS

Ethics and animal experimentation

All animal work was conducted in accordance with the German Tierschutzgesetz in der Fassung von 18. Mai 2006 (BGB1. I S. 1207), which implements the Directive 86/609/EEC from the European Union and the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes. Animal experiments performed at London School of Hygiene and Tropical Medicine were conducted under licence from the United Kingdom Home Office under the Animals (Scientific Procedures) Act 1986. All protocols were approved by the ethics committee of the Max Planck Institute for Infection Biology and the Animal Welfare and Ethics Review Board of the London School of Hygiene and Tropical Medicine. NMRI, CD-1 and C57BL/6 laboratory mouse strains were bred in house at LSHTM or purchased from Charles River Laboratories (Margate, UK or Sulzfeld, Germany). NMRI and CD-1 were used for cycling of parasites between vertebrate and mosquito hosts. Female C57BL/6 mice were used for immunology experiments at age 6-8 weeks.

Plasmodium berghei ANKA immunisation

P. berghei $CSP^{SIINFEKL}$ and $UIS4^{SIINFEKL}$ (strain ANKA clone c15cy1) parasites (Chapter 2: Müller and Gibbins, paper in preparation) were maintained by continuous cycling between murine hosts (NMRI or CD-1) and *Anopheles stephensi* mosquitoes. Infected mosquitoes were kept in incubators (Panasonic and Myrton) at 70-80% humidity and 20°C. Sporozoites were isolated from salivary glands no earlier than 18 days after infection. Mice were immunised with 10,000 sporozoites intravenously in the lateral tail vein. Sporozoites were either γ-irradiated at 1.2×10^4 cGy or administered under prophylactic azithromycin drug cover. Azithromycin (Pfizer) was administered at a dose of 240 mg/kg intraperitoneally on the same day as immunisation and one day after[16].

Quantification of SIINFEKL-specific CD8+ T cell responses

Spleens and livers perfused with 5ml PBS were harvested from immunised and naive mice. Lymphocytes were filtered by passing the organs through 70μm cell strainers (Corning). Red blood cells were lysed with PharmLyse (BD) and lymphocytes were resuspended in complete RPMI (cRPMI- RPMI + 10% FCS + 2% Penicillin-Streptomycin + 1% L-glutamine (Gibco)).

Cells were diluted 40x in Trypan Blue (ThermoFisher Scientific) and counted by microscopy using a Neubauer 'Improved' haemocytometer (Biochrom) or cells were counted using a MACSQuant flow cytometer (Miltenyi Biotec), using propidium iodide (PI) (Sigma Aldrich) and CD45.2-Alexa647 (Biolegend) to distinguish between hepatocytes and lymphocytes. 2-3x10^6 splenocytes or 0.5-1x10^6 liver cells were plated in flat bottom 96 well plates and incubated with peptides at final concentration 10μg/ml in the presence of Brefeldin A (eBioScience). Peptides SIINFEKL, SALLNVDNL[24] and VNYSFLYLF[24] were synthesised and purchased from Peptides and Elephants. Cells were incubated at 37°C, 5% CO_2 for 5-6 hours, before incubation at 4°C overnight. Cells were stained the following day for cell surface markers and intracellular IFN-γ. Cells were incubated for 1 hour at 4°C for cell surface marker staining first then intracellular staining. Between stainings cells were fixed with 4% paraformaldehyde and permeabilised using PermWash (BD). Data was acquired by flow cytometry using an LSRII or LSRFortessa (BD). Antibodies used for stainings were obtained from BD: CD3 (500A2) or eBioScience: CD8 (53-6.7), CD11a (M17/4), IFN-γ (XMG1.2).

Statistics

Data was analysed using FlowJo version 10.0.8 (Tree Star Inc., Oregon, USA), Microsoft Excel and GraphPad Prism v7 (GraphPad Software Inc., CA, USA). Statistics were calculated using one-way ANOVA with Tukey's multiple comparison test.

RESULTS

We first investigated the effect of different methods of sporozoite attenuation on the expansion of antigen-experienced CD8+ T cells, in the spleen and liver following immunisation, by flow cytometry. Previous work has shown that upregulation of CD11a and downregulation of CD8α is a durable and accurate phenotype for identifying infection or vaccine induced parasite-specific CD8 T cells[25-27]. We found that immunisation with RAS or those attenuated by azithromycin produced a similar proportion of CD11a^{hi} CD8+ T cells, around 8% in the spleen and 30% in the liver, 2 weeks after immunisation, determined post *ex vivo* stimulation with SIINFEKL peptide (Figure 1 and 2). This corroborates with previous work that shows both of these methods of attenuation induce comparable high levels of antigen-experienced cells in peripheral blood following immunisation[13].

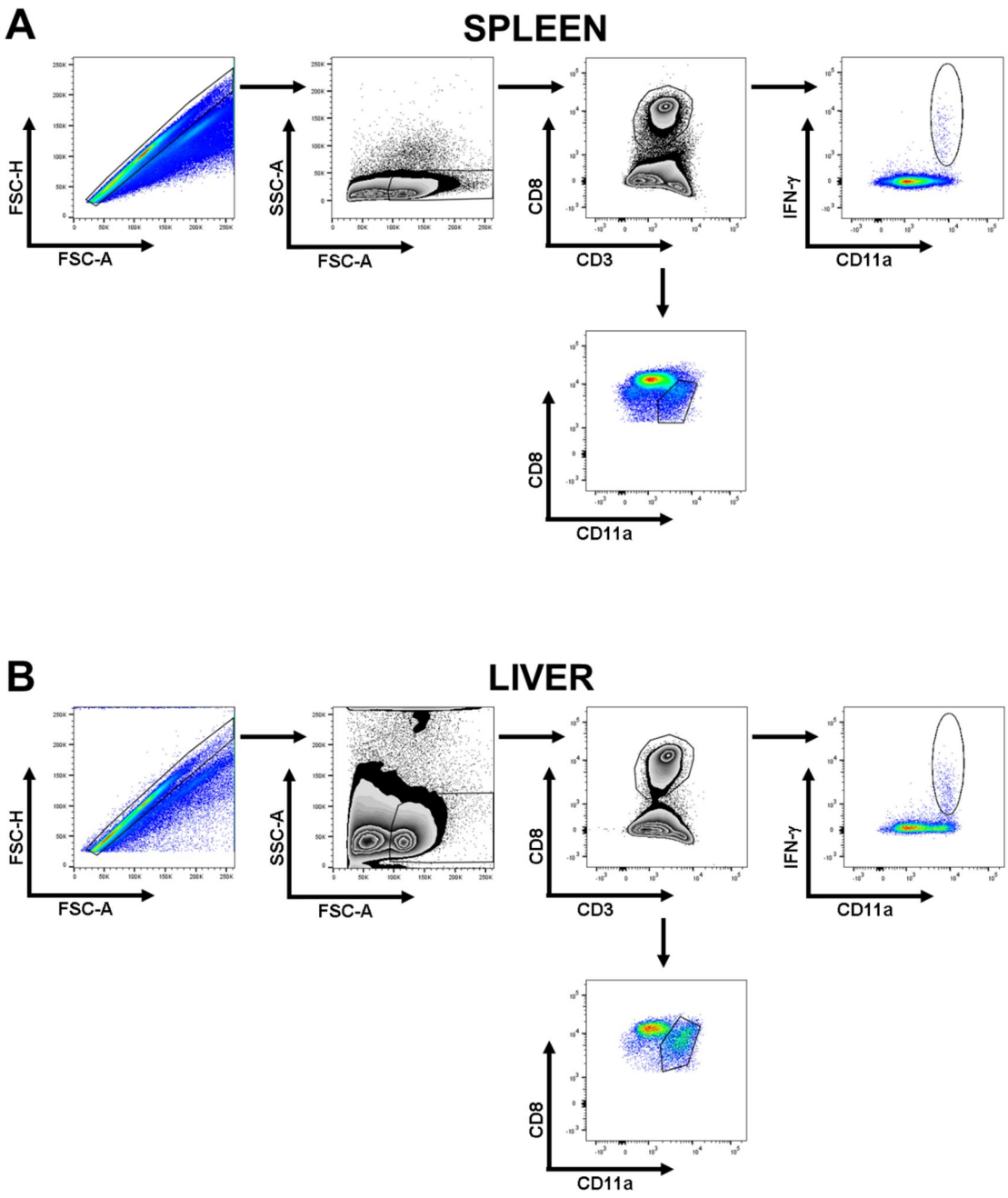


Figure 1 – Flow cytometry gating strategies

The flow cytometry gating strategy used to assess the proportion of antigen-experienced CD8+ T cells ($CD11a^{hi}$ $CD8^{lo}$) and IFN-γ producing antigen-specific CD8+ T cells (IFN-γ+ CD11a+) in the (A) spleen and (B) liver.

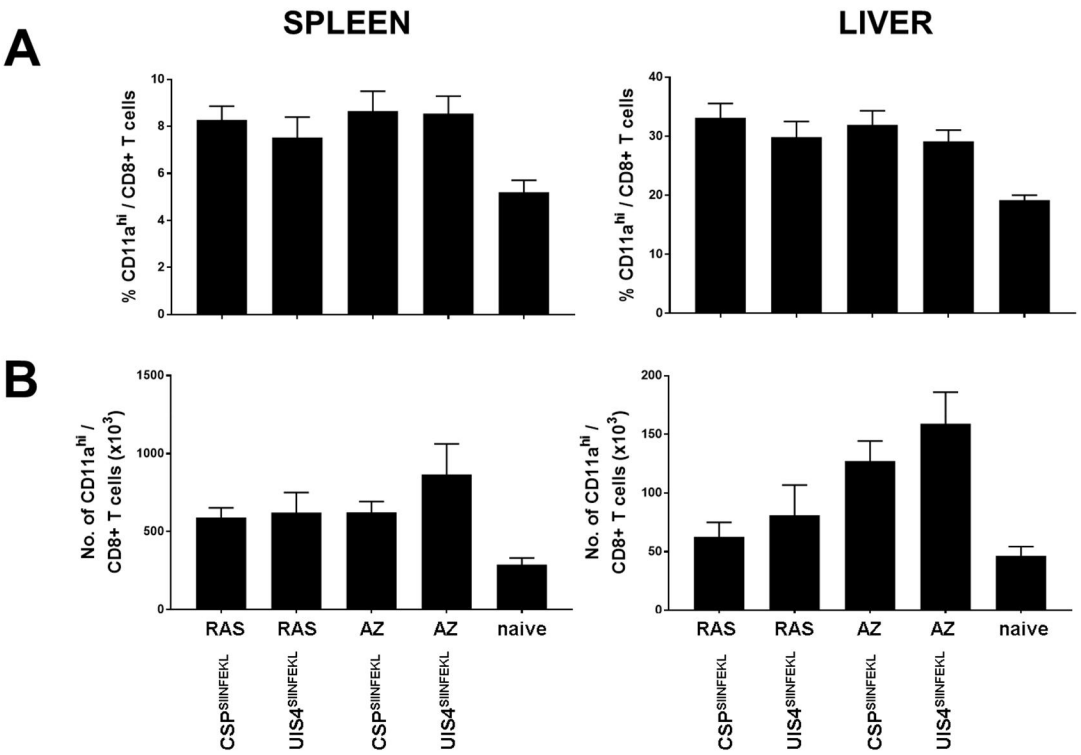


Figure 2 – Similar levels of antigen-experienced CD8+ T cells are induced in mice immunised with irradiated and drug attenuated sporozoites

Mice (n=3-5 per group) were immunised with $CSP^{SIINFEKL}$ or $UIS4^{SIINFEKL}$ RAS or sporozoites with azithromycin cover (AZ). Spleens and livers from immunised and naïve mice were harvested 14 days later. Lymphocytes were restimulated with SIINFEKL and stained for flow cytometry. The graphs show the (A) percentage and (B) absolute cell counts of $CD11a^{hi}$ $CD8^{lo}$ cells from the CD8+ T cell compartment in the spleen (left panel) and liver (right panel). Bar charts depict data pooled from three independently conducted experiments with mean values ± SEM shown.

We then compared antigen-specific CD8+ T cell responses by peptide *ex vivo* stimulation and found that the magnitude of responses to SIINFEKL in the context of UIS4 also did not change regardless of the attenuation method (Figure 3). The extension of UIS4 expression permitted by azithromycin administration did not alter the frequency and number of IFN-γ producing SIINFEKL-specific CD8+ T cells induced compared to when RAS were given. We also assessed the SIINFEKL-specific responses in the context of CSP expression and found the number of IFN-γ producing SIINFEKL-specific CD8+ T cell responses were significantly higher than those elicited against UIS4 as reported before (Chapter 2: Müller & Gibbins, prepared for publication). However, interestingly, SIINFEKL-specific CD8+ T cell responses in the spleen in the context of CSP are significantly lower when mice are immunised with sporozoites attenuated by azithromycin cover compared to RAS although this significance disappears when assaying liver infiltrating lymphocytes. Thus, we show that extending EEF development, and presumably vacuolar membrane antigen expression, does not amplify IFN-γ producing CD8+ T cell responses to EEF vacuolar membrane proteins constitutively expressed in the EEF.

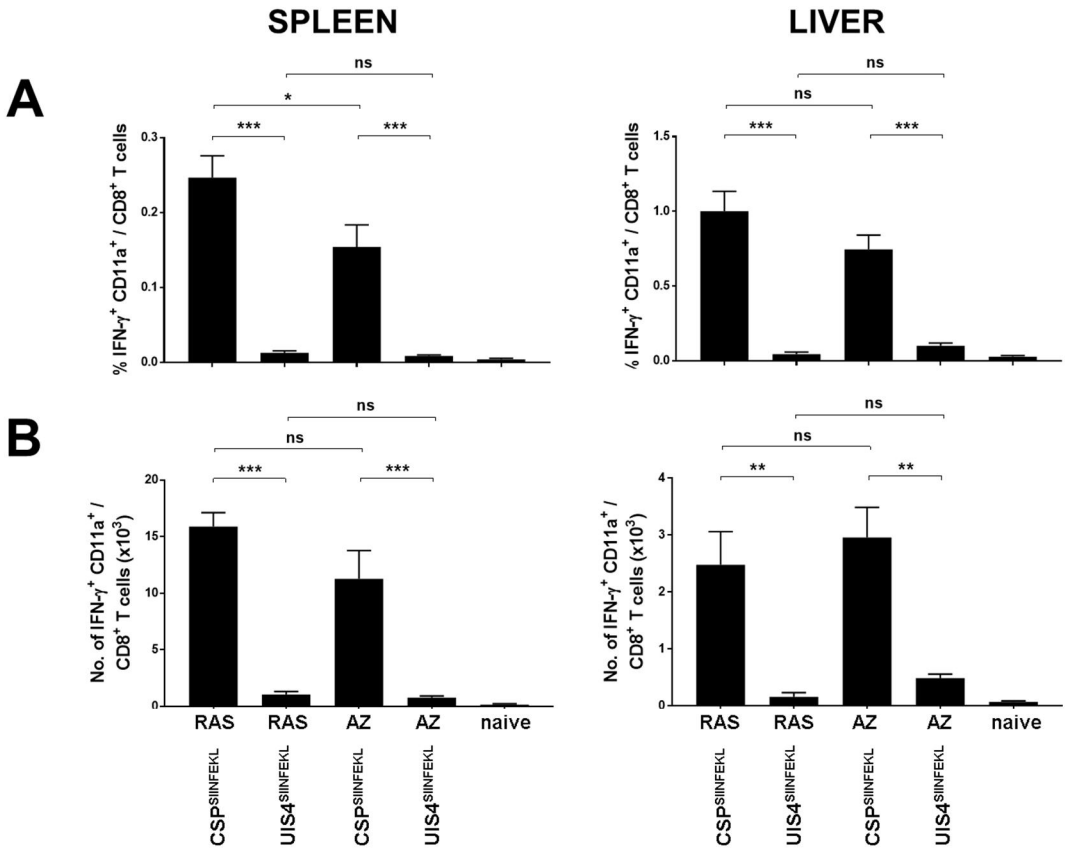
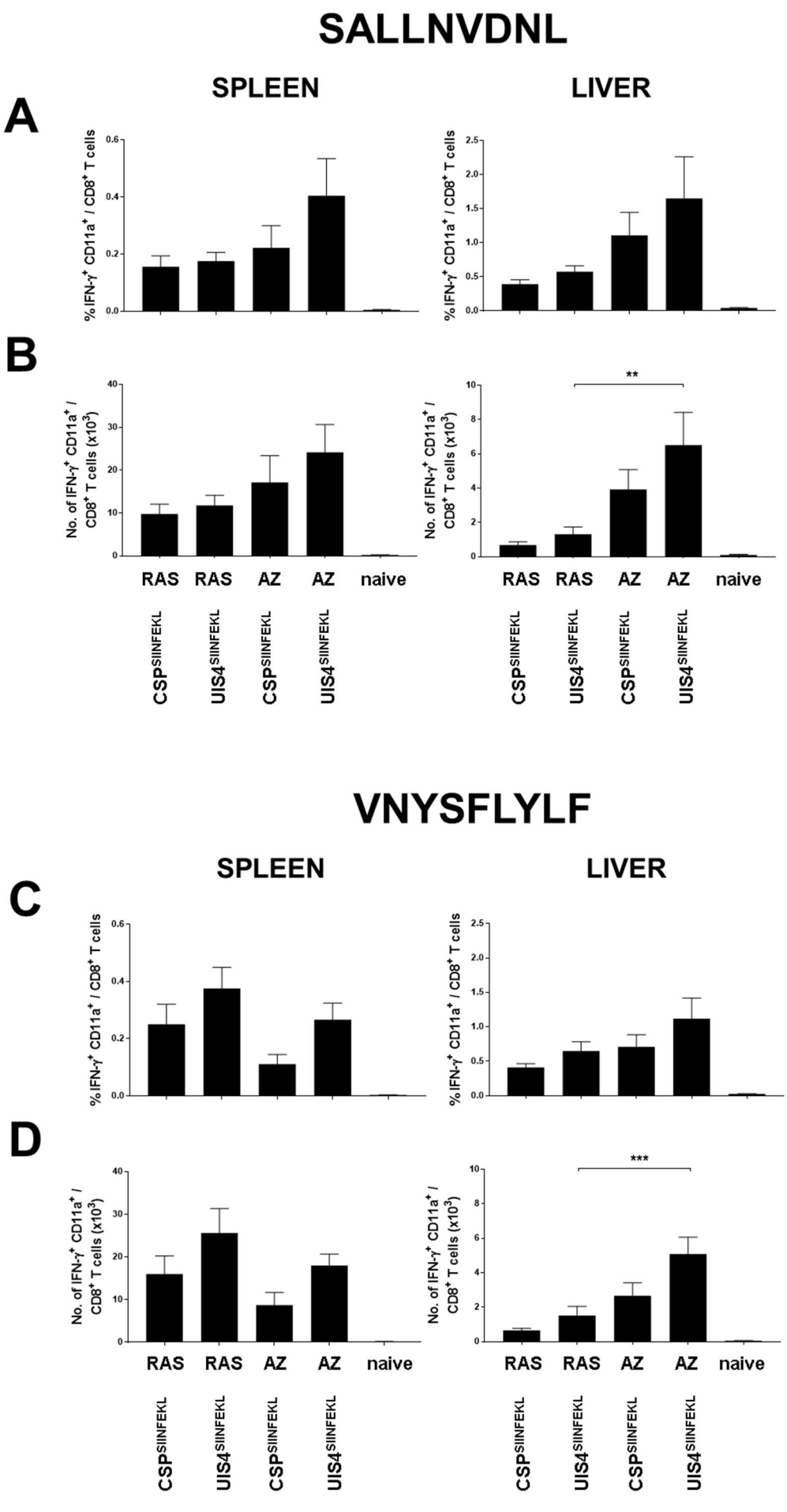


Figure 3 – Extending the duration of EEF development during immunisation does not enhance CD8+ T cell responses to EEF vacuolar membrane proteins

Mice (n=3-5) were immunised with $CSP^{SIINFEKL}$ or $UIS4^{SIINFEKL}$ RAS or sporozoites with azithromycin cover (AZ). Spleens and livers from immunised and naïve mice were harvested 14 days later. Lymphocytes were restimulated with SIINFEKL and stained for flow cytometry. The graphs show the (A) percentage and (B) absolute cell counts of CD8+ T cells co-expressing IFN-γ and CD11a in the spleen (left panel) and liver (right panel). Bar charts depict data pooled from three independently conducted experiments with mean values ± SEM shown (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; one-way ANOVA with Tukey's multiple comparison test).

To determine if the lack of effect of azithromycin on antigen-specific CD8+ T cell responses was specific to EEF antigens, we assessed endogenous CD8+ T cell responses to two sporozoites antigens, thrombospondin related anonymous protein (TRAP) and sporozoite-specific gene 20 (S20), that are not expressed during the liver stage. Following *ex vivo* restimulation with peptides corresponding to their CD8+ T cell epitopes[24] we found that similar levels of CD8+ T cell responses were induced irrespective of attenuation method (Figure 4) with the only exceptions being a difference in absolute cell numbers of TRAP- and S20-specifc CD8+ T cell responses observed in the livers of mice receiving UIS4^{SIINFEKL} parasites


SALLNVDNL
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No. of IFN-γ+ CD11a+ / CD8+ T cells (x10³)
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UIS4SIINFEKL

Figure 4 – CD8+ T cell responses to sporozoite antigens are not affected by an extended EEF development

Mice (n=3-5) were immunised with $CSP^{SIINFEKL}$ or $UIS4^{SIINFEKL}$ RAS or sporozoites with azithromycin cover (AZ). Spleens and livers from immunised and naïve mice were harvested 14 days later. Lymphocytes were restimulated with peptides corresponding to the immunogenic CD8+ T cell epitopes of TRAP protein (SALLNVDNL)[24] (A,B) and S20 protein (VNYSFLYLF)[24] (C,D) and stained for flow cytometry. The graphs show the (A,C) percentage and (B,D) absolute cell counts of CD8+ T cells co-expressing IFN-γ and CD11a in the spleen (left panel) and liver (right panel). Bar charts depict pooled data from three independently conducted experiments with mean values ± SEM (**, $p<0.01$; ***, $p<0.001$; one-way ANOVA with Tukey's multiple comparison test).

DISCUSSION

Azithromycin prophylaxis allows full EEF development of *Plasmodium* but inhibits apicoplast maturation and inheritance leading to non-infectious merozoites that fail to initiate erythrocytic infections[16]. Using azithromycin, we can immunise against antigens expressed throughout the pre-erythrocytic stages spanning from those antigens expressed by the sporozoite to those expressed very late on in the EEF prior to merozoite release into the blood. This would seem beneficial for increasing the pool of immunisation-induced CD8+ T cells that are specifically targeted against EEF antigens. Specifically looking at *P. berghei* UIS4, a PVM protein expressed constitutively in the liver following proper sporozoite invasion of a hepatocyte; azithromycin prophylaxis would allow UIS4 to continue to be expressed for the full 48-52 hours of EEF development in contrast to ~24 hours if RAS were used. Nonetheless, we show here that increasing the duration of UIS4 expression, facilitated by azithromycin, does not improve the magnitude of cognate CD8+ T cell responses against this vacuolar membrane protein. This is in concert with our previous finding that responses to UIS4 could also not be improved by increasing the dose of sporozoites used for immunisation (Chapter 2: Müller & Gibbins, prepared for publication). In addition, similar levels of antigen-experienced CD8+ T cells are induced following immunisation with RAS or sporozoites under azithromycin prophylaxis as previously shown[13]. Similar levels of memory CD8+ T cells following sporozoite immunisation under chloroquine cover compared to RAS have also been identified[28]. This is surprising considering that immunisation with late-arresting GAPs induce the greatest protection from sporozoite challenge[11, 12], thus a larger antigen-specific CD8+ T cell compartment would have been expected[11] if EEF development was extended. Alas, while CD8+ T cells have been shown to be crucial for pre-erythrocytic protection, the acquisition of protection following immunisation with diversely attenuated parasites (RAS, GAPs or drug prophylaxis) are likely different, which means the correlates of protection following each immunisation are also probably distinct. Further research is required to deduce the

differences in responses induced by different parasite immunisation strategies and how they mediate protection.

C57BL/6 mice immunised twice with *P. berghei* sporozoites under azithromycin cover have been shown to be better protected from sporozoite challenge than when they are immunised with RAS[13]. The authors also remark a non-significant but trending observation for better protection when assaying relative parasite load in the liver at 50 hours post challenge compared to 42 hours. At 42 hours there is no difference in parasite load in the liver, whereas 8 hours later, a trend appears for a lower parasite load in liver when mice receive sporozoite immunisation under azithromycin cover rather than RAS immunisation. This suggests, in concert with sterile protection data that azithromycin induces protective CD8+ T cells that are specific for late-expressed EEF antigens, which act in the later stages of EEF development[13]. However, under 10 days of chloroquine cover after both immunisations, parasite load in the liver was significantly higher than mice under azithromycin cover and sterile protection was comparable to RAS immunisation. This superior protection may be achieved because of increased immune responses to late expressed antigens on the non-invasive merozoite, which would never be presented using RAS or unlikely to be presented on the hepatocyte under chloroquine cover as merozoites do not arrest in the host hepatocyte but enter the bloodstream normally. We report similar results when assessing specific CD8+ T cell responses to mid-late expressed antigens LISP1 and LISP2 using the reporter epitope SIINFEKL as a proxy (Chapter 4: Gibbins et al., paper in preparation). Mice, vaccinated with adenovirus expressing ovalbumin, have high parasite loads in the liver compared to non-vaccinated controls at 40 hours post sporozoite challenge with parasites expressing SIINFEKL in the context of LISP1 or LISP2. However, when mice were followed for the onset of parasitaemia, 50% of mice exhibit sterile protection. This suggests that LISP1 and LISP2 induce CD8+ T cells that have some protective capability and may act very late following challenge, leading to the discrepancy between the parasite load in the liver data and sterile protection data

(Chapter 4: Gibbins et al, paper in preparation). Together, these data suggest that increasing the breadth of CD8+ T cell responses to include later expressed EEF antigens could lead to greater protection. Here we extended expression of UIS4 protein hypothesising that late expression of EEF antigens may still be important. However, extending EEF antigen expression via drug cover does not increase the number of cognate CD8+ T cell responses. More research is required to determine the kinetics of antigen-specific CD8+ T cell responses and other immune responses in the liver stage of malaria. Investigation into how to enhance liver stage responses to achieve protection and identification of antigens that are presented at this stage will be crucial in designing and developing new generation malaria vaccines.

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SECTION A – Student Details

Student	Matthew Paul Gibbins
Principal Supervisor	Julius Clemence R. Hafalla
Thesis Title	Dissecting the CD8+ T cell responses to pre-erythrocytic malaria antigens

If the Research Paper has previously been published please complete Section B, if not please move to Section C

SECTION B – Paper already published

Where was the work published?			
When was the work published?			
If the work was published prior to registration for your research degree, give a brief rationale for its inclusion			
Have you retained the copyright for the work?*	Choose an item.	Was the work subject to academic peer review?	Choose an item.

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SECTION C – Prepared for publication, but not yet published

Where is the work intended to be published?	
Please list the paper's authors in the intended authorship order:	Matthew P. Gibbins, Sylvie Briquet, Niculò Barandun, Liya Mathew, Arturo Reyes-Sandoval, Adrian V. S. Hill, Simon J. Draper, Olivier Silvie & Julius Clemence R. Hafalla
Stage of publication	**Not yet submitted**

SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	I generated and characterised the transgenic parasites, performed immunological and vaccination experiments, analysed data and performed the statistical analyses. I wrote the paper.

Student Signature: [redacted] Date: 17/09/18

Supervisor Signature: [redacted] Date: 17/09/18

CHAPTER 4

Late liver stage antigens confer partial protection against the pre-erythrocytic stages of malaria

Late liver stage antigens confer partial protection against the pre-erythrocytic stages of malaria

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ABSTRACT

Understanding the liver stage of *Plasmodium* infection is important for the development of future vaccines, however research on effective immune responses in the liver is lacking. Only recently have the intricacies of parasite development in the liver and the interactions with the host begun to be fully determined. CD8+ T cells are known to be critical immune cells in targeting the parasite hidden inside hepatocytes, however the antigen specificity of these cells is only just starting to come to light. Using genetically attenuated parasites it has been found that vaccination with parasites that arrest in the late stages of parasite development in the hepatocyte induce a greater level of anti-*Plasmodium* immunity and protection from sporozoite challenge. Using *Plasmodium berghei* parasites expressing a reporter epitope, we show that antigens expressed during mid-late liver stage development induce antigen-specific CD8+ T cell responses and offer partial protection against sporozoite challenge. With delayed antigen expression and presentation, but the ability to induce effective CD8+ T cell responses, this result emphasises the potential for including late liver stage antigens in new malaria vaccines.

INTRODUCTION

Malaria is a global health burden affecting more than around 216 million cases per year, resulting in around 445,000 deaths, with 80% of global cases occurring in 14 countries in sub-Saharan Africa and India[1]. However, with the rise and spread of drug resistant parasites[2], the road to elimination will be slow without the advent of efficacious vaccines.

A vaccine that targets the pre-erythrocytic stages of malaria is favourable because it would target a natural bottleneck in the life cycle of *Plasmodium*. Only a few hundred sporozoites are injected into the skin by an infected female *Anopheles* mosquito[3], gliding in the dermis[4] in a random pattern until they reach a blood vessel[5] where they travel to the liver, invade[6] and undergo replication within an hepatocyte. Only 25% of this small inoculum of sporozoites successfully make it out of the skin into the bloodstream[7]. So, if the few sporozoites that find the liver could be killed en route or whilst developing in the liver, then subsequent blood stages, which lead to both symptoms and transmission, would be prevented.

Vaccine research against the pre-erythrocytic stages of malaria to date, has broadly focused on sporozoite antigens. Two antigens in particular, circumsporozoite antigen (CSP) and thrombospondin-related anonymous protein (TRAP), were originally shown to induce strong immunological responses, in the form of anti-CSP antibody production[8] and anti-CSP CD4+ T cells[9-11] or anti-TRAP CD8+ T cells[12] following immunisation of humans with radiation attenuated sporozoites. This led to subunit vaccine development and thus CSP and TRAP remain the most described malaria vaccine candidates to date.

RTS,S/AS01 is the most advanced malaria vaccine to date. RTS,S/AS01 is a subunit vaccine based on the major surface antigen of sporozoites, CSP, which offered modest protection against clinical episodes of *Plasmodium falciparum* malaria in a multi-site Phase III study in Africa, however efficacy waned over time[13]. Vaccine-induced

protection was correlated with the induction of high-titres of anti-CSP antibodies and CSP-specific CD4+ T cells[14]. A Phase IIb trial in Kenya showed promising 67% efficacy against *P. falciparum* malaria infection for a viral vectored prime-boost ME-TRAP vaccine (multiple epitope (ME) string containing T cell epitopes of other pre-erythrocytic antigens in addition to TRAP), however longevity of efficacy over time could not be determined due to the short follow-up period[15]. Furthermore, combining 3 doses of RTS,S/AS01B and the viral vectored ME-TRAP prime-boost regimen gave promising sterile efficacy against malaria infection following CHMI (Controlled Human Malaria Infection) 12 weeks after first vaccination and repeat CHMI 6 months later in a UK based Phase I/IIa study[16]. Therefore, vaccines based on sporozoite antigens are successful at protecting individuals from malaria in endemic regions.

This focus on sporozoite surface antigens may be due to the perception that they are more accessible to the immune system than antigens expressed by liver stages or exo-erythrocytic forms (EEFs) of the parasite hidden away within a hepatocyte. However recently we have demonstrated that by expressing the model epitope of ovalbumin, SIINFEKL, in the context of different *P. berghei* proteins, the temporal and spatial effects of pre-erythrocytic antigen expression on CD8+ T cell responses could be determined by proxy (Chapter 2: Müller and Gibbins, paper in preparation). Previously, we generated two transgenic parasites which express the reporter epitope in the context of sporozoite surface protein CSP or EEF parasitophorous vacuole membrane (PVM)-associated upregulated in infectious sporozoites gene 4 (UIS4)[17]. We showed that despite the greater immunogenic qualities (CD8+ T cell cytokine production and proliferative capacity) of sporozoite antigens compared to EEF vacuolar membrane antigens, both antigens were equally protective when mice were vaccinated prior to sporozoite challenge. This finding highlighted two concepts- poor natural antigen-specific CD8+ T cell responses don't necessarily negate an antigen's suitability as a vaccine candidate and that EEF antigens can be protective. Following on from this discovery, we wanted to know at what stage of EEF development can the parasite still be detected, and the

infected hepatocyte destroyed. We hypothesised that later expressed EEF antigens would not provide the same level of protection as those expressed constitutively.

Here, our aim was to investigate CD8+ T cell responses and protection offered by proteins expressed later during EEF development. Given that development of *Plasmodium berghei* EEFs in mice only lasts for around 48-52 hours[18] before the first merozoites are released into the bloodstream, we chose two mid-late expressed PVM associated proteins to compare against constitutively expressed UIS4. UIS4 protein is constitutively expressed upon development of parasite within a hepatocyte and localises to the PVM[17, 19]. In contrast, Liver Specific Protein 1 (LISP1)[20] and Liver Specific Protein 2 (LISP2)[21] have similar mid-late EEF specific expression profiles, with mRNA and protein expression being absent during early EEF development and expression peaking at 48 hours *in vitro* and *in vivo*. LISP1 has been shown to be crucial for egress of merozoites from the PVM[20], while LISP2 is carried to the PVM by secretory vesicles and subsequently transported to the cytoplasm and nucleus of hepatocyte where it is suggested that it plays a role in modifying the running of the cell for its own devices[21]. We generated transgenic parasites expressing SIINFEKL in the context of LISP1 or LISP2 and here we show the CD8+ T cell responses launched against these mid-late expressed EEF antigens and the partial protection against the pre-erythrocytic stages that they induce.

MATERIALS AND METHODS

Ethics and animal experimentation

Animal procedures were performed in accordance with the Directive 2010/63/EU from the European Parliament and Council 'On the protection of animals used for scientific purposes'. Animal experiments performed at London School of Hygiene and Tropical Medicine were conducted under license from the United Kingdom Home Office under the Animals (Scientific Procedures) Act 1986. Protocols were approved by the Charles Darwin Ethics Committee of the University Pierre et Marie Curie, Paris, France and the Animal Welfare and Ethics Review Board of the London School of Hygiene and Tropical Medicine. Swiss Webster mice were purchased from Janvier (Saint Berthevin, France) and used to generate the transgenic parasites. CD-1 mice were bred in house at LSHTM and used for cloning by limiting dilution and for cycling parasites between murine and mosquito hosts. C57BL/6 mice were purchased from Charles River Laboratories (Margate, UK). OT-I mice were purchased from Charles River Laboratories (L'Arbresles, France) or spleens were kindly donated by James Cruickshank at the Babraham Institute, Cambridge. Female mice were used for experiments at age 6-8 weeks.

Parasites and mosquitoes

Transgenic parasites pLISP1-UIS4^{SIINFEKL}, pLISP2-UIS4^{SIINFEKL}, LISP1^{SIINFEKL} and LISP2^{SIINFEKL} were generated in a *Plasmodium berghei* ANKA strain which expresses GFP at the dispensable *p230p* locus[22]. Wild type *P. berghei* (clone 507) was used in comparison which expresses GFP at the elongation factor 1 alpha *(eef1a)* locus. Additional *P. berghei* CSP^{SIINFEKL} and UIS4^{SIINFEKL} parasites that do not express GFP (Chapter 2: Müller and Gibbins, paper in preparation) were used for immunological comparisons. *Anopheles stephensi* mosquitoes were reared and infected with these *P. berghei* parasites. Infected mosquitoes were kept incubators (Panasonic) at 22°C and 70% humidity. Infected mosquitoes were given a second naïve blood feed from

anaesthetised mice 7 days post infection[23]. Salivary gland sporozoites were dissected at least 21 days post infection.

For immunological experiments, mice were immunised intravenously or intradermally once with 10,000 sporozoites concomitantly with azithromycin (Pfizer) given at a dose of 240 mg/kg intraperitoneally on the same day as immunisation and one day after[24]. Viral vaccinated mice were challenged with 1,000 or 10,000 sporozoites intravenously for sterile protection and parasite load in the liver experiments respectively.

Transgenic parasite generation

pLISP1-UIS4^{SIINFEKL}, pLISP2-UIS4^{SIINFEKL}, LISP1^{SIINFEKL} and LISP2^{SIINFEKL} parasites were generated using In-Fusion (Clontech) technology to generate plasmids for transfection based on generation of a common plasmid[25] (MG1 - Figure 1). The similarities between the parasites include the addition of mCherry, SIINFEKL epitope and 3' UTR from UIS4 appended to the end of a designated protein. In the case of pLISP1-UIS4^{SIINFEKL} and pLISP2-UIS4^{SIINFEKL} (plasmids MG2b and MG2c respectively), the mCherry-SIINFEKL modification occurs at the C-terminus of an additional copy of the UIS4 protein. This modified ORF is under the promoter of LISP1 or LISP2, appearing downstream of the endogenous ORF at the UIS4 locus. In the case of LISP1^{SIINFEKL} (plasmid MG2e), the mCherry-SIINFEKL modification occurs at the C-terminus of the endogenous LISP1 protein. In the case of LISP2^{SIINFEKL}, the mCherry-SIINFEKL modification occurs at the C-terminus of the endogenous LISP2 protein. Plasmids were transfected into *Plasmodium berghei* ANKA expressing GFP at the dispensable *p230p*[22] by electroporation of merozoites using Nucleofector buffer and an AMAXA Nucleofector[26]. Swiss Webster mice were immediately injected with electroporated merozoites intravenously. Transgenic clones were isolated after limiting dilution and injection into CD-1 mice. Details of plasmid design, primers, cloning and genotyping of parasites can be found in Supplementary Experimental Procedures and Supplementary Table 1.

In vitro infection of hepatoma cells and fluorescent staining

In vitro liver EEF development was analysed in infected Huh7 hepatoma cells at 12, 24 and 48 hours. Duplicate Labtek (Nunc) wells were infected with 10,000 *P. berghei* WT, pLISP1-UIS4^{SIINFEKL}, pLISP2-UIS4^{SIINFEKL}, LISP1^{SIINFEKL} or LISP2^{SIINFEKL} sporozoites. Cells were fixed with 4% paraformaldehyde and stained for analysis by fluorescence microscopy using polyclonal anti-*Pb*UIS4 (SICGEN) or anti-DsRed/mCherry (Takara Bio) primary antibodies. Corresponding secondary antibodies conjugated to Alexa546 were used and nuclear staining was visualised using DAPI before mounting with Vectashield (Vector Labs). The stainings were analysed using an Eclipse Ti-E inverted microscope (Nikon).

Restimulation of splenic, liver infiltrating and peripheral blood lymphocytes

Spleens and livers perfused with PBS were harvested from immunised and naïve mice. Peripheral blood was acquired by tail vein puncture collected in Na^+ heparin capillary tubes (Brand) and assayed in 96-well flat bottom plates (Corning). Organs were homogenised using 70μm cell strainers (Corning), hepatocytes removed using a Percoll (GE Healthcare) gradient, and red blood cells lysed using PharmLyse (BD). Lymphocytes were resuspended in complete RPMI (cRPMI- RPMI + 10% FCS + 2% Penicillin-Streptomycin + 1% L-glutamine (Gibco)). Cells were counted by microscopy following 40x dilution with Trypan Blue (ThermoFisher Scientific) on a Neubauer 'Improved' haemocytometer (Biochrom). 2-3x10^6 splenocytes or 0.5-1x10^6 liver cells were incubated with SIINFEKL peptide (Peptides and Elephants) at final concentration of 10μg/ml in the presence of Brefeldin A (eBioScience) and incubated for 5-6 hours at 37°C and 5% CO_2 before incubation at 4°C overnight. CD8+ T cells were stained for flow cytometry using CD3 (500A2), CD8 (53-6.7) and CD11a (M17/4) and intracellular cytokine markers IFN-γ (XMG1.2). Antibodies were obtained from BD (CD3 only) or eBioscience. Spleen or liver cells were fixed using 4% paraformaldehyde and peripheral blood cells were fixed with 1% paraformaldehyde. Cells were permeabilised using

PermWash (BD). Data was acquired on an LSRII (BD).

Vaccination with OVA expressing recombinant adenovirus for assessment of parasite load in the liver and sterile protection

To assess parasite load in the liver and sterile protection, mice were vaccinated recombinant human adenovirus serotype 5 (AdHu5) expressing full-length chicken ovalbumin (AdOVA)[27]. Mice received $1x10^8$ infective units (ifu) diluted in ice cold PBS with 100µl of the virus administered subcutaneously (50µl into each thigh). Vaccinated and control mice also received $2x10^6$ OT-I splenocytes intravenously.

Quantitative real time PCR to determine parasite load in the liver

14 days after vaccination, vaccinated and control mice were challenged with 10,000 *P. berghei* ANKA sporozoites intravenously. 40hrs after the challenge, livers were harvested and homogenised in TRIzol (ThermoFisher Scientific) for total RNA isolation. Quantitative real-time PCR was performed using the 7500 Fast Real-Time PCR System and FastSYBR Green PCR Master Mix (Applied Biosystems). Relative liver parasite levels were quantified using the ΔΔCt method comparing levels of *P. berghei* 18S rRNA and normalised to levels of mouse *GAPDH* mRNA. Primers used can be found in Supplementary Table 1.

Sterile protection

14 days after vaccination, vaccinated and control mice were challenged with 1,000 *P. berghei* ANKA sporozoites intravenously. Parasitaemia in challenged mice was monitored by daily blood smears taken from day 3-14 after challenge, stained with Giemsa solution (VWR).

Statistics

Data was analysed using FlowJo version 10.0.8. (Tree Star Inc., Oregon, USA), Microsoft Excel and GraphPad Prism v7 (GraphPad Software Inc., CA, USA). We calculated statistics between two groups using Mann-Whitney U test or Welch's t-test for non-normally or normally distributed data, respectively, and one-way ANOVA with Tukey's multiple comparison test for comparing more than two groups.

RESULTS

Generation of LISP1^{SIINFEKL} and LISP2^{SIINFEKL} transgenic parasites

We developed *P. berghei* parasites that express the reporter CD8+ T cell epitope of ovalbumin, SIINFEKL, in the context of LISP1 and LISP2 which are expressed at similar times in during EEF development, with mRNA transcripts and protein only detectable after 24 hours[20, 21]. Using the common plasmid MG1 (Figure 1) a similar strategy to that used to generate UIS4^{SIINFEKL} (Chapter 2: Müller and Gibbins, paper in preparation) was employed. We modified the loci of LISP1 or LISP2 by appending the SIINFEKL epitope to the C-terminus of the LISP1 or LISP2 protein, as well as incorporating an mCherry tag prior to SIINFEKL. LISP1^{SIINFEKL} (Figure 2A) and LISP2 ^{SIINFEKL} (Figure 2B) thus expressed one copy of LISP1 or LISP2 which was mCherry-SIINFEKL tagged, regulated by the endogenous promoter region although the 3' UTR was from UIS4, not the gene specific 3' UTR. This was because we generated two sets of parasites but used the common plasmid MG1 (Figure 1) to generate both. We generated in parallel, pLISP1-UIS4^{SIINFEKL} and pLISP2-UIS4^{SIINFEKL} parasites that were modified at the UIS4 locus, so that an extra copy of UIS4, under the promoter of LISP1 or LISP2, was incorporated which would contain the mCherry-SIINFEKL tag (Figure 3A, B). These parasites were generated so that if the parasites LISP1^{SIINFEKL} and LISP2^{SIINFEKL} failed to express functional modified LISP1 or LISP2 tagged protein that did not associate with the PVM, a later expressed tagged UIS4 protein could be used to probe responses to a PVM protein that is expressed later than endogenous UIS4. The tagged UIS4 was placed under a separate 3' UTR of UIS4. It was necessary to engineer the tagged version of UIS4 as regulatorily separate from the endogenous copy, because a single copy of UIS4 strictly under the LISP1 or LISP2 promoter would prevent early EEF development. All four transgenic parasites were generated using the MG1 plasmid which contained a mCherry-SIINFEKL-UIS4 3' UTR cassette (Figure 1), hence why LISP1^{SIINFEKL} and LISP2^{SIINFEKL} contain this unconventional 3' UTR.

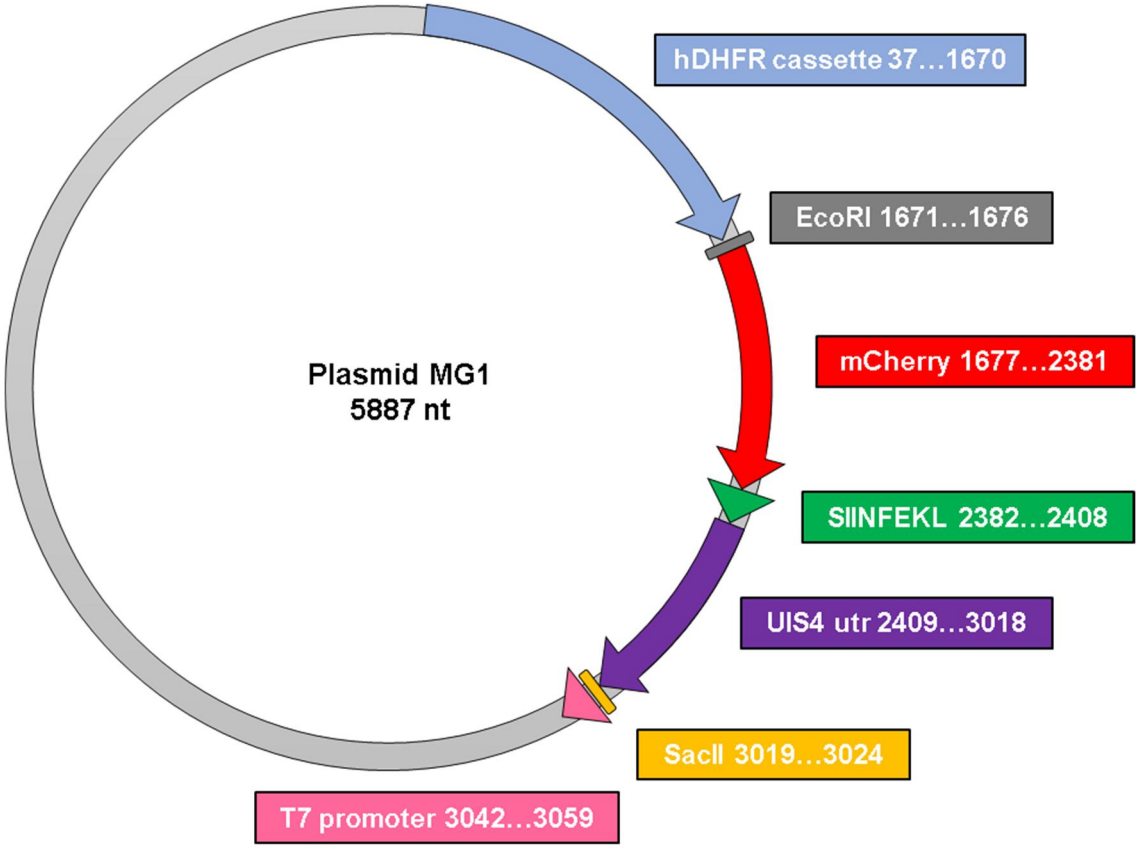


Figure 1

Generation of mCherry-SIINFEKL-UIS4 3' UTR containing plasmid

All parasites were based on MG1 starting plasmid. Full details of plasmid construction are described in the Supplementary Experimental Procedures. The hDHFR cassette (blue) provided the drug selectable resistance gene against pyrimethamine allowing determination of parasites incorporating the plasmid. MG1 was linearised by *EcoRI* (grey), with gene fragments for the other plasmids inserted here using In-Fusion (Clontech) technology. Ultimately, following insertion of gene fragments, genes would be appended 3' by the mCherry-SIINFEKL-UIS4 3' UTR sequences. Thus, following translation, proteins would be mCherry tagged (red) at the C-terminus followed by the SIINEKL CD8+ T cell target epitope (green). The 3' UTR of UIS4 (purple) would be the 3' regulatory region functioning to signal the end of transcription, though transcription may not be under the promoter region of UIS4, depending on the parasite in question. The sequence of the T7 promoter (pink) present in the plasmid was used in conjunction with a sequence from hDHFR to genotype parasites and identify episome formation.

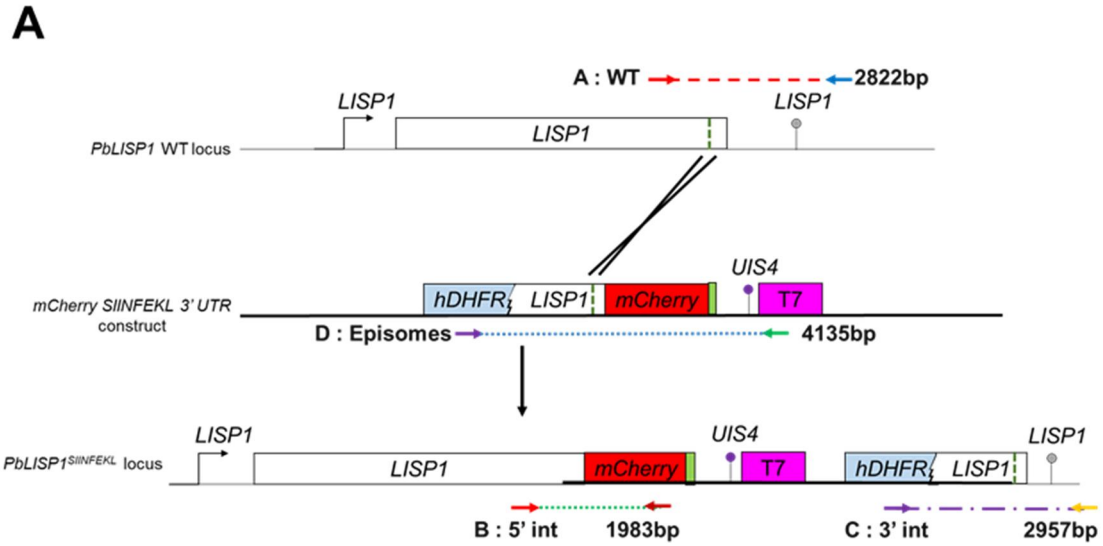
Transgenic parasites develop normally into sporozoites and EEFs with localisation of UIS4, LISP1 and LISP2 with the parasitophorous vacuole membrane

Recombinant parasites that had integrated the plasmid, containing the human dihydrofolate reductase drug selectable marker gene (*hDHFR*), were selected for by treatment with pyrimethamine, followed by limiting dilution to isolate clones. Genotyping PCRs were performed using primers designed to determine the presence of WT parasites, 5' and 3' integration and the presence of episomes (LISP1^{SIINFEKL}- Figure 2C; LISP2^{SIINFEKL}- Figure 2D; pLISP1/2-UIS4^{SIINFEKL}- Figure 3C). Primers were also required to determine the difference between pLISP1-UIS4^{SIINFEKL} and pLISP2-UIS4^{SIINFEKL} by amplifying the LISP1 or LISP2 promoters ahead of the UIS4-mCherry-SIINFEKL gene (Figure 3C). Clones for all four parasites were successfully acquired.

Transmission to mosquitoes is an important attribute with genetically modified *Plasmodium* parasites. All parasites were successfully transmitted to *Anopheles stephensi* mosquitoes, with comparable numbers of salivary gland sporozoites to WT (LISP1/2^{SIINFEKL}- Figure 2E; pLISP1/2-UIS4^{SIINFEKL}- Figure 3D) except pLISP1-UIS4^{SIINFEKL} where the average load of sporozoites dissected from salivary glands was consistently and drastically lower than the other parasites.

We infected Huh7 hepatoma cells with sporozoites to determine development of the transgenic parasites and also the subcellular localisation of LISP1 or LISP2 (LISP1/2^{SIINFEKL}- Figure 2F, G); pLISP1/2-UIS4^{SIINFEKL}- Figure 3E, F). All parasites developed with sizes comparable to WT parasites. We used anti-mCherry antibodies as a proxy to stain for our tagged proteins. UIS4 but not LISP1 nor LISP2 showed localisation to the PVM at 12 hours. All three proteins could be visualised at the PVM after 24 hours with a similar localisation pattern to UIS4 in our WT parasites, which we stained with anti-UIS4 because these parasites do not express mCherry. This confirmed that expression of LISP1 and LISP2 protein does not occur in the first 12 hours after hepatocyte invasion whereas UIS4 protein is expressed very soon after invasion[19].

Previously, weak LISP2 expression at 24 hours was described in parasites that express mCherry tagged LISP2 and visualised using mCherry antibodies as we have here[20]. Visualisation of LISP1 and LISP2 expression using LISP1 or LISP2 primary antibodies suggested that the protein was not visible until 36 or 24 hours respectively after invasion[20, 21]. These differences may be due to the increased sensitivity of the mCherry primary antibody. Nonetheless the proteins localise as reported previously[20, 21] both at 24 and 48 hours. In the same way we visualised expression of UIS4 under the promoters of LISP1 or LISP2 in pLISP1-UIS4^{SIINFEKL} and pLISP1-UIS4^{SIINFEKL} respectively with UIS4 localised as seen for WT with similar levels of expression at 24 hours and 48 hours.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LISP1
LISP1
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construct
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mCherry
T7
D : Episomes
4135bp
LISP1
UIS4
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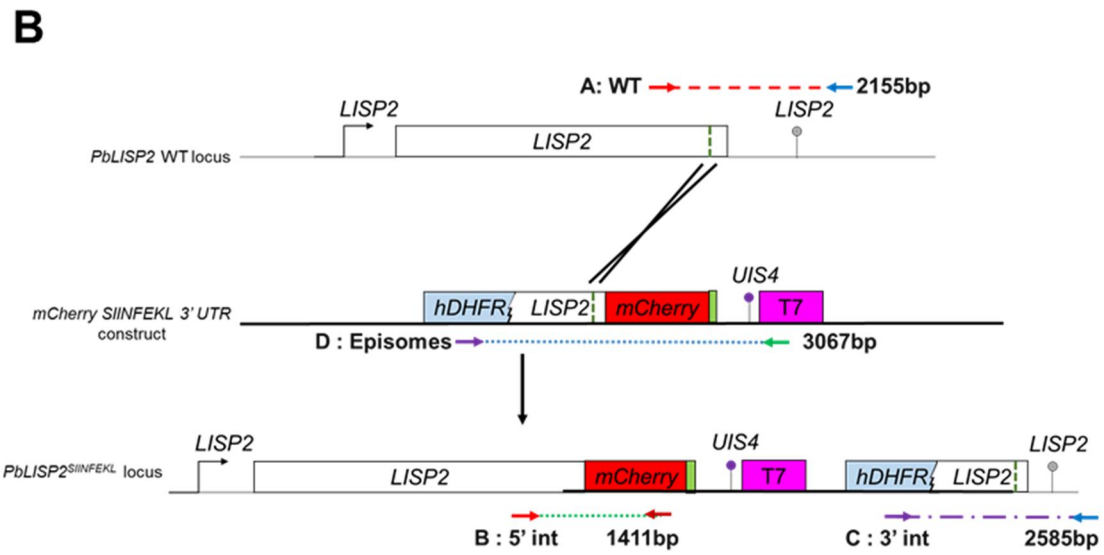
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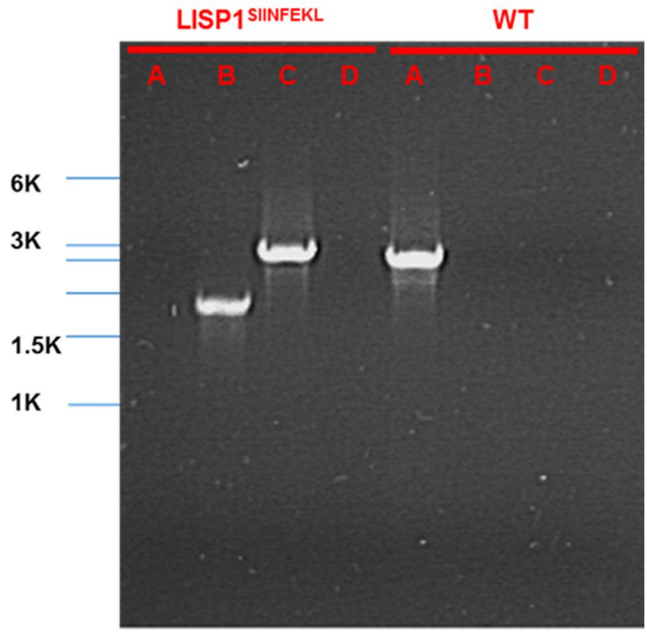
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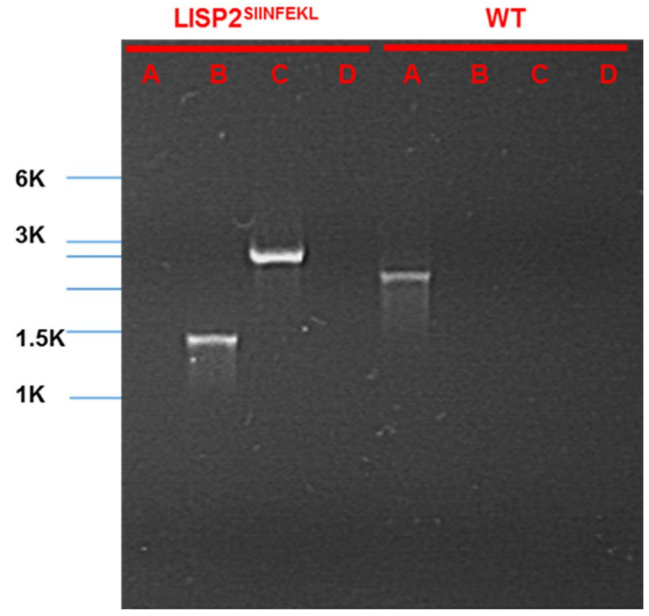
1.5K
1K

D


LISP2SIINFEKL
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1.5K
1K

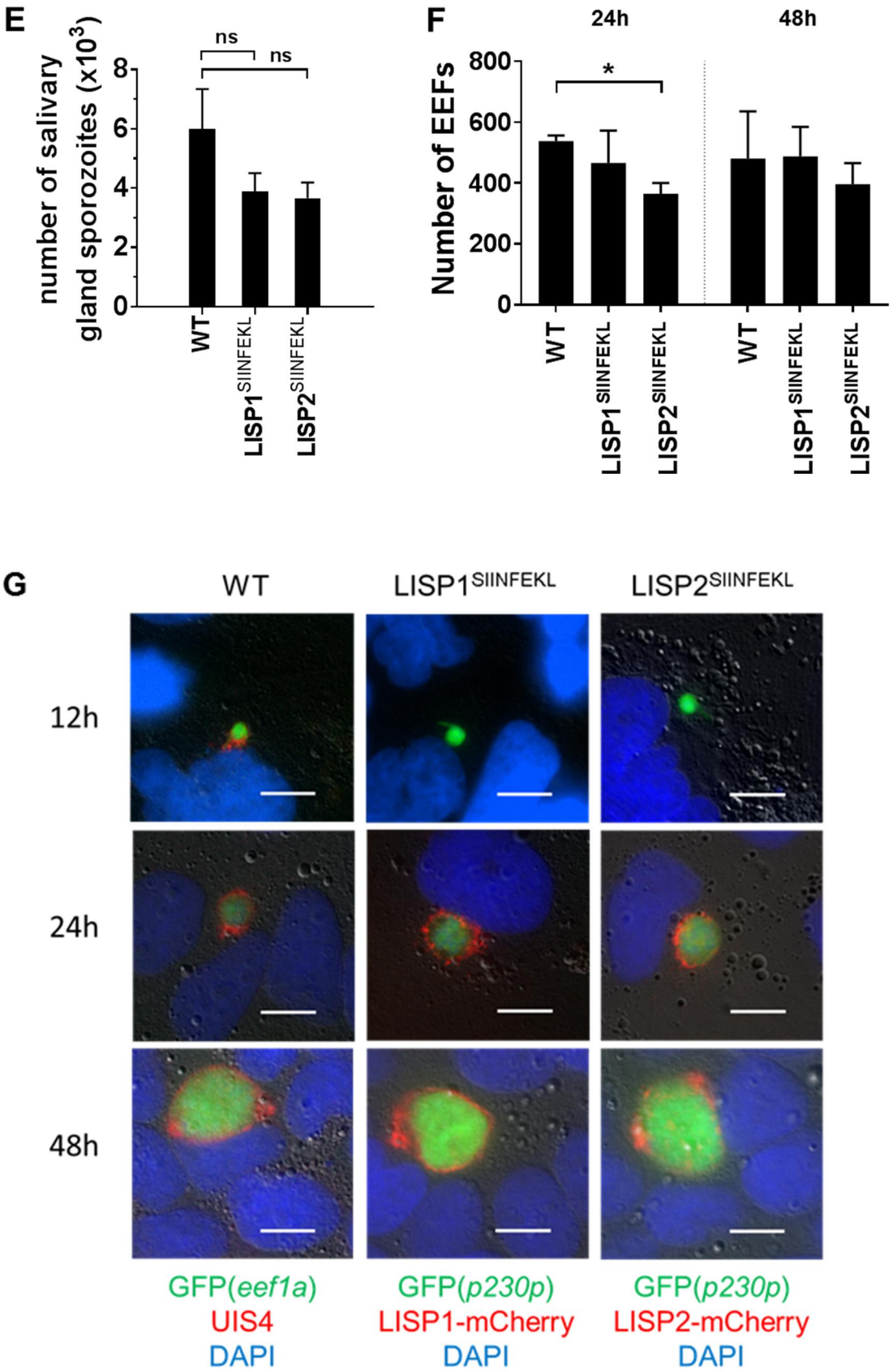
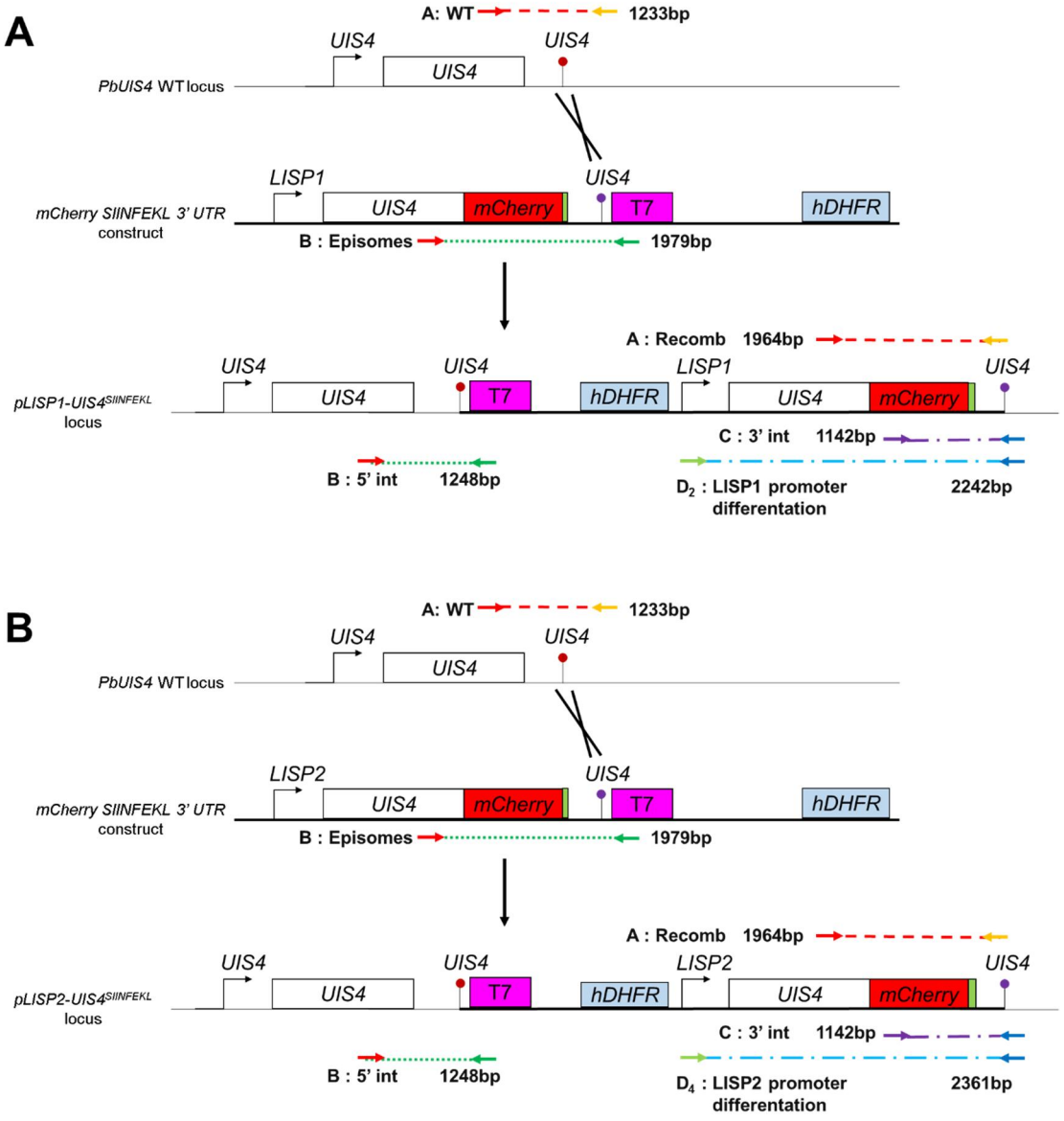
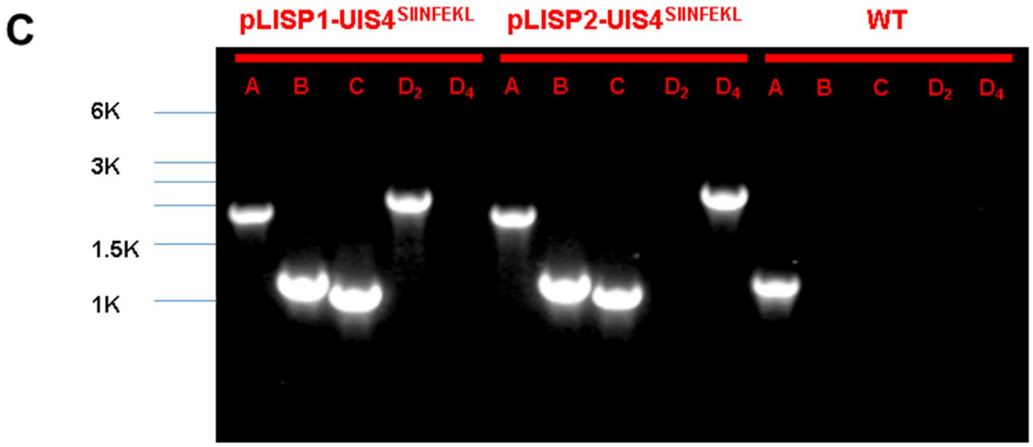

E
number of salivary gland sporozoites (x10^3)
ns
ns
8
6
4
2
0
WT
LISP1SIINFEKL
LISP2SIINFEKL
F
24h
48h
Number of EEFs
800
600
400
200
0
*
WT
LISP1SIINFEKL
LISP2SIINFEKL
WT
LISP1SIINFEKL
LISP2SIINFEKL
G
WT
LISP1SIINFEKL
LISP2SIINFEKL
12h
24h
48h
GFP(eef1a)
UIS4
DAPI
GFP(p230p)
LISP1-mCherry
DAPI
GFP(p230p)
LISP2-mCherry
DAPI

Figure 2

Generation and characterisation of LISP1^{SIINFEKL} and LISP2^{SIINFEKL} parasites

(A-B) The plasmids used to generate LISP1^{SIINFEKL} and LISP2^{SIINFEKL} parasites were based on MG1 starting plasmid (Figure 1) before In-Fusion (Clontech) technology was used to append short C-terminal regions of (A) LISP1 or (B) LISP2 ORFs before the mCherry-SIINFEKL sequences. Consequently, following correct integration at (C) LISP1 or (D) LISP2 locus, endogenous (A) LISP1 and (B) LISP2 would now have mCherry-SIINEFKL appended to the C-terminus. (C) LISP1^{SIINFEKL} and (D) LISP2^{SIINFEKL} Parasites were genotyped using PCR with specific primers to amplify regions to assess for the presence of WT parasites, 5' and 3' integration of the plasmid and episome formation. (E) The number of sporozoites dissected from salivary glands from mosquitos infected with WT, LISP1^{SIINFEKL} or LISP2^{SIINFEKL} transgenic parasites 18-27 days post infection from at least nine different infections per parasite. (F-G) EEF development of WT, LISP1^{SIINFEKL} and LISP2^{SIINFEKL} parasites in Huh7 hepatocytes *in vitro* at 12, 24 hours and 48 hours. (F) EEFs were counted from a minimum of 2 wells with experiments performed 2-3 times (G) Parasites expressing GFP (green) were also stained with anti-UIS4 or anti-mCherry (red) and DAPI for nuclear staining (blue). Images show representative EEF development. Scale bars: 10 μm. (E, F) Bar charts show mean values (±SEM) with statistics calculated by Welch's t-test (*, $p<0.05$).


A
A: WT
1233bp
UIS4
UIS4
UIS4
PbUIS4 WT locus
LISP1
UIS4
UIS4
mCherry SIINFEKL 3' UTR construct
UIS4
mCherry
T7
hDHFR
B : Episomes
1979bp
A : Recomb 1964bp
UIS4
UIS4
LISP1
UIS4
pLISP1-UIS4SIINFEKL locus
UIS4
T7
hDHFR
UIS4
mCherry
C : 3' int 1142bp
B : 5' int 1248bp
D2 : LISP1 promoter differentation 2242bp
B
A: WT
1233bp
UIS4
UIS4
UIS4
PbUIS4 WT locus
LISP2
UIS4
UIS4
mCherry SIINFEKL 3' UTR construct
UIS4
mCherry
T7
hDHFR
B : Episomes
1979bp
A : Recomb 1964bp
UIS4
UIS4
LISP2
UIS4
pLISP2-UIS4SIINFEKL locus
UIS4
T7
hDHFR
UIS4
mCherry
C : 3' int 1142bp
B : 5' int 1248bp
D4 : LISP2 promoter differentation 2361bp


C
pLISP1-UIS4SIINFEKL
pLISP2-UIS4SIINFEKL
WT
A B C D2 D4 A B C D2 D4 A B C D2 D4
6K
3K
1.5K
1K

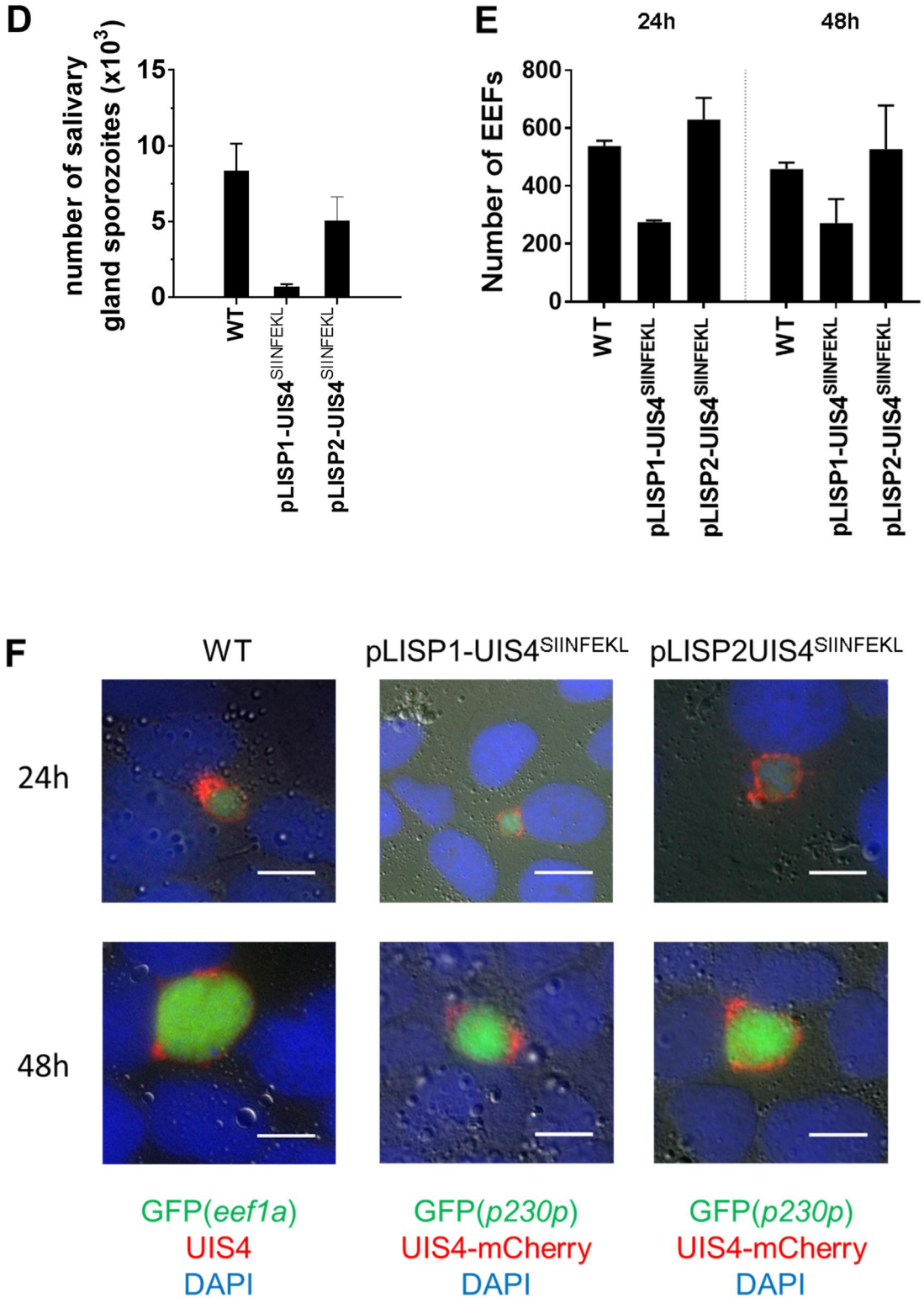

D
number of salivary gland sporozoites (x10^3)
15
10
5
0
WT
pLISP1-UIS4SIINFEKL
pLISP2-UIS4SIINFEKL
E
24h
48h
Number of EEFs
800
600
400
200
0
WT
pLISP1-UIS4SIINFEKL
pLISP2-UIS4SIINFEKL
WT
pLISP1-UIS4SIINFEKL
pLISP2-UIS4SIINFEKL
F
WT
pLISP1-UIS4SIINFEKL
pLISP2UIS4SIINFEKL
24h
48h
GFP(eef1a)
UIS4
DAPI
GFP(p230p)
UIS4-mCherry
DAPI
GFP(p230p)
UIS4-mCherry
DAPI

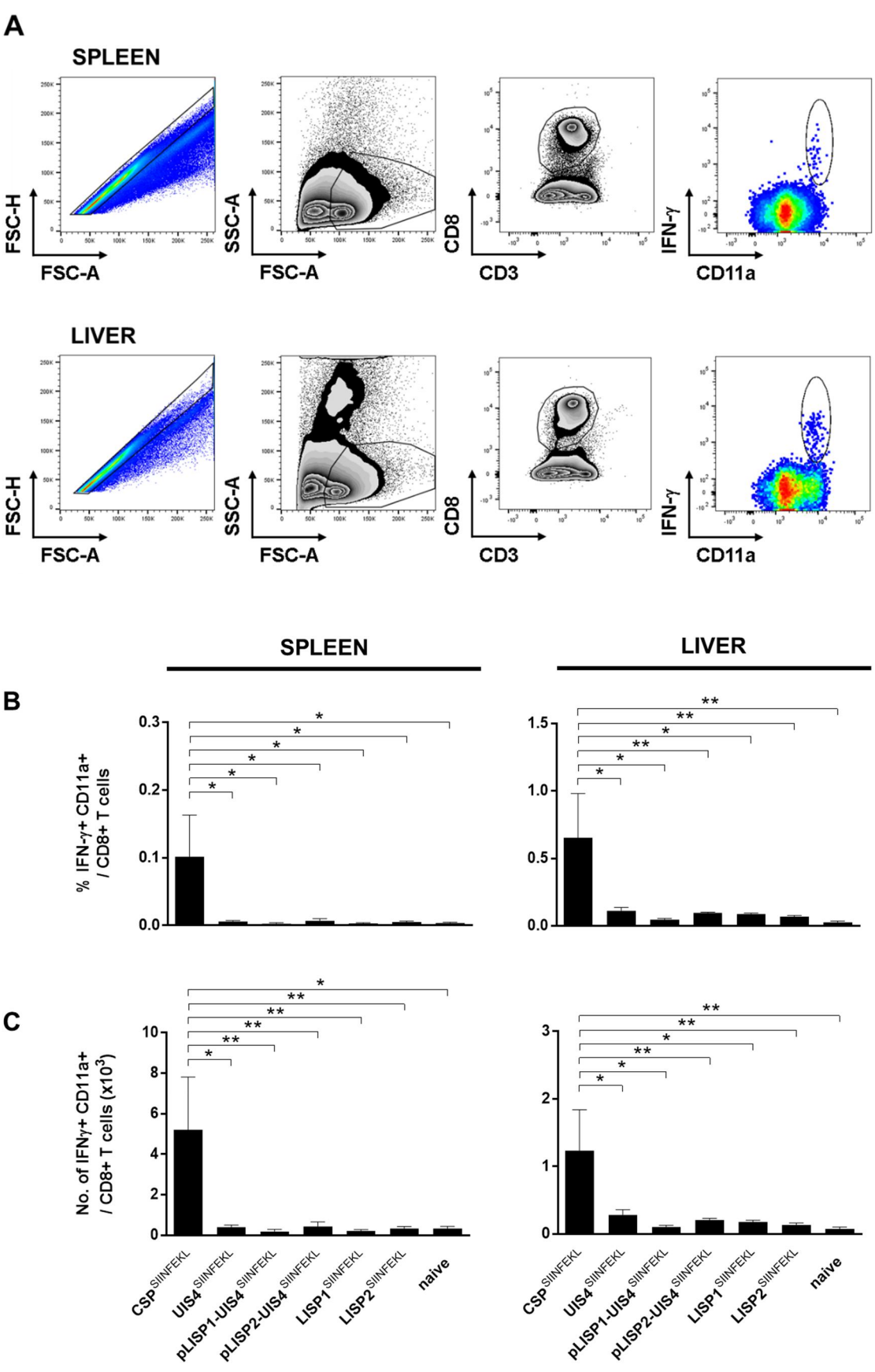
Figure 3

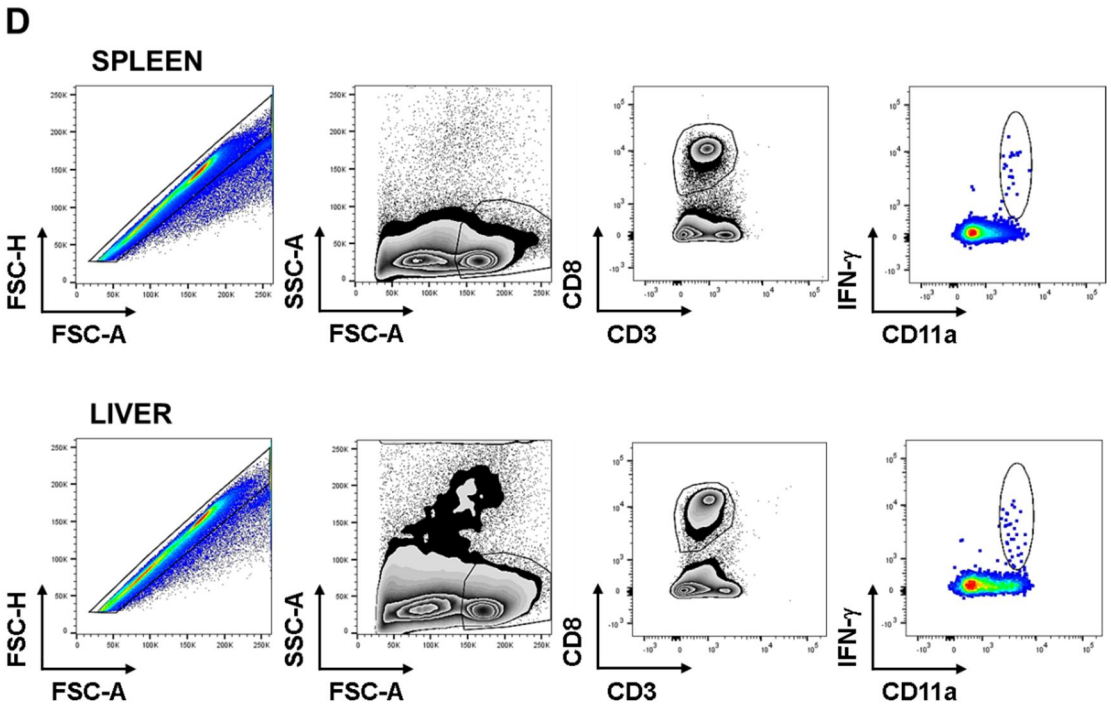
Generation and characterisation of pLISP1-UIS4^{SIINFEKL} and pLISP2-UIS4^{SIINFEKL} parasites

(A-B) Plasmids used to generate pLISP1-UIS4^{SIINFEKL} and pLISP2-UIS4^{SIINFEKL} parasites were based on MG1 starting plasmid (Figure 1) before In-Fusion (Clontech) technology was used to add fragments containing UIS4 ORF and (A) LISP1 or (B) LISP2 promoter regions before the mCherry-SIINFEKL sequences. (C) Parasites were genotyped using PCR with specific primers to amplify regions to assess for the presence of WT parasites, 5' integration and episome formation, 3' integration and to distinguish between the modified LISP1 and LISP2 promoters. (D) The number of sporozoites dissected from salivary glands from mosquitos infected with WT, pLISP1-UIS4^{SIINFEKL} or pLISP2-UIS4^{SIINFEKL} parasites 18-27 days post infection from at least seven different infections per parasite. (E-F) EEF development of WT, LISP1^{SIINFEKL} and LISP2^{SIINFEKL} parasites in Huh7 hepatocytes *in vitro* at 24 hours and 48 hours. (E) EEFs were counted from a minimum of 2 wells with experiments performed 2-3 times. (F) Parasites expressing GFP (green) were also stained with anti-UIS4 or anti-mCherry (red) and DAPI for nuclear staining (blue). Images show representative EEF development. Scale bars: 10 μm. (D-E) Bar charts show mean values ± SEM.

Early and mid-late EEF antigens induce similar CD8+ T cell responses

We immunised mice once with parasites intravenously (Figure 4A-C) or intradermally (Figure 4D-F) under azithromycin prophylaxis. Azithromycin prophylaxis was used to ensure that parasites developed fully inside hepatocytes and that arrest did not affect the normal expression patterns of the LISP1 and LISP2 promoters prior to merozoite release. We restimulated splenocytes and liver infiltrating lymphocytes with SIINFEKL *ex vivo* to determine proportions and numbers of SIINFEKL-specific effector cell CD8+ T cells with the capacity to produce IFN-γ. We co-stained with CD11a, which has been shown to be a robust and reliable marker of antigen experienced CD8+ T cells[28, 29]. More akin to responses to UIS4^{SIINFEKL} than CSP^{SIINFEKL}, LISP1^{SIINFEKL} and LISP2^{SIINFEKL} induced a similar proportion and number of IFN-γ producing SIINFEKL-specific CD8+ T cells. pLISP1-UIS4^{SIINFEKL} and pLISP2-UIS4^{SIINFEKL} (Figure 4A-C) also have a similar immunogenic profile to UIS4^{SIINFEKL}, LISP1^{SIINFEKL} and LISP2^{SIINFEKL} for inducing CD8+ T cells. pLISP1-UIS4^{SIINFEKL} and pLISP2-UIS4^{SIINFEKL} parasites were only used for assessing CD8+ T cell responses following intravenous immunisation and were not further investigated as LISP1^{SIINFEKL} and LISP2^{SIINFEKL} parasites express SIINFEKL in a more physiological context, with the native protein localising to the PVM as seen previously[20, 21] despite our previous concern. Together these results suggest that early and mid-late EEF antigens are equally poor immunogens.


INTRAVENOUS IMMUNISATION
A
SPLEEN
FSC-H
FSC-A
SSC-A
FSC-A
CD8
CD3
IFN-γ
CD11a
LIVER
FSC-H
FSC-A
SSC-A
FSC-A
CD8
CD3
IFN-γ
CD11a
B
SPLEEN
LIVER
% IFN-γ+ CD11a+ / CD8+ T cells
C
No. of IFNγ+ CD11a+ / CD8+ T cells (x10³)
CSP SIINFEKL
UIS4 SIINFEKL
pLISP1-UIS4 SIINFEKL
pLISP2-UIS4 SIINFEKL
LISP1 SIINFEKL
LISP2 SIINFEKL
naive


D
SPLEEN
FSC-H
FSC-A
SSC-A
FSC-A
CD8
CD3
IFN-γ
CD11a
LIVER
FSC-H
FSC-A
SSC-A
FSC-A
CD8
CD3
IFN-γ
CD11a

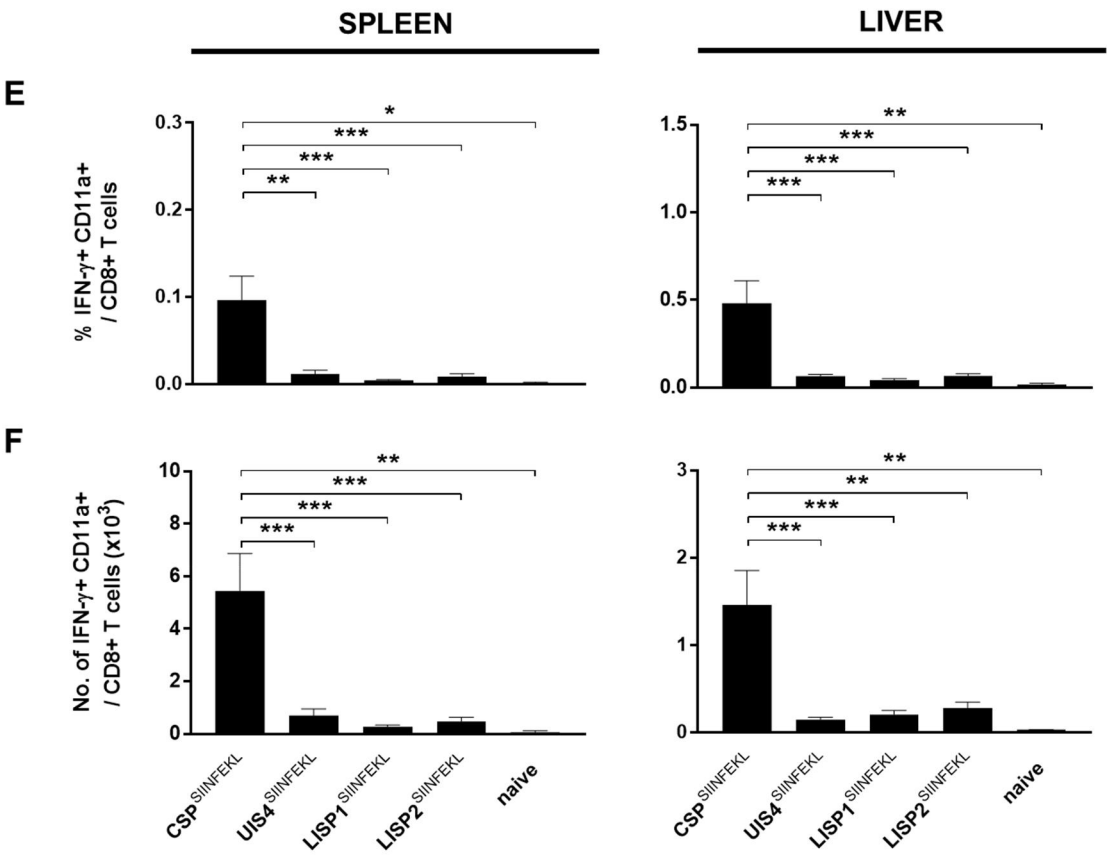

SPLEEN
LIVER
E
% IFN-γ+ CD11a+ / CD8+ T cells
F
No. of IFN-γ+ CD11a+ / CD8+ T cells (x10³)
CSP SIINFEKL
UIS4 SIINFEKL
LISP1 SIINFEKL
LISP2 SIINFEKL
naive

Figure 4

Early and mid-late expressed EEF proteins induce low frequencies of antigen specific CD8+ T cell responses when sporozoites are administrated intravenously or intradermally

(A-C) Mice (n=4-7 per group) were immunised intravenously once with 10,000 $CSP^{SIINFEKL}$, $UIS4^{SIINFEKL}$, pLISP1-$UIS4^{SIINFEKL}$, pLISP2-$UIS4^{SIINFEKL}$, $LISP1^{SIINFEKL}$ or $LISP2^{SIINFEKL}$ sporozoites under azithromycin prophylaxis. Splenocytes and liver infiltrating lymphocytes from immunised or naïve mice (n=4) were restimulated with SIINFEKL 14 days post immunisation and co-stained with CD11a and IFN-γ and CD8+ T cell populations were enumerated by flow cytometry. (A) Flow cytometry gating strategy used. (B) The percentage of all CD8+ T cells co-expressing IFN-γ and CD11a. (C) The absolute cell counts of all CD8+ T cells co-expressing IFN-γ and CD11a. Data shown is from one representative experiment of two experiments performed. (D-F) Mice (n=6-7 per group) were immunised intradermally once with 10,000 $CSP^{SIINFEKL}$, $UIS4^{SIINFEKL}$, $LISP1^{SIINFEKL}$ or $LISP2^{SIINFEKL}$ sporozoites under azithromycin prophylaxis. Splenocytes and liver infiltrating lymphocytes from immunised or naïve mice (n=2) were restimulated with SIINFEKL 14 days post immunisation and co-stained with CD11a and IFN-γ and CD8+ T cell populations were enumerated by flow cytometry. (D) Flow cytometry gating strategy used. (E) The percentage of all CD8+ T cells co-expressing IFN-γ and CD11a. (F) The absolute cell counts of all CD8+ T cells co-expressing IFN-γ and CD11a. Data shown is from one experiment performed. (B,C,E,F) Bar charts show mean values (±SEM) with statistics calculated using one-way ANOVA with Tukey multiple comparisons post-test (*, $p<0.05$; **, $p<0.01$, ***, $p<0.001$).

Mid-late EEF antigens offer partial protection

UIS4 was as poorly immunogenic as LISP1 and LISP2 at inducing CD8+ T cells responses which suggested that the CD8+ T cell response to an EEV PVM antigen is not improved by the duration for which the antigen is expressed. To assess the time scale within which an EEF antigen can be protective, we decided to determine if vaccination could show a protective role for LISP1 and LISP2, compared to that rendered by UIS4. Mice were vaccinated with adenovirus expressing full length ovalbumin and OT-I splenocytes which induced high levels of SIINFEKL-specific CD8+ T cells (Supplementary Figure 1) before mice were challenged 14 days later with our transgenic parasites. Vaccine efficacy was assessed by reduction in parasite load in the liver and sterile protection.

Challenge with LISP1^{SIINFEKL} and LISP2^{SIINFEKL} resulted in around ~70% reduction in parasite load in the liver, however vaccinated mice had a reduction of >99% when challenged with CSP^{SIINFEKL} and UIS4^{SIINFEKL} (Figure 5A, B). These data would suggest that the mice challenged with LISP1^{SIINFEKL} and LISP2^{SIINFEKL} would not be sterilely protected. However, LISP1 and LISP2 induced around 50% sterile protection (Figure 5C). Compared to the nearly 90% protection offered by CSP^{SIINFEKL} and UIS4^{SIINFEKL}, it contradicts the parasite load in the liver data as a two log difference in liver load would not normally lead to any mice being sterilely protected. Also, the onset of parasitaemia in challenged vaccinated mice that were not sterilely protected was delayed with some mice becoming visibly parasitaemic by blood smear, occurring up to four days later than those mice challenged with CSP^{SIINFEKL} or UIS4^{SIINFEKL} (Figure 5D).

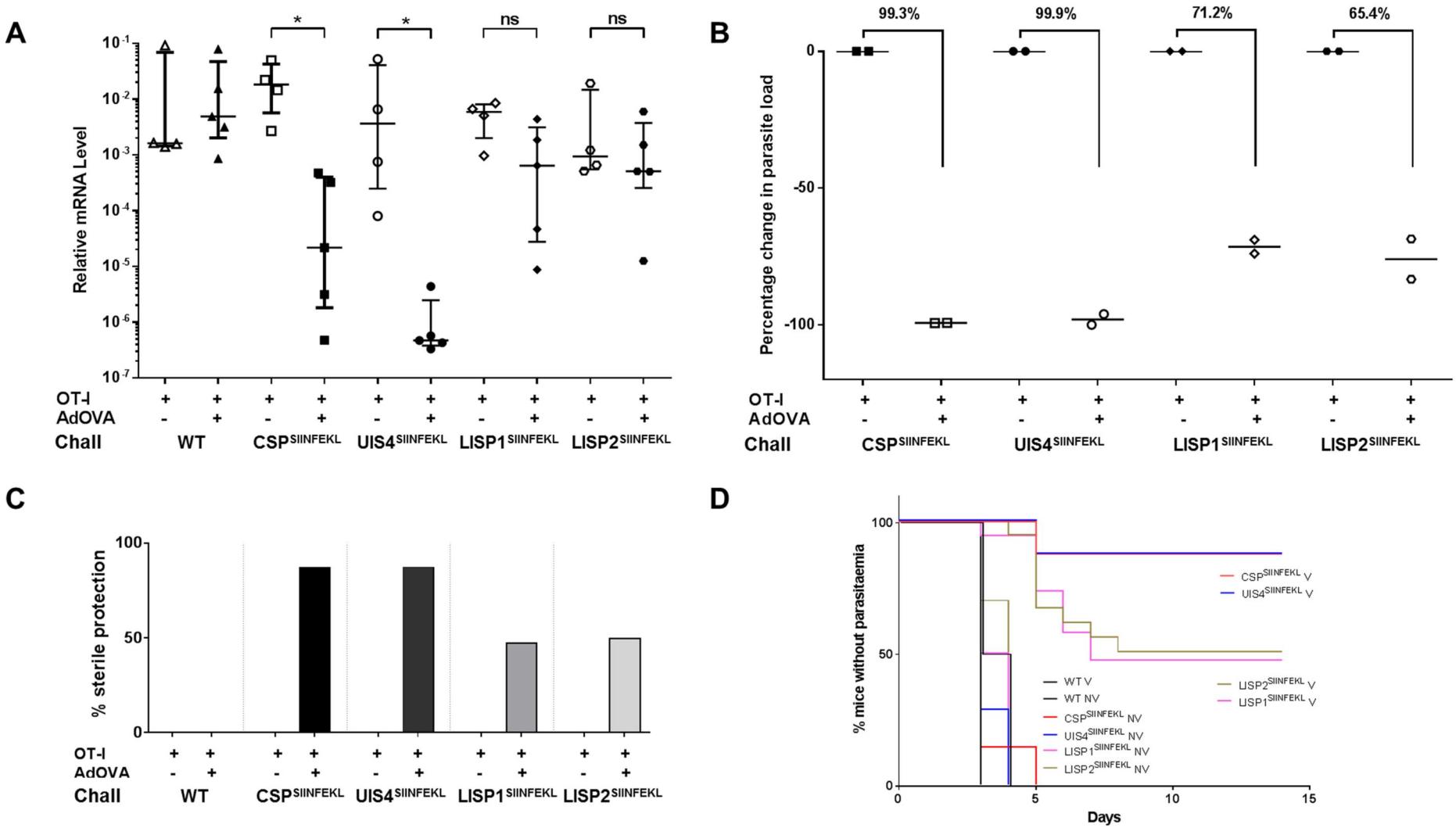

A
Relative mRNA Level
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
10^{-6}
10^{-7}
*
*
ns
ns
OT-I + + + + + + + + + +
AdOVA - + - + - + - + - +
Chall WT CSP^{SIINFEKL} UIS4^{SIINFEKL} LISP1^{SIINFEKL} LISP2^{SIINFEKL}
B
Percentage change in parasite load
0
-50
-100
99.3%
99.9%
71.2%
65.4%
OT-I + + + + + + + +
AdOVA - + - + - + - +
Chall CSP^{SIINFEKL} UIS4^{SIINFEKL} LISP1^{SIINFEKL} LISP2^{SIINFEKL}
C
% sterile protection
100
50
0
OT-I + + + + + + + + + +
AdOVA - + - + - + - + - +
Chall WT CSP^{SIINFEKL} UIS4^{SIINFEKL} LISP1^{SIINFEKL} LISP2^{SIINFEKL}
D
% mice without parasitaemia
100
50
0
0
5
10
15
Days
CSP^{SIINFEKL} V
UIS4^{SIINFEKL} V
WT V
WT NV
CSP^{SIINFEKL} NV
UIS4^{SIINFEKL} NV
LISP1^{SIINFEKL} NV
LISP2^{SIINFEKL} NV
LISP2^{SIINFEKL} V
LISP1^{SIINFEKL} V

Figure 5

Despite the poor immunogenicity of mid-late EEF antigens, following vaccination they induce a reduction in parasite load in the liver and unlikely levels of sterile protection

(A-D) Vaccinated mice received AdOVA and OT-I cells (n=5) and control mice received just OT-I cells (n=4). (A-B) Mice were challenged with 10,000 sporozoites two weeks after vaccination. (A) The relative parasite load in the liver for individual mice comparing the difference between the concentration of mouse *GAPDH* mRNA and *P. berghei* 18S rRNA. Data is from one representative experiment of two independent qPCR experiments performed. The mean + interquartile range is shown with statistics calculated by Mann Whitney U-test (*, $p<0.05$). (B) The average percentage reduction in parasite load in the liver of mice in the vaccinated group, compared with a normalised 0 value for mice in the non-vaccinated group. Each data point is the average from each experiment. (C-D) Vaccinated (n=3-11) and control mice (n=2-7) were challenged with 1,000 sporozoites. (C) The percentage of mice which did not become parasitaemic following challenge with sporozoites. (D) Kaplan-Meier curve showing the onset of parasitaemia in vaccinated (V) and control mice (NV) following challenge with WT (black), $CSP^{SIINFEKL}$ (red), $UIS4^{SIINFEKL}$ (blue), $LISP1^{SIINFEKL}$ (pink) or $LISP2^{SIINFEKL}$ (green). (C-D) Data are pooled from two experiments performed.

DISCUSSION

Much research has emerged with a focus on improving the efficacy of current malaria vaccines in trials with further antigens. Many have come to the conclusion that, unlike a single protein subunit vaccine against a virus or bacteria, many antigens from different life stages of the parasite will be required to evoke long-lasting vaccine efficacy against *Plasmodium*[30, 31]. Here we have presented evidence that antigen specific CD8+ T cell responses induced by the mid-late EEF antigens LISP1 and LISP2 are as poor as those induced by constitutively expressed EEF antigens UIS4. However, following viral vaccination, LISP1 and LISP2 induce a small reduction in parasite load in the liver but around 50% sterile protection, highlighting their potential for use in next-generation malaria vaccines.

Our previous work (Chapter 2: Müller and Gibbins, paper in preparation) had shown that UIS4, a PVM protein, can be as protective as CSP. Using a reporter epitope marker, CSP induced a larger frequency of antigen-specific CD8+ T cells than UIS4, however both proteins were successful in their ability to reduce parasite load in the liver following vaccination and challenge. This suggests that both proteins were presented on infected hepatocytes, inducing recognition by effector memory CD8+ T cells and the killing of the developing parasite inside the hepatocyte. CSP has been shown previously to be presented on infected hepatocytes to antigen-specific CD8+ T cells and be protective[32, 33], but the protective capability of EEF antigens has not been investigated.

The liver stage infection in mice infected with *Plasmodium berghei* is relatively short with merozoite release starting 48-52 hours post infection[18]. CD8+ T cells have been shown to peak in cytolytic activity against peptide coated cells in culture between 20-24 hours[34, 35] and CD8+ T cells from *Toxoplasma gondii* immunised mice have been shown to lyse peptide labelled cells within 4 hours with a peak at 16 hours *in vivo*[36]. UIS4 protein translation in the liver is constant, starting soon after sporozoite invasion of the

hepatocyte up until merozoite release[17, 19]. Thus, the potential for recognition of UIS4 protein presented on the hepatocyte surface can occur very soon after sporozoite invasion, with MHC being present on the cell surface of infected and uninfected hepatocytes from 3 hours with a large upregulation between 12 and 15 hours[37]. When vaccinated mice were challenged, which possessed a large proportion of antigen-specific CD8+ T cells in the blood, UIS4-targeted responses reduced the parasite load in the liver by more than 99% despite UIS4 inducing a weak endogenous CD8+ T cell response following sporozoite immunisation. Thus, if a liver-PVM associated antigen expressed constitutively during EEF development can protect in a 48-hour window, what would happen if this period was shortened? Essentially at what point is the parasite still vulnerable to attack in the liver?

LISP1 and LISP2 are highly expressed proteins that associate with the PVM but their expression profiles peak later than UIS4; nonetheless they are all required for effective EEF development. UIS4 is required for absolute development in the liver; without it, EEF development does not occur. LISP1 is associated with rupturing of the PVM; LISP1-KO merozoites inside the merosome are infective, but ten-fold less merozoites are released by the merosome due to an impairment with PVM lysis[20]. LISP2-KO parasites arrest as late merozoites which is proposed to be because LISP2 modulates a change in host hepatocyte environment, which is critical for merogony[21]. We show that expression of LISP1 and LISP2 is absent 12 hours after infection but appears at 24 hours and induce weak CD8+ T cell responses following sporozoite immunisation. However, the degree of partial sterile protection induced by LISP1 and LISP2, despite the poor CD8+ T cell responses they induce following sporozoite immunisation and the delay in their protein expression, highlights the importance and rapidity of CD8+ T cell mediated attack in the liver. Yet the discrepancy between the lack of reduction of parasite load in the liver, as determined by relative expression *of P. berghei* 18S rRNA, in vaccinated mice following sporozoite challenge and the apparent partial protection from blood stage parasitaemia remains to be explained.

18S rRNA is a very stable nucleic acid structure with a half-life of 3-7 days in cells grown *in vitro*[38-40] and 5 days *in vivo*[41] and there is >1000x greater concentration of 18S rRNA in sporozoites compared to their genomic coding rDNA[42]. It is the structural nucleic acid of the 40S small ribosomal subunit in eukaryotes and is often used as an internal control for reverse transcription PCR. Killing of the EEF leads to destruction of the parasite and hepatocyte proteins and nucleic acids. It has been shown that DNA from the genome of non-viable (freeze-thawed) *Plasmodium chaubaudi* AS merozoites is detectable in the host blood 24 hours after injection but not after 48 hours, suggesting non-viable parasite DNA in the blood is turned over between 24 and 48 hours[43]. Focusing on the liver; in mice receiving late arresting genetically attenuated *P. yoelii* parasites, arrested EEFs observed by microscopy start to lose their viability (as determined by PVM integrity) from 36 hours, with the majority being compromised at 48 hours (when WT parasites are starting the process of merozoites egress)[44]. While EEF abundance in the liver was no different to WT at 44 hours prior to merozoite egress, no genetically attenuated EEFs could be observed after 60 hours, suggesting that the parasites had been removed from the system over this time[44]. These data suggest that dead or arrested parasites are quickly turned over in the host. With regard to 18S rRNA; following intravenous injection of *P. yoelii* sporozoites into the tail vein of mouse, subsequent blood spot samples from the tail were taken to show that parasite 18S rRNA from sporozoites does not persist in the blood following hepatocyte invasion[42]. It was shown that genomic 18S rDNA is constantly detectable at the site of inoculation[42, 45] (but not a spatially different site)[42], suggesting the qPCR was detecting locally deposited, residual parasite contamination[42]. However, 18S rRNA could not be detected in blood after 30 mins, indicating a more rapid turnover of rRNA compared to rDNA in the absence of the parasite[42]. In a plant system, victorin toxin, produced by the plant fungus *Cochliobolus victoriae,* induces programmable cell death of *Avena sativa* (oats) which has characteristics of animal apoptosis[46]. Leaves incubated with victorin toxin suffered severe and then complete loss of leaf viability following four and then six hours incubation with the toxin[46]. A time-course of incubation with the toxin showed that specific cleavage and degradation of 18S rRNA

was observable from 2 hours and a reduction in intact 18S rRNA observable from 6 hours[46]. This further shows that 18S rRNA is broken down following death of cells. While it is not fully understood how CD8+ T cells kill the parasite inside hepatocytes, these data do suggest that 18S rRNA is rapidly broken down following death or arrest of EEF parasites with turn over quicker than that of genomic DNA in non-viable parasites[43].

In our experiments, in vaccinated mice challenged with $CSP^{SIINFEKL}$ or $UIS4^{SIINFEKL}$, antigen-specific responses are assumed to have removed the majority of EEFs over a period of 40 hours, as a significant reduction in parasite liver load was observed and that in a parallel experiment, most mice were sterilely protected. With complete removal of arrested EEFs from the liver observed over a 26 hour period[44], it is possible that the 18S rRNA observed in those mice receiving $LISP1^{SIINFEKL}$ or $LISP2^{SIINFEKL}$ challenge did not reflect the level of EEF killing that was starting or about to start at 40 hours post-infection, the point at which livers were harvested. LISP1 and LISP2 protein expression peaks in the final stages of EEF development, which may ensure the final stages of merozoite development and release occur correctly[20, 21] but we do not observe protein expression in the first 12 hours. With a 12-hour delay in protein expression compared to CSP and UIS4, the critical period of killing of infected hepatocytes induced by LISP1 and LISP2 may not have yet happened by 40 hours. The minimal reduction in parasite load in the liver we report would not normally have yielded sterilely protected mice. By performing qPCR at 40 hours, we cannot determine parasite prevalence of $LISP1^{SIINFEKL}$ and $LISP2^{SIINFEKL}$ parasites in the last 8-12 hours of the liver stage before merozoite release, with killing possibly affected by the minimum 12-hour delay in antigen presentation and recognition. We suggest there is a level of killing occurring in this timeframe which is sufficient to provide some mice with sterile protection. The delay in patency, as shown here in the Kaplan-Meier curves, also suggests that $LISP1^{SIINFEKL}$ and $LISP2^{SIINFEKL}$ merozoites are reduced in number as a result of induced immune responses. To investigate this stage, it would be pertinent to take liver sections from vaccinated and non-vaccinated mice that have been challenged to look for parasite viability and

morphology at various time points to determine when different parasites are being killed and determine the cause of delayed patency in non-protected vaccinated mice.

Additionally, while the percentage of antigen experienced, IFN-γ producing SIINFEKL-specific CD8+ T cells in the blood following vaccination averaged around 8% of all CD8+ T cells, within the liver this may be a different story. Tissue-resident memory CD8+ T cells have been described in the sinusoid of the liver in RAS-immunised and vaccinated mice exhibiting a patrolling phenotype which function in parasite surveillance[47]. The authors show that with a vaccination method using PbT-I cells, from a T cell receptor transgenic mouse which produce CD8+ T cells specific for a malaria antigen expressed in the sporozoite and the blood stages[48], a large T_{RM} population in the liver can be induced and mice are protected from challenge with sporozoites one month later. They also show that inducing T_{RM} CD8+ T cells through a liver centric vaccination strategy using hepatocyte targeting viruses can achieve better protection from challenge than using radiation-attenuated sporozoites. It would be interesting to investigate the differences in T_{RM} expression molecules on our SIINFEKL-specific CD8+ T cells induced by different liver antigens to determine if antigen expression timing affected the liver residence of CD8+ T cells and if this correlated with protection. The expression of MHC class I molecules has also been shown to be reduced following very late EEF development[37], so it would be interesting to know if merozoite proteins would still be protective, given their late expression and down-regulation of MHC class I molecules.

Historically, the liver stage has not been investigated as much as the blood stage for instance, possibly because of the complexity of the models used and the curious tolerogenic nature of the liver environment. However, antigens and epitopes are starting to be identified against this stage of the parasite life cycle. Recently Speake et al. identified six novel antigens that, when given as DNA vaccines prior to challenge with sporozoites, induced a reduction in parasite load in the liver[49]. One of these genes included LISP1, which when combined with a CSP DNA vaccine further reduced parasite

load in the liver. This corroborates our data to highlight the importance of LISP1. Later, Pichugin et al. went on to identify a novel CD8+ T cell epitope in a *Plasmodium berghei* "Middle domain of eukaryotic initiation factor 4G(MIF4G)-like-protein" EEF protein[50], which had previously been identified in the DNA vaccine study[49]. Murphy et al. also identified that an epitope presented by BALB/c mice from L3 60S ribosomal protein from *Plasmodium yoelii* (PY05881/PY17X_0513000) expressed throughout the liver stage and blood stages, which induced strong CD8+ T cell responses but did not ultimately protect mice on its own[51]. Speake et al. suggest that those antigens expressed throughout and with increasing magnitude in the EEF are those that show greater protection[49]. This has been echoed in genetically attenuated parasite studies, where parasites that arrest later during EEF development induce the most protection following challenge[44, 52]. It has also been suggested that fewer doses of sporozoites are required to protect mice when immunised with chloroquine prophylaxis because of the extended potential repertoire of antigen expressed[53]. We concur with our data here and previously (Chapter 3: Gibbins et al., paper in preparation) showing that longer antigen expression in EEF does not increase the number of antigen-specific CD8+ T cells and that greater protection is likely achieved through generation of a wide repertoire of CD8+ T cells. We also propose that while viral vaccination can induce large numbers of antigen-specific CD8+ T cells, there must be sufficient time for the antigen to be presented and recognised by its cognate CD8+ T cell for parasite destruction to occur. This would explain why LISP1 and LISP2 are not as protective as UIS4, as LISP1 and LISP2 have at least a 12-hour delay in protein expression in the EEF and thus their potentiality for presentation is delayed.

Nonetheless. we have shown that mid-late expressed EEF antigens are presented to the immune system and can induce killing of infected hepatocytes in a CD8+ T cell dependent manner. This further enhances the argument that more research should be conducted on determining the role of EEF antigens in the induction of pre-eyrthrocytic

stage immunity and be considered for development in next generation malaria vaccines, most likely in combination with other pre-erythrocytic antigens.

AUTHOR CONTRIBUTIONS

O.S. and J.C.R.H. designed the experiments; M.P.G., S.B. and O.S. generated the transgenic parasites; M.P.G. characterised the transgenic parasites; M.P.G., N.B. and L.M. performed immunology and vaccination experiments, analysed data and performed statistics; A.R.-S., A.V.S.H. and S.J.D. provided the adenovirus AdOVA; M.P.G. wrote the paper.

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COMPETING INTERESTS

S.J.D. is a named inventor on patent applications relating to malaria vaccines, adenovirus vaccines and immunisation regimens.

SUPPLEMENTARY INFORMATION

SUPPLEMENTARY EXPERIMENTAL PROCEDURES

Transgenic parasite generation

The common plasmid MG1 was generated from a starting plasmid (OS-*DHFR-mCherry*) containing a T7 promoter, *Toxoplasma gondii* dihydrofolate reductase/thymidylate synthase (*TgDHFR/TS*) pyrimethamine resistance cassette and mCherry cassette. The mCherry cassette was released by excision using restriction enzymes *EcoRI* and *SacII* and the vector backbone was purified. MG1 was generated from this backbone by adjoining two fragments called mChOVA and OVAutr using In-Fusion technology. mChOVA was generated by amplifying the mCherry region of the OS-*DHFR-mCherry* plasmid and incorporating SIINFEKL coding sequence at the 3' end. OVAutr was generating by amplifying the 3' UTR of UIS4 from *Plasmodium berghei* WT genomic DNA and appending the SIINFEKL encoding region to the 5' end of the fragment. In-Fusion (Clontech) technology works such that fragments are designed to have 15 base overhangs so that in the presence of In-Fusion Enzyme, DNA fragments with complementary 15mer overhangs are fused together and fused into the linearised vector backbone. To generate MG1, the two fragments would then have complementary regions in the SIINFEKL coding region and with the 5' and 3' region of the restricted plasmid. MG1 was then ready to be modified further by incorporated more fragments 5' to the mCherry coding sequence which following transfection of parasites would lead to the mCherry-SIINFEKL-UIS4 3' UTR being incorporated to the 3' end of the ORF of the gene of interest. XL10 competent *E. coli* were transformed with MG1 and plasmid amplified and retrieved by miniprep and plasmid verified by sequencing.

Additional fragments were generated by designing primers to amplify LISP1 and LISP2 promoters and ORFs for UIS4, LISP1 and LISP2 following PCR of *Plasmodium berghei* WT genomic DNA.

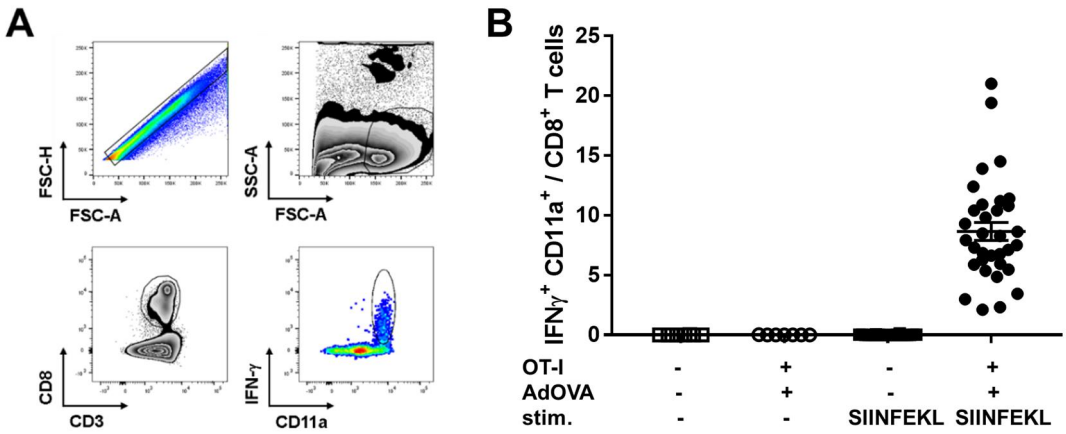
Generation of MG2b-pLISP1-UIS4^{SIINFEKL}, MG2c-pLISP2-UIS4^{SIINFEKL}, MG2e-LISP1^{SIINFEKL} and MG2f-LISP2^{SIINFEKL} plasmids was achieved by digestion of MG1 with *EcoRI* and In-Fusion with gene specific promoter and ORF DNA fragments.

For MG2b-pLISP1-UIS4^{SIINFEKL}, fragments consisting of the promoter of LISP1 (L1prom) and UIS4 ORF (U4orf) were fused into MG1. For MG2c-pLISP2-UIS4^{SIINFEKL}, fragments consisting of the promoter of LISP2 (L2prom) and UIS4 ORF (U4orf) were fused into MG1. For MG2e-LISP1^{SIINFEKL}, two overlapping fragments corresponding to a 3' region and end of the LISP1 ORF (L1Aorf and L1Borf) were fused into MG1. For MG2f-LISP2^{SIINFEKL}, two fragments corresponding to a 3' region and end of the LISP2 ORF (L2Aorf and L2Borf) were fused into MG1. In this way, when the corresponding plasmid is incorporated into the genome at the UIS4 locus by single cross-over homologous recombination, MG2b-pLISP1-UIS4^{SIINFEKL} and MG2c-pLISP2-UIS4^{SIINFEKL} will result in an extra UIS4 gene being inserted which was appended by the mCherry-SIINFEKL-UIS4 3' UTR cassette. For MG2e-LISP1^{SIINFEKL} and MG2f-LISP2^{SIINFEKL}, following single cross-over homologous recombination at the LISP1 or LISP2 locus respectively, the endogenous gene would now be appended by the mCherry-SIINFEKL-UIS4 3' UTR cassette.

Following miniprep and verification by sequencing, plasmids were transfected into *Plasmodium berghei* ANKA expressing GFP at the dispensable *p230p*[22] by electroporation of merozoites using Nucleofector buffer and an AMAXA Nucleofector[26]. Briefly, *Plasmodium berghei* blood stage parasites were generated in Swiss Webster mice and blood taken late in the day by cardiac puncture when parasitaemia was around 5%. Red blood cells were incubated in RPMI with 20% FCS overnight at 36.5°C with shaking at 70 rpm to slow the asexual cycle and allow for isolation of viable, mature, synchronised schizonts the following morning. Nycodenz was used to isolate the schizonts by density gradient centrifugation. Isolated schizonts were resuspended in Nucleofector buffer and DNA plasmid to rupture the RBCs and release merozoites. Merozoites were electroporated with the plasmid using AMAXA Nucleofector (program U33) and Swiss Webster mice were injected with electroporated merozoites

intravenously. Mice were provided with pyrimethamine (7μg/ml) in drinking water the day after injection. Mice were monitored and were bled by cardiac puncture when their parasitaemia was at 1.5%. Genomic DNA was extracted using PureLink Genomic DNA Kits (Invitrogen) and primers were designed to genotype the parasites, assessing for the presence of WT and recombinant parasites, 5' and 3' integration of the plasmid and presence of episomes. Upon appearance of recombinant populations with the correct integration of the plasmid, transgenic clones were generated by limiting dilution into CD-1 mice. Clones were verified by genotyping PCR (FastStart Taq, Roche). PCR products were run on 1.2% agarose gels with SybrSafe (ThermoFisher Scientific) intercalatant.

SUPPLEMENTARY FIGURES



Supplementary Figure 1

Vaccination with AdOVA and OT-I cells

(A) Flow cytometry panel used to assess (B) the magnitude of SIINFEKL-specific CD8+ T cells in the blood of non-vaccinated and vaccinated mice following restimulation with SIINFEKL peptide. Scatter plot shows mean ±SEM.

	Oligonucleotide	Sequence 5' --> 3'
Generation of plasmid MG1	mChOVAfor	taccgagctcGAATTCGTGAGCAAGGGCGAGGAGGATAACATGG
	mChOVArev	TTTTTCAAAGTTGATTatactCTTGTACAGCTCGTCCATGCC
	OVAutrFor	ATCAACTTTGAAAAActgtaaatataattcattatgagtagtgtaattcagaaagag
	OVAutrRev	AATTGGAGCTCCACCGCGGacacatatgtaaaaaagttgcatatacgg
Generation of plasmid MG2b	L1promFor	taccgagctcGAATTCattattattggcttattgtcctttatgcc
	L1promRev	TGTGGTTTTCATttcaaattaaaataaaataaaacaataatagtaagatgg
	U4orfRev	aaaATGAAAACCACATACGTTTCTCTATTTTTTATCATATTCACTAATTTTTCGG
	U4orfRev	ccttgctcacgaattcTATGTATGGGCCGAATGATTTATTTTCC
Generation of plasmid MG2c	L2promFor	taccgagctcGAATTCattattattggcttattgtcctttatgcc
	L2promRev	TGTGGTTTTCATttatagaatgtgaaaaatgaaagaagaagaatattactcc
	U4orfFor	aaaATGAAAACCACATACGTTTCTCTATTTTTTATCATATTCACTAATTTTTCGG
	U4orfRev	ccttgctcacgaattcTATGTATGGGCCGAATGATTTATTTTCC
Generation of plasmid MG2e	L1AorfFor	taccgagctcGAATTCAAAGTGATAAAACTGAACAACTAATCGCCAC
	L1AorfRev	atagagctagcattAAAGAAGATATAATATCATTACATTCTTCC
	L1BorfFor	aaatgctagctctatATTTTGGAGAAATAGTATATTAGGATTTATAGC
	L1BorfRev	ccttgctcacgaattcGAATATGGAAATAGAAAATCAATAACAGCC
Generation of plasmid MG2f	L2AorfFor	taccgagctcGAATTCTCTTGCCATATTCTAATCCATATCTCATGC
	L2AorfRev	ttattgctagcattaTAATTTTTCTTTTTCTTCTATAAATATATTATGC
	L2BorfFor	taatgctagcaataaAATAAAAAGTATATTTGATATTCAAGAAAAATC
	L2BorfRev	ccttgctcacgaattcTTGTTTTCTTCTTGGAATAATACCTTCTTTCTC
Genotyping of LISP-1^{SIINFEKL}	LISP1^{SIINFEKL} A: WT For	TCATTTCCCAGCTACTATTAGAACAATTCG
	LISP1^{SIINFEKL} A: WT Rev	CATTGTTTAGCAGAGAAATCTTTGAA
	LISP1^{SIINFEKL} B: 5' int For	TCATTTCCCAGCTACTATTAGAACAATTCG
	LISP1^{SIINFEKL} B: 5' int Rev	TCGTGGCCGTTCACGGAGCCCTCC
	LISP1^{SIINFEKL} C: 3' int For	AAACTGCATCGTCGCTGTGTCC
	LISP1^{SIINFEKL} C: 3' int Rev	GCATGGGAATATGCACATACGATTTGCTG
	LISP1^{SIINFEKL} D: Episomes For	AAACTGCATCGTCGCTGTGTCC
	LISP1^{SIINFEKL} D: Episomes Rev	GTAATACGACTCACTATAGGGC
Genotyping of LISP-2^{SIINFEKL}	LISP2^{SIINFEKL} A: WT For	TTAATAAAACAAGCGATAATGCAAAAGG
	LISP2^{SIINFEKL} A: WT Rev	ATATCACAATAGTCTAAGCAACCA
	LISP2^{SIINFEKL} B: 5' int For	TTAATAAAACAAGCGATAATGCAAAAGG
	LISP2^{SIINFEKL} B: 5' int Rev	TCACCTTCAGCTTGGCGGTCTGG
	LISP2^{SIINFEKL} C: 3' int For	TGTTCTCTCTGATGTCCAGGAG
	LISP2^{SIINFEKL} C: 3' int Rev	ATATCACAATAGTCTAAGCAACCA
	LISP2^{SIINFEKL} D: Episomes For	TGTTCTCTCTGATGTCCAGGAG
	LISP2^{SIINFEKL} D: Episomes Rev	GTAATACGACTCACTATAGGGC
Genotyping of pLISP1-UIS4^{SIINFEKL} and pLISP2-UIS4^{SIINFEKL}	pLISP1/2-UIS4^{SIINFEKL} A: WT For	ACCCATTGATGAGACAAACGATTCAAAACC
	pLISP1/2-UIS4^{SIINFEKL} A: WT Rev	TTCGTAAAAAATAAAAACGCATGTTATGC
	pLISP1/2-UIS4^{SIINFEKL} B: 5' int and Episomes For	ACCCATTGATGAGACAAACGATTCAAAACC
	pLISP1/2-UIS4^{SIINFEKL} B: 5' intand Episomes Rev	GTAATACGACTCACTATAGGGC
	pLISP1/2-UIS4^{SIINFEKL} C: 3' int For	CTGCAGGACGGCGAGTTCATCTACAAGG
	pLISP1/2-UIS4^{SIINFEKL} C: 3' int Rev	ACCAACAATTTTTATGAAAAAGTCTTTAGG
	pLISP1/2-UIS4^{SIINFEKL} D_2: LISP1 promoter differentiation For	TGTTTGTTTATTTTGGCGCAGAACC
	pLISP1/2-UIS4^{SIINFEKL} D_2: LISP1 promoter differentiation Rev	ACCAACAATTTTTATGAAAAAGTCTTTAGG
	pLISP1/2-UIS4^{SIINFEKL} D_4: LISP2 promoter differentiation For	TATACATGCATGTATATTCCCCTGC
	pLISP1/2-UIS4^{SIINFEKL} D_4: LISP2 promoter differentiation Rev	ACCAACAATTTTTATGAAAAAGTCTTTAGG
qPCR - *Mus musculus GAPDH*	mGAPDH For	TGAGGCCGGTGCTGAGTATGTCG
	mGAPDH Rev	CCACAGTCTTCTGGGTGGCAGTG
qPCR - *Plasmodium berghei 18S rRNA*	Pb18S For	AAGCATTAAATAAAGCGAATACATCCTTAC
	Pb18S Rev	GGAGATTGGTTTTGACGTTTATGTG

Supplementary Table 1

Table of primers for plasmid generation, parasite genotyping and parasite load in the liver qPCR

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SECTION A – Student Details

Student	Matthew Paul Gibbins
Principal Supervisor	Julius Clemence R. Hafalla
Thesis Title	Dissecting the CD8+ T cell responses to pre-erythrocytic malaria antigens

If the Research Paper has previously been published please complete Section B, if not please move to Section C

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Where is the work intended to be published?	Infection and Immunity
Please list the paper's authors in the intended authorship order:	Matthew P. Gibbins, Maya Glover, Jasmine Liu, Karolis Bauza, Arturo Reyes-Sandoval, Katja Müller, Kai Matuschewski, Olivier Silvie, Julius Clemence R. Hafalla
Stage of publication	**Not yet submitted**

SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	I performed immunological experiments, analysed data and performed the statistical analyses. I wrote the first draft of the paper.

Student Signature: [redacted] Date: 17/09/18

Supervisor Signature: [redacted] Date: 17/09/18

CHAPTER 5

The importance of the immunodominant CD8+ T cell epitope of *Plasmodium* circumsporozoite protein in parasite- and vaccine-induced protection

The importance of the immunodominant CD8+ T cell epitope of *Plasmodium* circumsporozoite protein in parasite- and vaccine-induced protection

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Running Head: Immunodominant malaria CS Protein CD8+ T cell epitope

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ABSTRACT

The circumsporozoite protein (CSP), the surface coat of sporozoites, has been at the forefront in malaria pre-erythrocytic stage vaccine development for the last 30 years. CSP has been shown to induce robust CD8+ T cell responses that are capable of eliminating the developing parasites in hepatocytes resulting in protective immunity. In this study, we characterised the importance of SYIPSAEKI, the immunodominant CSP-derived epitope of *Plasmodium berghei* in both sporozoite- and vaccine-induced protection in murine infection models. In BALB/c mice, where SYIPSAEKI is efficiently presented in the context of the major histocompatibility complex class I (MHC-I) molecule H-2-K^d, we establish that epitope-specific CD8+ T cell responses contribute to parasite killing following sporozoite immunisation. Yet, sterile protection is achieved in the absence of this epitope confirming that other antigens are crucial for parasite-induced protective immunity. Moreover, we demonstrate that SYIPSAEKI-specific CD8+ T cell responses elicited by viral-vectored CSP-expressing vaccines effectively target parasites in hepatocytes and the resulting sterile protection strictly relies on the expression of SYIPSAEKI. We further show that in C57BL/6 mice, which expresses an irrelevant MHC-I and therefore unable to express the immunodominant epitope, CSP-based vaccines do not confer protection. These findings further demonstrate the importance of CSP in protection against malaria pre-erythrocytic stages and that a significant proportion of the protection against the parasite is mediated by CD8+ T cells that are specific for the immunodominant epitope of this sporozoite surface protein.

INTRODUCTION

Malaria is caused by a protozoan parasite of the genus *Plasmodium* and remains a major global health challenge in tropical and subtropical countries(1). A vaccine that reduces the burden of disease and prevents malaria transmission remains an ultimate goal for malaria elimination programmes. As a gold standard in malaria vaccination, multiple immunisations of γ-radiation-attenuated *Plasmodium* sporozoites (RAS) can completely protect against sporozoite challenge(2-4). This parasite-induced protection targets the developing exo-erythrocytic forms in the liver and completely abrogates blood stage infection. Antibodies and T cells have been implicated as important mechanisms of protection(5). In murine infection models, CD8+ T cells are the prime mediators of protective immunity(6, 7).

The circumsporozoite protein (CSP), the major surface coat protein of the malaria sporozoite, has been at the head of vaccination studies for more than 30 years – being the basis of RTS,S/AS01, the most progressed malaria vaccine candidate to date(8). Immunisation of BALB/c mice with *Plasmodium berghei* (*Pb*) or *P. yoelii* (*Py*) RAS evokes immunodominant major histocompatibility complex class I (MHC-I) H-2-K^d-restricted $CD8^+$ T cell responses against distinct CSP epitopes: SYIPSAEKI for *Pb*(9) and SYVPSAEQI for *Py*(10). Indeed, the measurement of responses to these epitopes have become the standard in fundamental immunological studies in BALB/c mice. Furthermore, numerous vaccination studies involving different viral-vectored CSP- or CSP epitope-expressing vaccines – used alone or in combination as part of prime-boost regimens – have corroborated that CSP is a highly protective antigen in the BALB/c infection model(11-17). In these studies, elevated levels of either SYIPSAEKI- or SYVPSAEQI-specific CD8+ T cell responses correlated with protection.

Several studies have interrogated the immunological relevance of whole CSP in parasite-induced protection. These studies emanated from observations that in naturally exposed humans, T cell responses to CSP are scarce(18). Moreover, multiple immunisations are

required to elicit CD8+ T cell-dependent protective immunity in various mouse strains, particularly where no other CSP-derived CD8+ T cell epitopes have been identified(19). In *Py*CSP-transgenic BALB/c mice which are tolerant to *Py*CSP, complete protection can be achieved by *Py* RAS immunisation(20). In good agreement, BALB/c mice immunised with *Pb* WT parasites were completely protected when challenged with transgenic parasites where the endogenous CSP has been swapped with the *P. falciparum* CSP(21). Taken together, these studies indicate that immune responses to CSP are dispensable for protection, and that other antigens are important to elicit protective immunity.

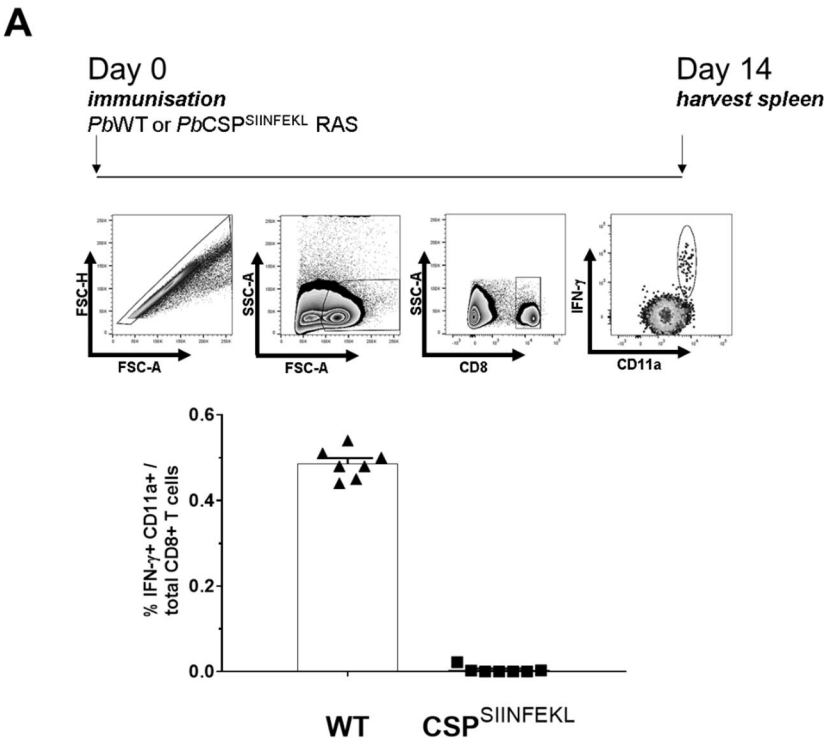
In this study, we extend previous work by dissecting the relevance of a single CSP-derived immunodominant epitope in parasite- and vaccine-induced protection in BALB/c mice, by utilising transgenic *Pb* parasites lacking SYIPSAEKI for immunisation and challenge experiments. In addition, we highlight the level of protection achieved by CSP-based vaccines, in mice expressing the relevant (BALB/c) [or irrelevant (C57BL/6)] MHC-I that is needed to present a single CSP-derived immunodominant epitope.

RESULTS AND DISCUSSION

Sporozoite-induced SYIPSAEKI-specific CD8+ T cell responses contribute to parasite killing but are dispensable for the development of sterile immunity. First, we interrogated the role that SYIPSAEKI, the H-2-K^d-restricted immunodominant epitope of *Pb*CSP, plays in protective immunity induced after sporozoite immunisation. For this purpose, *Pb*CSP^{SIINFEKL} RAS, where the SYPSAEKI sequence has been replaced with SIINFEKL (the H-2-K^b-restricted epitope of ovalbumin), were used to immunise H-2-K^d-expressing BALB/c mice. There are no other reported H-2-K^d-restricted *Pb*CSP epitopes. As shown in Figure 1A, *Pb*CSP^{SIINFEKL} RAS parasites elicited no SYIPSAEKI-specific CD8+ T cell responses in BALB/c mice, whilst these parasites evoke robust SIINFEKL-specific responses in H-2-K^b-expressing C57BL/6 mice (Chapter 2: Müller and Gibbins et al., paper in preparation).

To ascertain whether SYIPSAEKI contributes to parasite-induced protection, BALB/c mice were immunised once with either *Pb*WT or *Pb*CSP^{SIINFEKL} RAS. 14 days later, immunised mice were challenged with *Pb*WT sporozoites and protection was determined by measuring the parasite loads in the liver 40 hours later. As shown in Figure 1B, a significant reduction in parasite load – up to 4-log difference as compared to naïve challenge mice – was observed in mice immunised with *Pb*WT RAS and challenged with *Pb*WT parasites. In contrast, protection was reduced in mice immunised with *Pb*CSP^{SIINFEKL} RAS (Figure 1B). We observed similar results following two immunisations (data not shown) indicating that a substantial degree of protection in *Pb*WT RAS-immunised mice, measured by reduction in parasite load in the liver over two orders of magnitude, can be attributed to SYIPSAEKI-specific CD8+ T cell responses. These results highlight the notion that within *Pb*CSP, the SYIPSAEKI epitope has a critical and immunodominant contribution to protecting BALB/c mice after one or two immunisations with RAS.

However, at least three immunisations with RAS are required to induce sterile protection. To establish whether the development of sterile immunity is dependent on SYIPSAEKI-specific CD8+ T cell responses, BALB/c mice were immunised thrice with *Pb*CSP^{SIINFEKL} RAS one week apart; 14 days after the last immunisation, mice were challenged with *Pb*WT sporozoites. As shown in Figure 1C, all mice were protected from blood stage infection compared to the naïve controls.


A
Day 0
immunisation
PbWT or PbCSPSIINFEKL RAS
Day 14
harvest spleen
FSC-H
FSC-A
SSC-A
FSC-A
SSC-A
CD8
IFN-γ
CD11a
% IFN-γ+ CD11a+ / total CD8+ T cells
0.6
0.4
0.2
0.0
WT
CSPSIINFEKL

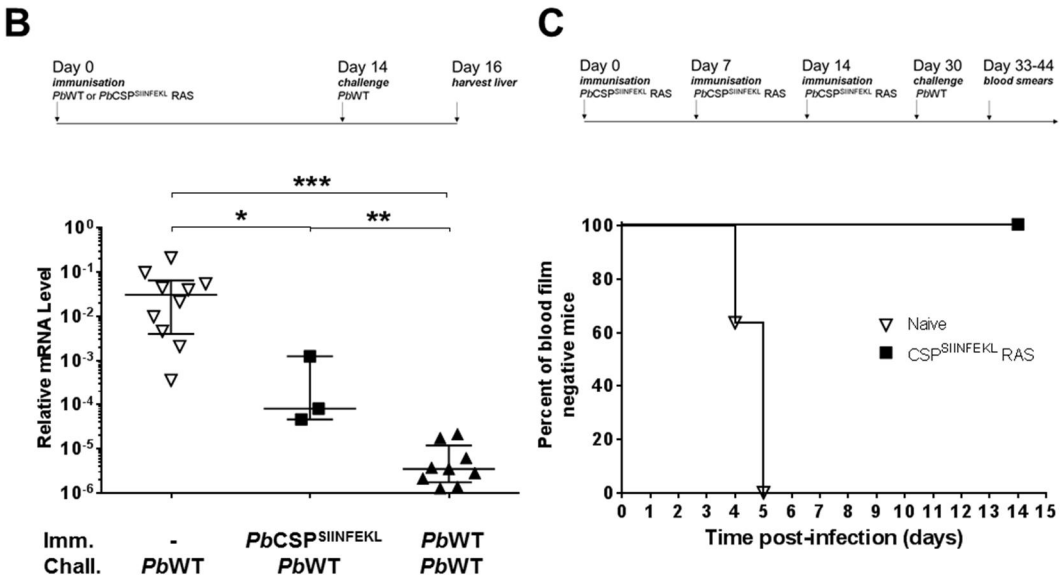

B
Day 0
immunisation
PbWT or PbCSPSIINFEKL RAS
Day 14
challenge
PbWT
Day 16
harvest liver
***
*
**
Relative mRNA Level
10^0
10^-1
10^-2
10^-3
10^-4
10^-5
10^-6
Imm.
Chall.
-
PbWT
PbCSPSIINFEKL
PbWT
PbWT
PbWT
C
Day 0
immunisation
PbCSPSIINFEKL RAS
Day 7
immunisation
PbCSPSIINFEKL RAS
Day 14
immunisation
PbCSPSIINFEKL RAS
Day 30
challenge
PbWT
Day 33-44
blood smears
Percent of blood film negative mice
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Time post-infection (days)
Naive
CSPSIINFEKL RAS

FIGURE 1

SYIPSAEKI is dispensable for RAS immunisation but predominates protection with fewer immunisations. (A) BALB/c mice (n=2-3) were immunised once with 10^4 *Pb*WT or *Pb*CSP^{SIINFEKL} RAS. Spleens were removed after two weeks. Splenocytes from the mice were pooled and restimulated with SYIPSAEKI peptide. IFN-γ production was assessed by ICS and flow cytometry. Each point represents the result from one well, with data pooled from two independent experiments. The results of this experiment were additionally confirmed using another transgenic parasite also lacking the SYIPSAEKI epitope of CSP (unpublished and data not shown).

(B) Groups of BALB/c mice were immunised once with $1.5x10^4$ *Pb*WT RAS (n=9) or *Pb*CSP^{SIINFEKL} RAS (n=3). Immunised mice and BALB/c naïve controls (n=10) were challenged with 10^4 *Pb*WT parasites two weeks after the last immunisation. Livers were harvested 40 hours post-challenge and relative liver parasite levels were quantified using the $\Delta\Delta$Ct method comparing levels of *P. berghei* 18S rRNA and levels of mouse *GAPDH* mRNA. Median values + interquartile ranges are shown with statistics calculated using the Mann-Whitney U-test (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).

(C) BALB/c mice (n=12) were thrice immunised with 10^4 *Pb*CSP^{SIINFEKL} RAS with immunisations one week apart. Immunised mice and BALB/c naïve controls (n=11) were challenged with $5x10^3$ *Pb*WT parasites 16 days after the last immunisation. Blood smears were taken on day 3-14 days after challenge. Smears were stained with Giemsa and parasitaemia was assessed by microscopy. Data shown is pooled from two independent experiments.

Prime-boost vaccination with CSP-expressing viruses induces strong anti-CSP antibody and CD8+ T cell responses but SYIPSAEKI is the key mediator of protection. Next, we probed the requirement for SYIPSAEKI presentation in protection elicited by viral-vectored CSP-expressing vaccines administered in a prime-boost regimen. Priming with adenovirus (Ad) carrying a foreign antigen and boosting with orthopoxvirus modified vaccinia Ankara (MVA) expressing the same antigen has consistently been shown to induce strong CD8+ T cell responses capable of inducing high levels of efficacy against intracellular pathogens including malaria pre-erythrocytic stages(14, 17).

Chimpanzee adenovirus serotype 63 (AdCh63) and MVA vaccines expressing *Pb*CSP were used to vaccinate BALB/c mice with a two-week resting period between priming and boosting (Figure 2A). Two weeks after boosting, whole blood was collected and was restimulated *ex vivo* with SYIPSAEKI peptide. The frequencies of IFN-γ secreting CD8+ T cells were enumerated by flow cytometry (Figure 2B) and as shown in Figure 2C, Ad-MVA *Pb*CSP-vaccinated mice elicited ~12% SYIPSAEKI-specific circulating CD8+ T cells. Serum samples were also collected from the vaccinated animals and were used in an immunofluorescence assay against air-dried *Pb* sporozoites. As shown in Figure 2D, Ad-MVA *Pb*CSP-vaccinated BALB/c mice induced strong anti-CSP antibody tires ($1:10^4$). These data indicate that Ad-MVA *Pb*CSP vaccination elicit both high frequencies of SYPSAEKI-specific CD8+ T cells and high titres of CSP-specific antibodies. It is probable that the vaccination regimen induced CD8+ T cell and antibody responses to other unidentified CD8+ T cell epitopes of CSP.

Two weeks after boosting, Ad-MVA *Pb*CSP-vaccinated mice were challenged with *Pb*WT or *Pb*CSP^{SIINFEKL} parasites. Protection was assessed by two complementary assays; (i) determination of the reduction of parasite load in the liver and (ii) induction of sterile protection. As shown in Figure 2E, parasite load in the liver of Ad-MVA *Pb*CSP-vaccinated mice was not significantly different to non-vaccinated mice when challenged

with *Pb*CSP^{SIINFEKL} sporozoites in contrast to challenge with *Pb*WT sporozoites. As shown in Figure 2F, vaccinated mice challenged with *Pb*CSP^{SIINFEKL} sporozoites were patent for parasitaemia by day 5, whereas vaccinated mice challenged with *Pb*WT sporozoites remained completely protected. These results denote that vaccine-induced effector SYIPSEAKI-specific CD8+ T responses efficiently target parasites expressing the cognate epitope. Parasites lacking the SYIPSAEKI epitope are not eliminated despite high levels of CSP-specific antibodies evoked by vaccination.

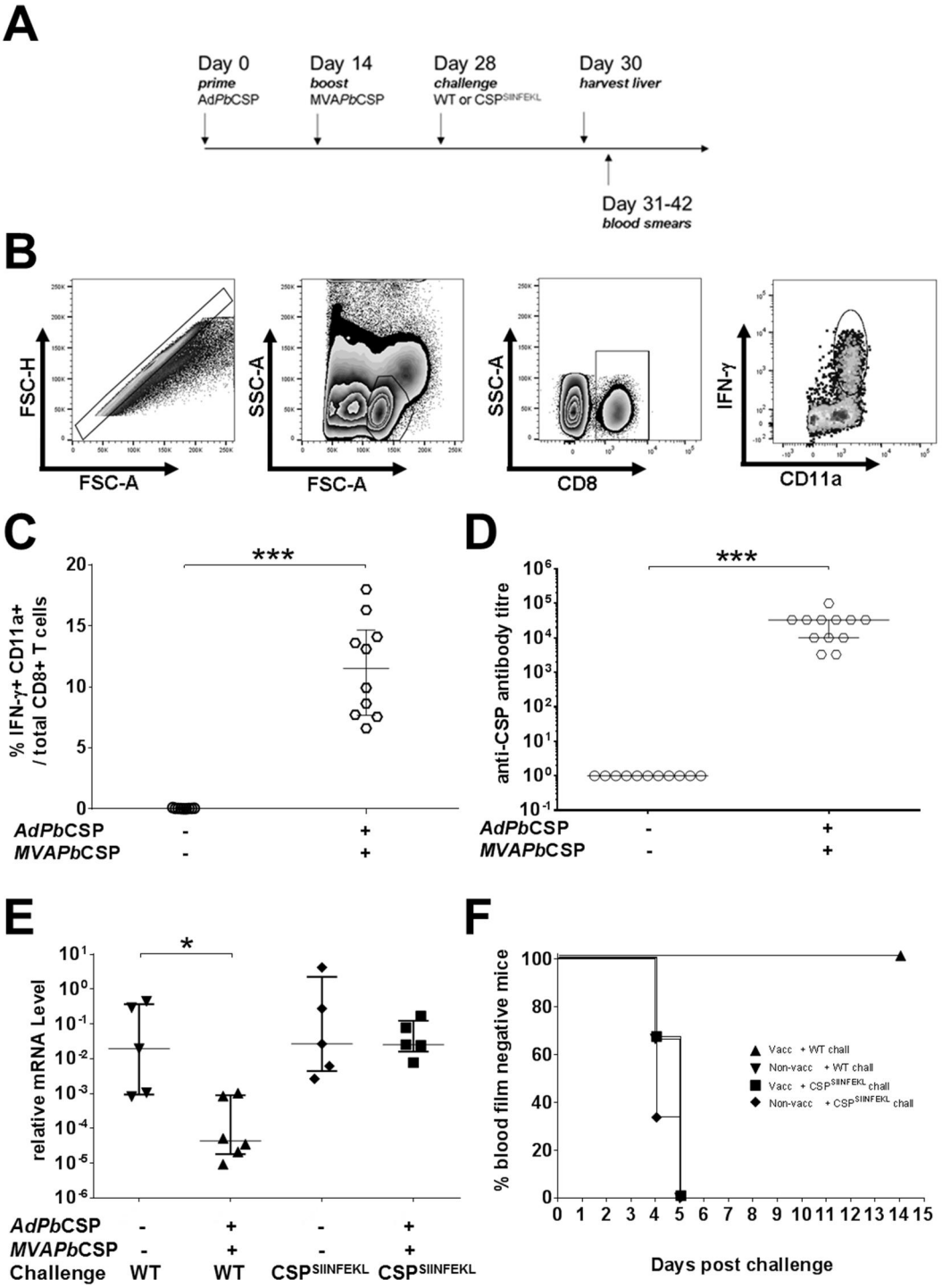

A
Day 0
prime
AdPbCSP
Day 14
boost
MVAPbCSP
Day 28
challenge
WT or CSPSIINFEKL
Day 30
harvest liver
Day 31-42
blood smears
B
FSC-H
FSC-A
SSC-A
FSC-A
SSC-A
CD8
IFN-γ
CD11a
C
% IFN-γ+ CD11a+ / total CD8+ T cells
***
AdPbCSP
MVAPbCSP
D
anti-CSP antibody titre
***
AdPbCSP
MVAPbCSP
E
relative mRNA Level
*
AdPbCSP
MVAPbCSP
Challenge
WT
WT
CSPSIINFEKL
CSPSIINFEKL
F
% blood film negative mice
Vacc + WT chall
Non-vacc + WT chall
Vacc + CSPSIINFEKL chall
Non-vacc + CSPSIINFEKL chall
Days post challenge

FIGURE 2

Prime-boost vaccination with CSP expressing viruses induces strong anti-CSP antibody and CD8+ T cell responses but SYIPSAEKI-specific CD8+ T cell responses are absolutely required for protection. (A) BALB/c mice were vaccinated with AdCh63 and MVA vaccines expressing the mammalian codon-optimised fragment of *Pb*CSP and challenged with 10^4 *Pb*WT or *Pb*CSP^{SIINFEKL} sporozoites as shown. (B) The flow cytometry gating strategy used to determine proportions of IFN-γ+ CD11a+ CD8+ T cells. (C) Blood drawn from the tail from naïve (n=9) and vaccinated mice (n=10) two weeks after boost and restimulated with SYIPSAEKI and stained for CD8, CD11a surface markers, and IFN-γ by ICS. (D) Serum from naïve (n=11) and vaccinated mice (n=12) was isolated two weeks after boost and CSP specific antibody titres were measured by immunofluorescent antibody assay. (E) Livers from vaccinated mice challenged with *Pb*WT (n=6) or *Pb*CSP^{SIINFEKL} sporozoites (n=5) and non-vaccinated mice challenged with *Pb*WT (n=5) or *Pb*CSP^{SIINFEKL} sporozoites (n=5) were harvested 42 hours post-challenge and relative liver parasite levels were quantified using the ΔΔCt method comparing levels of *P. berghei* 18S rRNA and levels of mouse *GAPDH* mRNA. (F) Groups of vaccinated and non-vaccinated mice (n=6) were challenged with $5x10^3$ *Pb*WT or *Pb*CSP^{SIINFEKL} sporozoites. Vaccinated mice challenged with *Pb*WT (triangles) or *Pb*CSP^{SIINFEKL} (squares) and non-vaccinated mice challenged with *Pb*WT (inverted triangles) or *Pb*CSP^{SIINFEKL} (diamonds) had daily tail smears taken from day 3-14 post challenge. Slides were stained with Giemsa and parasitaemia was assessed by microscopy. (C-E) Each data point represents one mouse with median values + interquartile ranges with statistics calculated using the Mann-Whitney test (*, $p<0.05$; ***, $p<0.001$).

CSP-based vaccines do not elicit sterile immunity in C57BL/6 mice. To further investigate the requirement of SYIPSAEKI as the necessary protective epitope of CSP, mice unable to present this epitope were vaccinated with Ad and MVA expressing *Pb*CSP with an interval of 2 weeks between vaccines, followed by challenge with either *Pb*WT or *Pb*CSP^{SIINFEKL} parasites (Figure 3A). C57BL/6 mice were used because SYIPSAEKI is an H-2-K^{d} restricted epitope, an MHC-I allele which they do not express. Thus, SYIPSAEKI would fail to be presented by infected hepatocytes. As before, blood and serum were derived 2 weeks after boost (Figure 3C,D). As expected, no SYIPSAEKI-specific CD8+ T cells were observed in Ad-MVA *Pb*CSP-vaccinated C57BL/6 mice (Figure 3C), but strong anti-CSP antibody tires (1:10^{4}) were elicited (Figure 3D). As shown in Figure 3E, Ad-MVA CSP-vaccinated C57BL/6 mice challenged with either *Pb*WT or *Pb*CSP^{SIINFEKL} parasites had comparable parasite load in the liver (Figure 3E) and no mice from any groups were sterilely protected (data not shown).

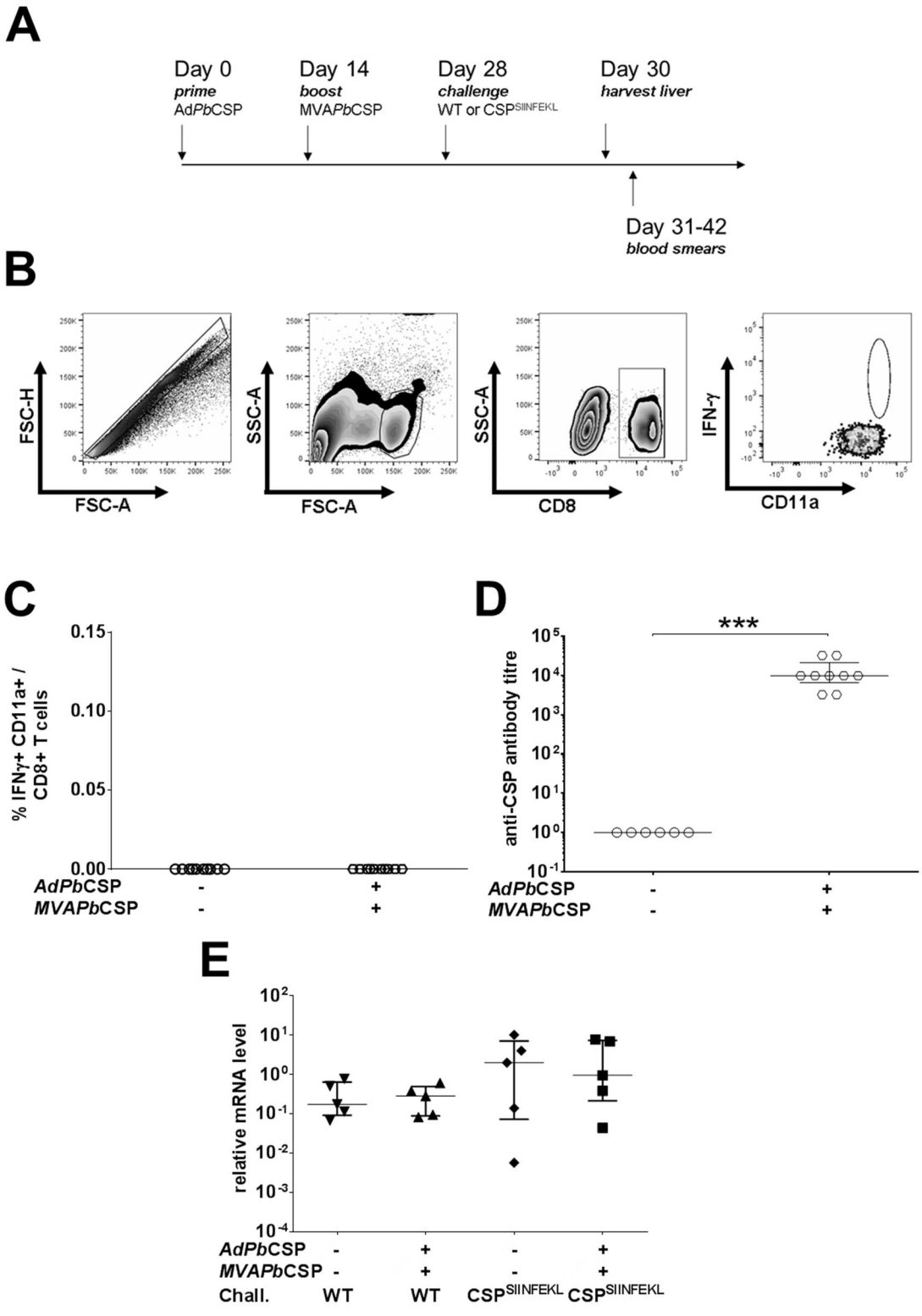

A
Day 0
prime
AdPbCSP
Day 14
boost
MVAPbCSP
Day 28
challenge
WT or CSPSIINFEKL
Day 30
harvest liver
Day 31-42
blood smears
B
FSC-H
FSC-A
SSC-A
FSC-A
SSC-A
CD8
IFN-γ
CD11a
C
% IFNγ+ CD11a+ / CD8+ T cells
0.15
0.10
0.05
0.00
AdPbCSP - +
MVAPbCSP - +
D
***
anti-CSP antibody titre
AdPbCSP - +
MVAPbCSP - +
E
relative mRNA level
AdPbCSP - + - +
MVAPbCSP - + - +
Chall. WT WT CSPSIINFEKL CSPSIINFEKL

FIGURE 3

Prime-boost vaccination with CSP expressing viruses does not protect C57BL/6 mice, irrespective of induced antibody titres. (A) C57BL/6 mice were vaccinated with AdCh63 and MVA vaccines expressing the mammalian codon-optimised fragment of *Pb*CSP and challenged with 10^4 *Pb*WT or *Pb*CSP^{SIINFEKL} sporozoites as shown. (B) The flow cytometry gating strategy used to determine proportions of IFN-γ+ CD11a+ CD8+ T cells. (C) Blood drawn from the tail from naïve (n=10) and vaccinated mice (n=10) two weeks after boost was restimulated with SYIPSAEKI and stained for CD8, CD11a surface markers, and IFN-γ by ICS. (D) Serum from naïve (n=6) and vaccinated mice (n=9) was isolated two weeks after boost and CSP specific antibody titres were measured by immunofluorescent antibody assay. (E) Livers from groups of 5 mice per condition were harvested 42 hours post-challenge and relative liver parasite levels were quantified using the ΔΔCt method comparing levels of *P. berghei* 18S rRNA and levels of mouse *GAPDH* mRNA. (C-E). Each data point represents one mouse with median values + interquartile ranges shown with statistics calculated using the Mann-Whiney test (***$p<0.001$).

Our data further support the notion that CSP is an immunodominant antigen following RAS immunisations in BALB/c mice(20). In addition, a single epitope, SYIPSAEKI is the immunodominant CD8+ T cell epitope of CSP and is responsible for the antigen's protective capacity against parasites in the liver. Following RAS immunisation, CD8+ T cell responses to SYIPSAEKI contribute to the reduction in parasite load in the liver following sporozoite challenge because when RAS-immunised mice are challenged with *Pb*CSP^{SIINFEKL}, transgenic parasites lacking SYIPSAEKI, reduced anti-*Plasmodium* activity in the liver is observed. Nonetheless, complete protection is achievable in the absence of SYIPSAEKI-specific CD8+ T cell responses, demonstrating that responses to other epitopes contribute to parasite-killing. It is conceivable that these epitopes are encoded by the hundreds of other *Plasmodium* genes expressed in malaria pre-erythrocytic stages.

Our findings also emphasise the importance of SYIPSAEKI-specific CD8+ T cell responses for promoting protective immunity when using CSP-based vaccines in BALB/c mice. These vaccines are aimed at generating high levels of epitope-specific CD8+ T cells but rely on the expression of relevant MHC-I in the vaccinated host and the presence of the cognate epitope in the parasite used for challenge. Notably, despite high levels of antibodies elicited following Ad-MVA *PbCSP* vaccination, sterile protection was not achieved following challenge of C57BL/6 mice, which cannot present SYIPSAEKI, nor when challenging BALB/c or C57BL/6 mice with a parasite lacking SYIPSAEKI.

These results have significance for the development of next generation malaria vaccines. We have demonstrated the significance of a single epitope of CSP in mediating protective CD8+ T cell responses while also recapitulating that protection can be achieved in the absence of responses to CSP(20, 21). In BALB/c mice, SYIPSAEKI-specific CD8+ T cell responses offered protection, however, to achieve complete sterile protection: either multiple sporozoite immunisations or viral vaccines, which induced large populations of SYIPSAEKI-specific CD8+ T cells, were required. Multiple

immunisations likely induced a wide range of immune responses and multiple high-dose immunisations with RAS in humans has been shown to induce dose-dependent anti-sporozoite CD8+ T cell responses in addition to dose dependent anti-sporozoite antibody and CD4+ T cell responses(4). In line with this, our findings lead us to suggest that future pre-erythrocytic malaria vaccine research should not only focus on inducing strong CD8+ T cell responses against one or a few antigens but should try to target a broad array of antigens to offer the best protection possible. The identification of novel antigens and epitopes that contribute to protection will aid this development. While RTS,S, the leading subunit malaria vaccine based on CSP, seems to offer some protection against *P. falciparum* re-infection(8) probably by the action of anti-sporozoite antibodies(22-24), peripheral blood CD8+ T cell responses were not identified to provide a role following sporozoite challenge. Similar findings from whole sporozoite vaccination challenge studies(4, 25, 26) show protected individuals exhibit variable peripheral blood CD8+ T cell responses, which may indicate that the protective CD8+ T cells are restricted to the liver. With evidence from mouse(27, 28) and non-human primates(29) studies indicating an association between liver-resident CD8+ T cells and protection, the generation of vaccines that can induce efficacious liver resident CD8+T cell populations that target the parasite in the liver would likely also be advantageous. Whilst it will probably be difficult to directly assess these responses in humans, a population of liver-resident CD8+ T cells with broad antigen specificities will surely be pivotal in contributing to protection against malaria.

MATERIALS AND METHODS

Ethics and animal experimentation. Animal procedures were performed in accordance with the German 'Tierschutzgesetz in der Fassung vom 18. Mai 2006 (BGBl. I S. 1207)' which implements the directive 2010/6 3/EU from the European Union. Animal experiments at London School of Hygiene and Tropical Medicine were conducted under license from the United Kingdom Home Office under the Animals (Scientific Procedures) Act 1986. CD-1 mice were bred in-house at LSHTM while NMRI, C57BL/6 and BALB/c laboratory mouse strains were purchased from either Charles River Laboratories (Margate, UK or Sulzfeld, Germany) or Janvier (Saint Berthevin, France). Female mice of 6-8 weeks of age were used in the experiments.

***Plasmodium* parasites and immunisation.** The transgenic *P. berghei* ANKA $CSP^{SIINFEKL}$ (*Pb*$CSP^{SIINFEKL}$) parasite was generated with the immunodominant CSP CD8+ T cell epitope SYIPSAEKI (252-260aa) being replaced with the H-2-^{b} restricted *Gallus gallus* ovalbumin CD8+ T cell epitope SIINFEKL (258-265aa) via double homologous recombination (Chapter 2: Müller and Gibbins et al., paper in preparation). Wild-type *Plasmodium berghei* ANKA (clone c115cy1) (*Pb*WT) and *Pb*$CSP^{SIINFEKL}$ were maintained by continuous cycling between murine hosts (NMRI or CD-1) and *Anopheles stephensi* mosquitos. Infected mosquitos were kept in incubators (Panasonic and Mytron) at 80% humidity and 20°C temperature. Sporozoites were isolated from the salivary glands and attenuated by γ-irradiation at $1.2x10^4$cGy. Mice were immunised with 10^4 sporozoites administered intravenously with multiple doses given 1 week apart unless otherwise stated. For challenge infections, $5x10^3$ or 10^4 live sporozoites were administered intravenously to assess sterile protection and parasite load in the liver respectively.

Viral-vectored CSP-expressing vaccines. AdCh63 and MVA vaccines expressing the mammalian codon-optimised fragment of *Pb*CSP were constructed and propagated based on previously published viral vectors(30, 31). The viral vectors were administered

intramuscularly in endotoxin-free PBS at a concentration of 10^5 viral particles for Ad*Pb*CSP to prime and 10^6 viral particles MVA*Pb*CSP to boost.

Immunofluorescent antibody assay. 10^4 sporozoites were spotted onto glass slides with marked rings (Medco), dried at room temperature and stored at -20°C. Thawed slides were fixed in acetone, dried and rehydrated with PBS before incubation in 10% FCS supplemented DMEM (Gibco) for 1 hour at 37°C in a humid chamber. Serum at concentrations 1:10^3, 1:3.3x10^3, 1:10^4, 1:3.3x10^4, 1:10^5 (and additionally 1:3.3x10^5 and 1:10^6 for C57BL/6 serum) were added to the ring wells and incubated for 1 hour at 37°C in a humid chamber. Slides were washed and stained with a mouse anti-CSP(32) primary antibody and a respective fluorescently labelled secondary antibody. Nuclei were stained with DAPI or DRAQ5 before a further 1-hour humid incubation. Slides were washed and mounted with 'Fluoromount-G' (Southern Biotech) and analysed by fluorescent microscopy (Zeiss Axio Observer).

Quantification of SYIPSAEKI-specific CD8+ T cell responses. Spleens were harvested and lymphocytes were derived by passing spleens through 40μm cell strainers (Corning). Peripheral blood was drawn from the tail vein and collected in Na^+ heparin capillary tubes (Brand) and assayed in 96-well flat bottom plates (Corning). Red blood cells were lysed using PharmLyse (BD) and lymphocytes were resuspended in 10% FCS, 2% Penicillin-Streptomycin and 1% L-glutamine supplemented RPMI 1640 (Gibco). Splenocytes were counted using a 40x dilution with Trypan Blue (ThermoFisher Scientific) and a Neubauer 'Improved' haemocytometer (Biochrom). 2x10^6 splenocytes and the lysed blood samples were prepared in 96 well plates and incubated with a final concentration of 10μg/ml of SYIPSAEKI peptide in in the presence of Brefeldin A (eBioScience) for 5-6 hours at 37°C and 5% CO_2. For staining of cell surface markers and intracellular cytokines, cells were incubated for 1 hour at 4°C for each staining. Cells were stained for CD8 (53-6.7) and CD11a (M17/4) (eBioscience). Splenic cells were

fixed with 4% paraformaldehyde and peripheral blood cells were fixed with 1% paraformaldehyde before staining for IFN-γ (XMG1.2) (eBioscience). Data was acquired by flow cytometry using an LSRFortessa or LSRII (BD) and analysed using Flowjo9.5.2 (Tree Star, Inc.).

Quantification of parasite load in the liver. Livers were harvested 40-42 hours after sporozoite challenge and total RNA was extracted following homogenisation using TRIzol (ThermoFisher Scientific). cDNA was generated using the RETROScript Kit (Ambion). Quantitative real-time PCR was performed using the StepOnePlus Real-Time PCR System and Power SYBR Green PCR Master Mix (Applied Biosystems). Relative liver parasite levels were quantified using the ΔΔCt method comparing levels of *P. berghei 18S* rRNA using specific primers and normalised to levels of mouse *GAPDH* mRNA(33).

Assessment of parasitaemia. Sterile protection was assessed by daily blood smears, taken from mice 3-14 days post sporozoite challenge, stained with Giemsa's stain improved solution (VWR) to determine the presence of blood stage parasites.

Statistical analysis. Statistical analysis was performed using GraphPad Prism v7 (GraphPad Software, Inc.). Statistics were calculated using the Mann-Whiney U test.

AUTHOR CONTRIBUTIONS

O.S. and J.C.R.H. designed the experiments in the laboratory of K.Matuschewski; O.S. generated the transgenic parasite CSP^{SIINFEKL}; M.P.G., K.Müller, M.G. and J.L. performed experiments and analysed data; K.B. and A.R.-S. generated the CSP-expressing viruses Ad*Pb*CSP and MVA*Pb*CSP; M.P.G. and J.C.R.H. wrote the paper.

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SECTION A – Student Details

Student	Matthew Paul Gibbins
Principal Supervisor	Julius Clemence R. Hafalla
Thesis Title	Dissecting the CD8+ T cell responses to pre-erythrocytic malaria antigens

If the Research Paper has previously been published please complete Section B, if not please move to Section C

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Stage of publication	**Not yet submitted**

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For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	I performed bioinformatic analysis and the immunological experiments and analysed data. I wrote the paper.

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Supervisor Signature: [redacted] Date: 17/09/18

CHAPTER 6

Identification of novel CD8+ T cell epitopes from the pre-erythrocytic stages of malaria

Identification of novel CD8+ T cell epitopes from the pre-erythrocytic stages of malaria

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ABSTRACT

CD8+ T cells are known to be important immune cells in providing protection against the liver stage of malaria. However, the identity of the parasite proteins which induce protective CD8+ T cells remain largely unknown. Using bioinformatics neural networks, which predict peptides that bind strongly to MHC class I molecules, we have identified novel CD8+ T cell epitopes that induce strong IFN-γ responses against proteins expressed in the sporozoite and liver stages following sporozoite immunisation of mice under azithromycin prophylaxis. Feature analysis of these derivative proteins also highlights a link between secreted or cell surface proteins and immunogenicity of antigens expressed in the pre-erythrocytic stages of malaria. A quarter of the induced CD8+ T cell response were found to express an antigen-experienced phenotype following immunisation and up to 20% of these cells produced IFN-γ following restimulation with pools of peptides. We propose that the majority of antigens that induce CD8+ T cell responses against the pre-erythrocytic stages of malaria still remain unknown.

INTRODUCTION

Malaria is a formidable, yet ancient, disease which continues to burden the human population[1]. Today it still causes significant morbidity and mortality, with around 216 million cases per year, resulting in around 445,000 fatalities, with the vast majority of death occurring in sub-Saharan Africa[2].

Efforts to identify efficacious vaccine targets against malaria have been ongoing for many years. First isolated from murine infective *Plasmodium berghei* in the early 1980s, circumsporozoite protein (CSP)[3-5], became the basis of the most advanced malaria vaccine candidate to date, RTS,S/AS01, inspired by the discovery that anti-CSP monoclonal antibodies could protect mice from sporozoite challenge[4, 6]. RTS,S/AS01 is a sub-unit vaccine based on the central repeat and C-terminal regions of CSP conjugated with hepatitis B virus surface antigen. In a multisite Phase III trial in Africa, RTS,S/AS01 showed an efficacy of preventing clinical malaria episodes of 28.3% in children and 18.3% in infants following a 3 dose immunisation, which increases to 36.3% and 25.9% respectively following a booster dose, 18 months after the last dose[7]. However, vaccine efficacy of RTS,S/AS01 wanes over time and does not realise a long lasting and robust effect. Thus, the hunt continues to find better vaccine targets and develop a more efficacious vaccine; a key requirement for malaria eradication.

An alternative vaccination approach showed that rodents[8], primates[9] and humans[10, 11] can be protected from infectious sporozoite following multiple immunisation with irradiated sporozoites. This protection was shown to be mediated by CD8+ T cells, which have been shown to have an essential role in combating liver stage infection in mice[12-14] and primates[15]. However, only a handful of targets of these CD8+ T cells have been identified.

A vaccine that targets the pre-erythrocytic stages of malaria is an attractive prospect because only around 100 sporozoites are injected into the skin by an infected mosquito[16]

and not all of these reach the liver[17, 18] and develop into liver stage exo-erythrocytic forms (EEFs). Blocking this narrow bottleneck in the parasite life cycle has the potential to prevent the subsequent blood stages which cause all the symptoms associated with malarial disease.

Using reverse immunological approaches, we set out to identify novel immunogenic CD8+ T cell epitopes against the pre-erythrocytic stages of malaria using MHC class I-peptide binding prediction algorithms. These algorithms seek to predict the affinity with which peptides bind strongly to MHC binding grooves using experimental binding data. It has been shown that peptides predicted to bind strongly to MHC class I (MHC-I) molecules induce strong T cell responses following interaction with the cognate T cell receptor[19]. Using this general idea, 178 novel immunogenic *Yersinia pestis* CD8+ T cell epitopes[20] and 10 immunogenic *Trypanosoma cruzi* CD8+ T cell epitopes[21] have been identified recently. In this work, we attempted to achieve a similar feat with *Plasmodium*. Previously in our group, Hafalla et al. were able to identify two highly immunogenic CD8+ T cell epitopes by predicting CD8+ T cell epitopes from salivary gland sporozoite and liver stage datasets, genome wide surveying and previously described antigenic *P. falciparum* proteins[22]. Here, we used a similar approach focusing on more recent and extensive proteomic and transcriptomic sporozoite and liver stage expression studies[23-27].

Additionally, the “rules” on what makes an epitope/ antigen immunogenic and potentially protective in a disease setting are not well defined, hindering the rapid discovery of new vaccines. Here we sought to address this and provide supplementary information that could further improve bioinformatics prediction algorithms while further informing immunological fields to immunogenic antigens, vital for vaccine developments. Looking at different pathogens, some protein features have been proposed to induce strong CD8+ T cell responses. Characteristics such as antigen secretion[28] and position within a protein[29] have been suggested to be responsible for enhanced CD8+ T cell responses.

We performed a feature analysis on the proteins in our dataset, determining a wide range of predicted features and characteristics, to look for the enrichment of features that correlated with immunogenicity. Given the paucity of CD8+ T cell epitopes from pre-erythrocytic antigens we also assessed how much of the antigen repertoire of the effector CD8+ T cell population we could account for using peptide pools from our screens.

Using the notoriously hard to protect *Plasmodium* berghei-C57BL/6 model, we report herein novel CD8+ T cell epitopes against the pre-erythrocytic stages of malaria and discuss the idea of features affecting the immunogenicity of antigens.

MATERIALS AND METHODS

Ethics and animal experimentation

Animal procedures were performed either at the Max Planck Institute of Infection Biology, Berlin or the London School of Hygiene and Tropical Medicine. Procedures were carried out following the approval by the institutional ethics review boards. Procedures were carried out in accordance with the German 'Tierschutzgesetz in der Fassung vom 18. Mai 2006 (BGBl. I S. 1207)', or under license from the United Kingdom Home Office under the Animals (Scientific Procedures) Act 1986 both which implement the directive 86/609/EEC from the European Union and the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes. Female C57BL/6 mice and NMRI were purchased from Charles River (Sulzfeld, Germany or Margate, UK) and CD-1 mice were bred in-house at LSHTM.

Parasites

Plasmodium berghei ANKA (clone 507) parasites were continuously cycled between CD-1 or NMRI mice and *Anopheles stephensi* mosquitoes. Adult mosquitos were kept in incubators (Panasonic) at 20°C and 70-80% humidity. Sporozoites were dissected from salivary glands no earlier than 18 days after infection. Mice were immunised intravenously with 10,000 sporozoites concomitantly with azithromycin (Pfizer) given at a dose of 240 mg/kg intraperitoneally on the same day as immunisation and one day after[30]. Mice were immunised with sporozoites twice with immunisations one week apart unless stated otherwise.

CD8+ T cell epitope prediction and feature analysis

The dataset of genes expressed in the sporozoite and liver stages was culminated from published suppression subtractive hybridisation experiments[23, 24], mass spectrometric data[25-27], published[27] and unpublished microarray data (Olivier Silvie, personal communication). Genes derived from experiments using *P. yoelii* and *P. falciparum* were converted to *P. berghei* orthologous genes and protein sequences were downloaded

using PlasmoDB[31]. Sequences were ran through NetMHCpan[32, 33] and NetMHCcons[34] prediction software to predict peptides with strong binding affinity to MHC class I (MHC-I) molecules H-2-D^b and H-2-K^b, the alleles expressed by C57BL/6 mice, using IC50 values and %Rank scores as guides[35]. 586 peptides were chosen and synthesised by Mimotopes (Melbourne, Australia) at batch 'as synthesised' purity for first-pass screening (purity range 4.89-98.6%, mean 62.95%, median 66.62%) and 95 peptides were resynthesised at greater purity (range 69-99%, mean 90.7%, median 94%). When performing NetMHCcons predictions, amino acids N at position P1 of the peptide and C at position P2 were mutated to V and S, respectively. This correction was required to address the lack of experimental data with the NC configuration, which we proposed may overly penalise candidate epitopes starting with the dipeptide NC. The models, servers and algorithms employed for feature analysis are listed in Table 1.

Splenocyte isolation and peptide restimulation

Spleens were harvested 14 days after the last immunisation. Splenocytes were isolated using a 70μm cell strainer (Falcon) and red blood cells lysed using PharmLyse (BD). $2x10^6$ splenocytes in complete RPMI (10% FCS, 2% Penicillin-Streptomycin, 1% L-glutamine (Gibco)) were restimulated with a final peptide concentration of 10μg/ml unless otherwise stated. We assessed the immunogenicity of novel predicted peptides against a panel of 10 published peptides[36] ($TRAP_{130-138}$ – SALLNVDNL[22]; $S20_{318-326}$ – VNYSFLYLF[22], $GAP50_{40-48}$ – SQLLNAKYL[37]; $RPA1_{227-234}$ – EIYIFTNI[38]; $RFC1_{651-658}$ – LLPHFSIL[38]; *$LSG_{119-126}$ – LSGRYNDL[38]; $RNR_{402-409}$ – WGDEFEKL[38]; $ApiAP2_{1898-1905}$ – YYYDYDKI[38]; $BLN_{592-599}$ – IITDFENL[39]; *$NCY_{397-404}$ – NCYDFNNI[40]). Cells were incubated in the presence of 0.6μg Brefeldin A for 5-6 hours at 37°C, 5% CO_2. Cells were stained the following day with extracellular stains for CD3 (clone 500A2) (BD); CD8 (clone 53-6.7) and CD11a (M17/4) (eBioscience), and intracellular stain for IFN-γ (clone XMG1.2) (eBioscience). Cells were fixed using 4% paraformaldehyde. Flow cytometric analysis of samples was acquired using an LSRII or LSRFortessa (BD).

Data and statistical analysis

Flow cytometric data was analysed using FlowJo version 9.5.3 (Tree Star Inc., Oregon, USA). Finite mixture model calculations were performed using Stata 15 (StataCorp). Graphs were produced in GraphPad Prism version 7 (GraphPad Software Inc., CA, USA). Statistical analysis for the feature analysis and boosting was performed using R (Foundation for Statistical Computing, Vienna, Austria). Statistics in the feature analysis were calculated assessed by a 10,000 resample comparison with significance considered as a p-value of <0.05. Statistics for boosted responses was determined by empirical Bayes t-test[41] with Benjamini-Hochberg adjustment to control for false discovery rate below 5%[42].

RESULTS

Novel CD8+ T cell epitopes to *P. berghei* pre-erythrocytic antigens can be identified purely through their MHC-peptide binding affinity

Our study focused on the sporozoite and liver stage forms of *Plasmodium* and we used several published and unpublished proteomic and transcriptomic expression datasets of these life stages to identify new CD8+ T cell epitopes. These included mass spectrometric datasets of *P. yoelii*[26] and *P. falciparum*[25, 26] salivary gland sporozoite proteomes and *P. yoelii* liver stage proteomes[27]. Genes were also chosen from a comprehensive microarray experiment assessing multiple stages of pre-erythrocytic infection in *P. yoelii*[27], an unpublished microarray experiment comparing WT and *slarp* KO transgenic *P. berghei* sporozoites (Olivier Silvie, personal communication), as well as suppression subtractive hybridisation experiments comparing *P. yoelii* salivary gland sporozoites and merozoites[23] and *P. berghei* oocyst sporozoites versus salivary gland sporozoites[24]. Our experimental model used *P. berghei*, a rodent infective species of *Plasmodium*, thus all the genes were transformed using PlasmoDB[31] to generate a degenerate list of *P. berghei* sporozoite and liver stage specific genes.

Our approach involved immunisation of C57BL/6 mice, the most difficult mouse strain to protect from *P. berghei* infection[43], which present antigen to CD8+ T cells in the context of H-2-^{b} MHC-I molecules. Thus the subsequent set of 2976 *P. berghei* antigens were run through MHC-I epitope prediction servers NetMHCpan[32, 33] and NetMHCcons[34], returning peptides with predicted binding affinities to H-2-D^{b} and H-2-K^{b} molecules (Figure 1). We chose peptides that were predicted to bind strongly to H-2-D^{b} and H-2-K^{b} molecules as well as peptides with a lower predicted affinity to test the doctrine that peptides that bind strongly to MHC-I molecules are more likely to be immunogenic than those that do not[19]. We chose predicted strong binders according to their IC50 values and %Rank scores[35]. IC50 values are a measure of predicted affinity of the peptide to the MHC-I molecule in the form of a dissociation concentration at which half of a reference peptide would be displaced by the predicted peptide. %Rank score returns a

% rank of the predicted peptide to a set of 200,000 random natural peptides[35], indicating the relative binding strength of the predicted peptide compared to many others associating with the same MHC-I molecule. Thus, we chose strong binders with IC50 values of less than 50nM and a %Rank score of 0.05 or lower. This corroborates with published CD8+ T cell epitopes against malaria, for example the epitopes from sporozoite antigens thrombospondin related anonymous protein ($TRAP_{130-138}$) and sporozoite-specific gene 20 ($S20_{318-326}$) induce strong CD8+ T cell responses in sporozoite immunised mice[22] with low IC50 values of 3.58nM and 4.88nM respectively, and a %Rank scores of 0.01. In contrast, the *$NCY_{397-404}$ peptide also induces strong CD8+ T cell responses in sporozoite immunised mice[36, 40]; however, the IC50 value and %Rank score are much poorer than one would expect (IC50: 3786.74nM, %Rank score:9). We hypothesize this discrepancy may be due to the paucity of peptides containing cysteine at position 2 in the training sets of NetMHCcons, thus preventing the algorithm to incorporate this preference. Given this, predictions for candidate epitopes starting with the dipeptide NC were repeated by replacing these residues with the more favoured V at P1 and S at P2. When our protein dataset was re-assessed, the top predicted strong binders with an original NC in position 1 and 2 (n=20) or C in position 2 (n=34) were also chosen for synthesis.

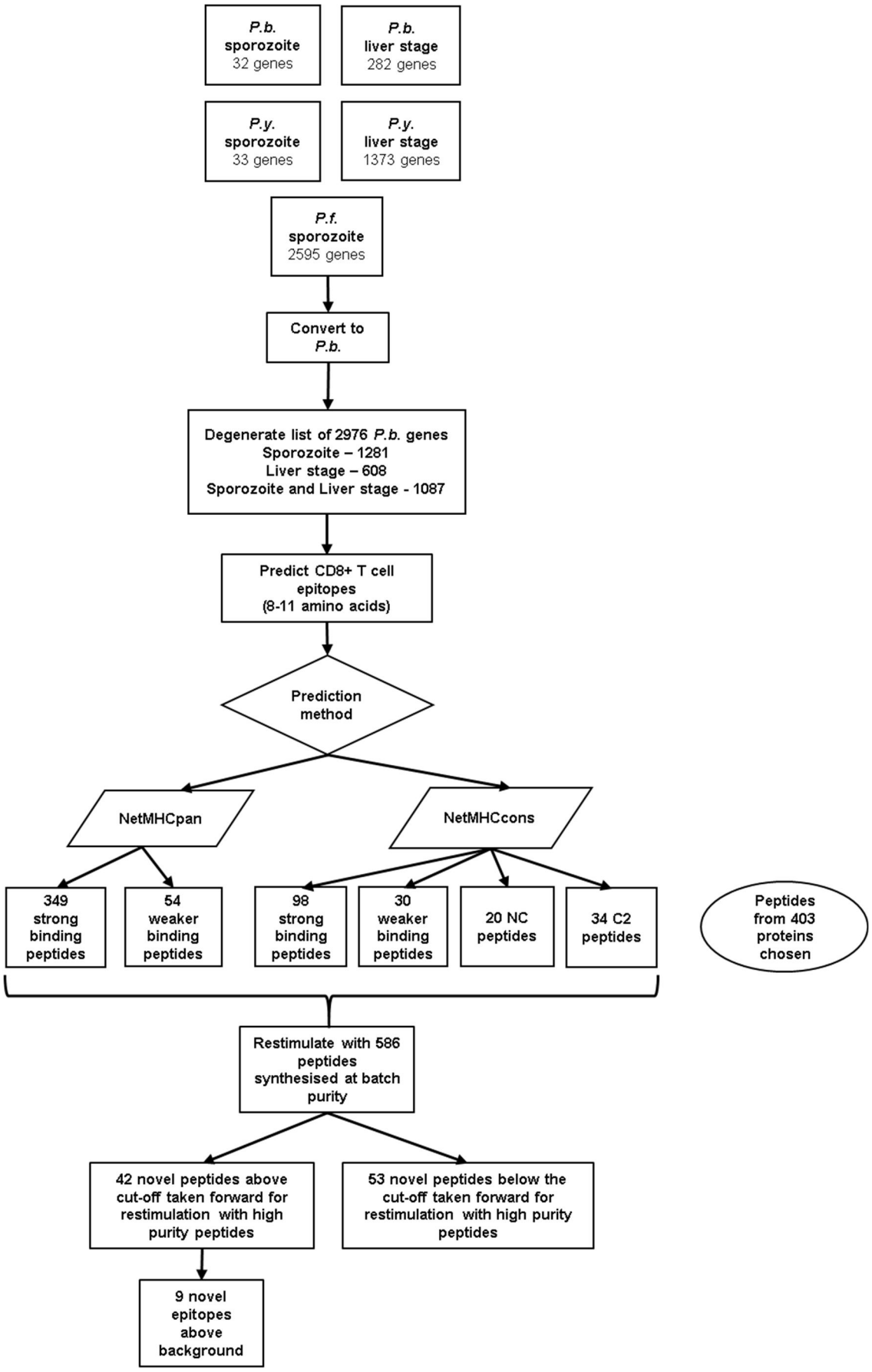
P.b. sporozoite 32 genes
P.b. liver stage 282 genes
P.y. sporozoite 33 genes
P.y. liver stage 1373 genes
P.f. sporozoite 2595 genes
Convert to P.b.
Degenerate list of 2976 P.b. genes
Sporozoite – 1281
Liver stage – 608
Sporozoite and Liver stage - 1087
Predict CD8+ T cell epitopes (8-11 amino acids)
Prediction method
NetMHCpan
NetMHCcons
349 strong binding peptides
54 weaker binding peptides
98 strong binding peptides
30 weaker binding peptides
20 NC peptides
34 C2 peptides
Peptides from 403 proteins chosen
Restimulate with 586 peptides synthesised at batch purity
42 novel peptides above cut-off taken forward for restimulation with high purity peptides
53 novel peptides below the cut-off taken forward for restimulation with high purity peptides
9 novel epitopes above background

Figure 1 – Flow chart for CD8+ T cell epitope discovery

Genes upregulated in the sporozoite and liver stages of *P. berghei*, *P. yoelii* and *P. falciparum* were retrieved and converted into *P. berghei* through PlasmoDB. From the 2976 genes, 586 novel peptides were selected for batch synthesis based on a number of parameters. Using NetMHCpan in the first instance we chose peptides that were predicted to bind strongly to H-2-D^b and H-2-K^b molecules with an IC50 affinity of under 22nM and a %Rank score of 0.05 or below (n=349) and additionally, 54 weaker binding peptides with affinity IC50 values ranging from 22.381-388.44, but always a %Rank score of 0.5 or below. We later used NetMHCcons for prediction of MHC-I epitopes, choosing peptides with an affinity of under 22nM and a %Rank score of 0.05 or below (n=98) and additionally, 30 weaker binding peptides with predicted IC50 values ranging from 23.05-531.38, also with a %Rank score of 0.5 or below. Additionally, with reference to the previously shown immunogenic *$NCY_{397-404}$ peptide[36, 40], which during the project returned a very high IC50 value and %Rank score, NetMHCcons predictions were repeated to consider NC in positions 1 and 2 as not so deleterious for binding by mutating for a more favourable amino acid binding motif, VS. When our dataset was re-assessed by the modified algorithm, the top predicted strong binders with NC in position 1 and 2 (n=20) or C in position 2 (n=34) were also chosen for synthesis. From 586 synthesised peptides, 43 peptides induced IFN-γ responses above a cut-off calculated by finite mixture model based on mean + 3 S.D. of the negative population. These 43 peptides and an extra 52 peptides from throughout the remaining responding peptides were chosen for synthesis at higher purity. 9 peptides induced IFN-γ responses above a cut-off calculated by assessing responses to the irrelevant peptide SIINFEKL, derived from *Gallus gallus* ovalbumin.

Thus, 586 novel peptides from 403 *P. berghei* proteins, were synthesised and tested. Peptides were synthesised for first pass screening and used for *ex vivo* restimulation of splenocytes from mice twice immunised with *P. berghei* sporozoites under azithromycin cover. Azithromycin was used to achieve the greatest repertoire of antigen expression by liver stage parasite in the liver[30], without induction of a blood stage infection, to aid identification of novel liver stage epitopes. Antigen experienced CD8+ T cells, expressing the surrogate marker CD11a[44], were assessed for IFN-γ production by intracellular cytokine staining and flow cytometry (Figure 2A,B). A finite mixture model (FMM) was used to determine which peptides induced positive responses and should be resynthesised at a higher purity. We used an FMM to separate all the 586 peptides into a positive and a negative population based on the IFN-γ responses they induced. Following this, a cut-off was derived, consisting of the mean + 3 S.D. of the negative population. This led to 43 novel peptides rising above this cut-off (Figure 2C and Appendix 1). These peptides we were sure were true positives based on the initial peptide screens. Given that the difference in response of the lowest positive peptides (of the 43 above the cut-off) and those in the negative population was quite small (Figure 2B), we wanted to ensure against false negative assignment. An additional 52 novel peptides were carried forward, drawing peptides at random from throughout the list of negative hits below the cut off, to be re-synthesised and re-tested. Choosing at least the same number of peptides from below the cut-off as positive peptides would also allow us to demonstrate that our experimental strategy of peptide restimulation was reliable and could identify responsive CD8+ T cell epitopes effectively.

During re-screening, the capacity of these 95 novel peptides to stimulate their cognate CD8+ T cells to produce IFN-γ was assessed by comparison with a series of positive controls: CD8+ T cell epitopes from published papers. Previously, only 5 papers had been published which identified 10 novel CD8+ T cell epitopes from *P. berghei* with a H-2-^{b} MHC-I allele restriction[22, 37-40]. Only 2 of these epitopes were identified in the pre-

erythrocytic stage (from sporozoites[22]), while the remainder were discovered in the blood stages[37-40, 45]. However, we show in a recent paper that 4 of the epitopes identified from blood stage studies, also have cross-stage reactivity with pre-erythrocytic stages[36], in corroboration with evidence of cross-stage reactivity against the *$NCY_{397-404}$ epitope[40]. From 95 novel peptides, we identified nine peptides that report consistent production of IFN-γ from antigen specific CD8+ T cells, above background responses using a cut-off based on the response to an irrelevant H-2-K^b restricted peptide, SIINFEKL (Figure 2D and Appendix 1). Interestingly the highest responding epitope is a refinement of the $S20_{318-326}$ epitope[22]. Originally published as a 9mer, the 8mer epitope we describe here lacks a phenylalanine at the C-terminus but induces a higher response than the 9mer. 3 further completely novel epitopes also give convincing comparable responses to those induced by the published $GAP50_{40-48}$[37], $RPA1_{227-234}$[38] and $S20_{318-326}$[22] peptides (Figure 2E). Our data suggests that azithromycin may have aided our identification of novel epitopes from proteins expressed in the liver stage, as six out of the nine highest responders are from proteins expressed during the liver stages[27]. Additionally, we reiterate that trained algorithms, which predict peptides that will bind strongly to MHC-I molecules, are a successful and useful tool in identifying novel CD8+ T cell epitopes.

We also assessed the responses to our novel epitopes, following a single sporozoite immunisation to see if responses were boostable. At an individual level, seven novel epitopes and three published peptides induced higher IFNγ production from CD8+ T cells following a second immunisation, though none survived Benjamini-Hochberg adjustment for false discovery (Figure 3).

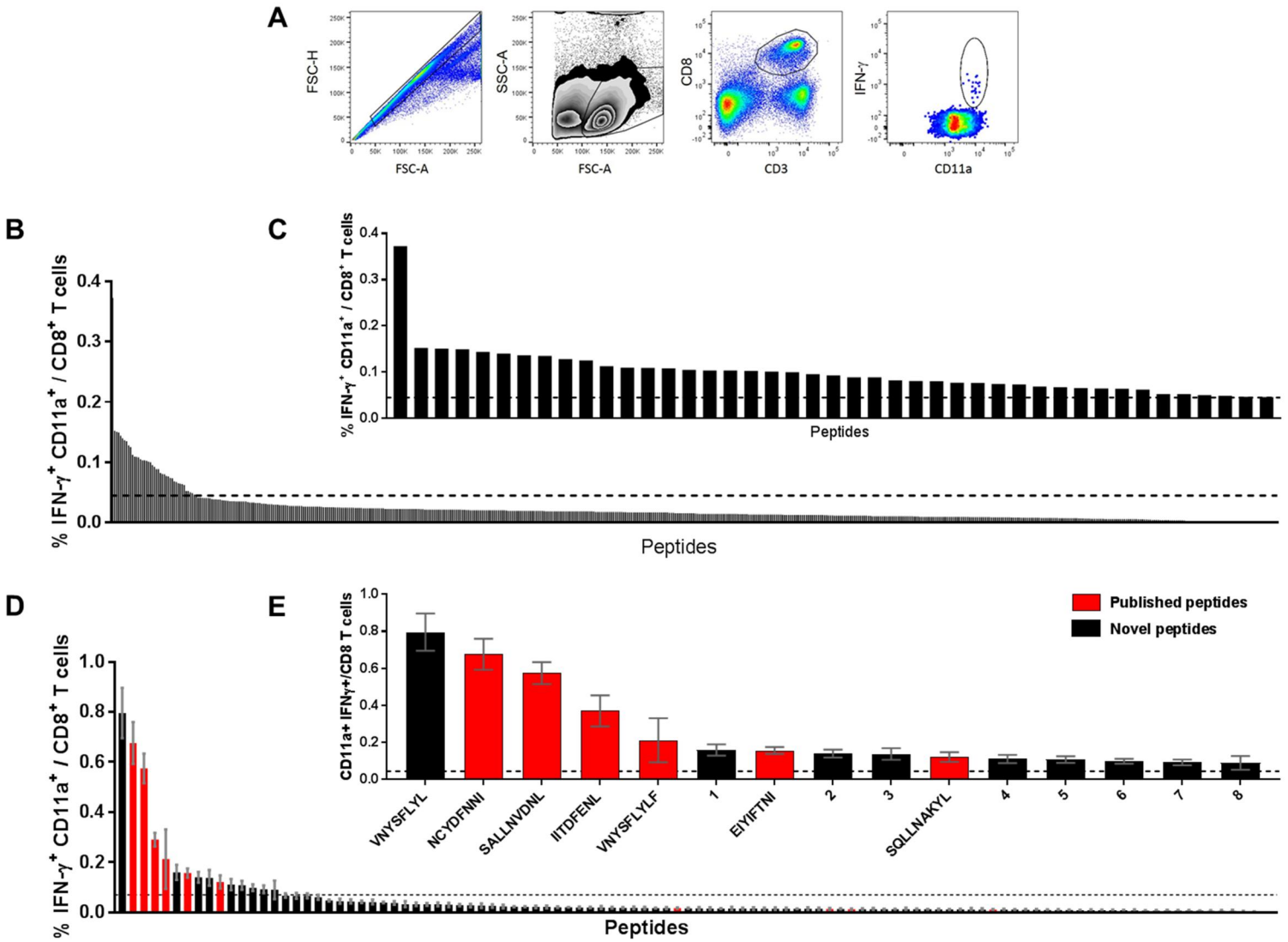
A
FSC-H
FSC-A
SSC-A
FSC-A
CD8
CD3
IFN-γ
CD11a
B
% IFN-γ+ CD11a+ / CD8+ T cells
Peptides
C
% IFN-γ+ CD11a+ / CD8+ T cells
Peptides
D
% IFN-γ+ CD11a+ / CD8+ T cells
Peptides
E
CD11a+ IFN-γ+/CD8 T cells
Published peptides
Novel peptides
VNYSFLYL
NCYDFNNI
SALLNVDNL
IITDFENL
VNYSFLYLF
1
EIYIFTNI
2
3
SQLLNAKYL
4
5
6
7
8

Figure 2 – Novel peptide restimulation of splenocytes from mice immunised with sporozoites under azithromycin prophylaxis

Mice (n=3-6) were immunised twice with *P. berghei* ANKA sporozoites under azithromycin prophylaxis. Splenocytes were harvested 14 days after the last immunisation and pooled. $2x10^6$ pooled splenocytes were restimulated with peptides at a final concentration of 10μg/ml in the presence of Brefeldin A for 5-6 hours. Splenocytes were stained for assessment by flow cytometry to identify CD8+ T cells expressing IFN-γ and CD11a as a marker of antigen experience. (A) Flow cytometry gating strategy used to gate CD8+ T cells. (B) The magnitude of responses induced by 586 novel peptides synthesised at batch (as synthesised) purity level in terms of percentage of IFN-γ+ CD11a+ CD8+ T cells. The dashed line shows the cut-off derived by finite mixture model calculation (mean + 3 S.D. of negative population). Each peptide was assayed in at least two separate experiments. (C) The 42 peptides that induced responses above the cut-off. (D) The magnitude of responses induced by 95 novel peptides (black bars) and published peptides (red bars) synthesised at a purity of >70% batch. The dashed line shows a cut-off defined by using an irrelevant peptide (mean + 3 S.D. of responses induced by SIINFEKL). Results shown are mean results ± SEM for each peptide with each peptide assayed in at least two separate experiments. (E) The top nine novel peptides (black bars) and top six published peptides (red bars) that induced responses above the cut-off (as shown in D).

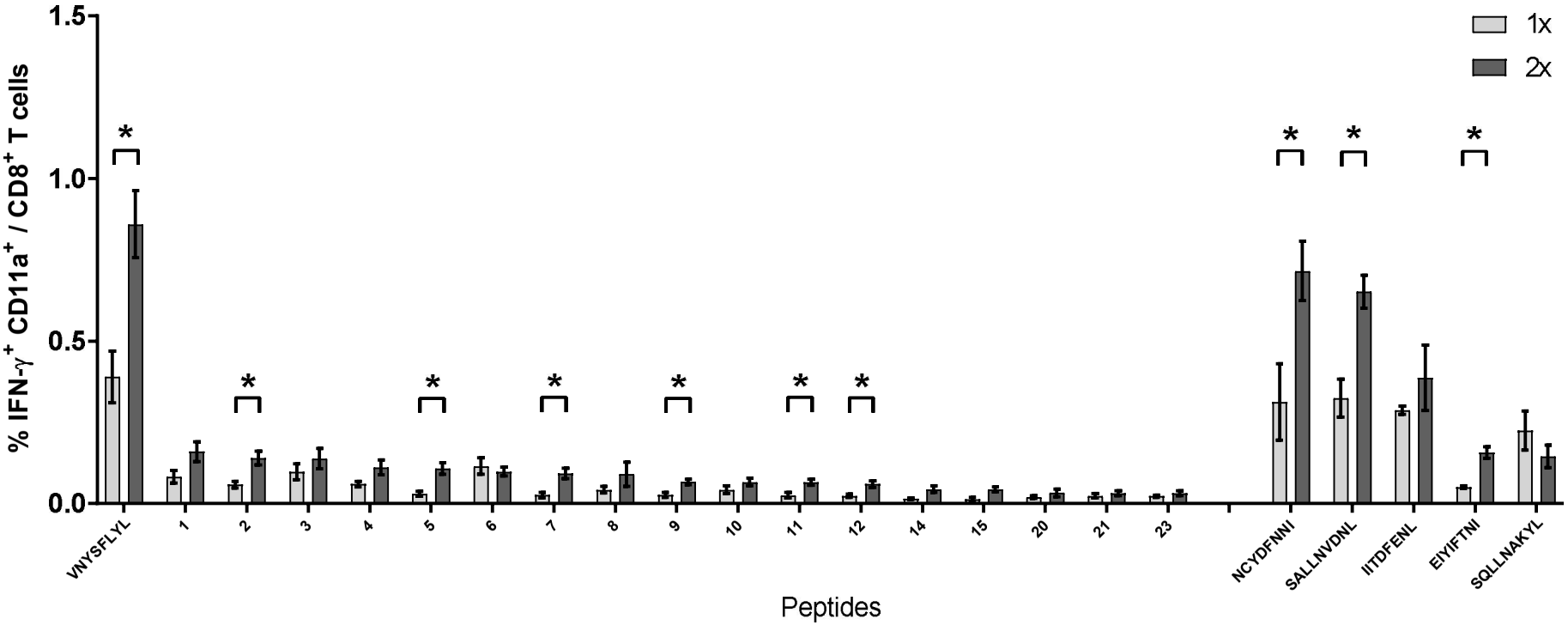
1.5
1.0
0.5
0.0
% IFN-γ+ CD11a+ / CD8+ T cells
1x
2x
*
VNYSFLYL
1
2
3
4
5
6
7
8
9
10
11
12
14
15
20
21
23
NCYDFNNI
SALLNVDNL
IITDFENL
EIYIFTNI
SQLLNAKYL
Peptides

Figure 3 – CD8+ T cell responses to novel epitopes are not significantly boosted by a secondary homologous immunisation

Mice (n=3-6) were immunised once (light grey bars) or twice (dark grey bars) with *P. berghei* ANKA sporozoites under azithromycin prophylaxis. $2x10^6$ pooled splenocytes harvested 14 days after the last immunisation and restimulated with 18 high responding novel peptides and 5 high responding published peptides at a final concentration of 10μg/ml in the presence of Brefeldin A for 5-6 hours. Splenocytes were stained for assessment by flow cytometry to identify the magnitude of CD8+ T cell responses through expression of IFN-γ and CD11a as a marker of antigen experience as before. Results shown are mean results ± SEM for each peptide pooled from at least two experiments. P-values are from unadjusted empirical Bayes t-test[41]; with Benjamini-Hochberg[42] false discovery (5%) no q-values were statistically significant (*, <0.05).

What makes a peptide immunogenic?

To help define the hallmarks of immunogenic CD8+ T cell epitopes, we performed a feature analysis comparing the proteins containing immunogenic epitopes with the remainder of the dataset. 14 different derivative proteins, within which our 15 highest responding epitopes (nine novel and six published) can be found, made up the immunogenic dataset. The features of these 14 proteins were compared to 10,000 groups of 14 proteins selected at random from the remaining 2,857 proteins in the data set. The 105 largest proteins (sizes above 4500aa) were excluded from analysis as some of the prediction software programs introduce errors when sizes exceed this threshold. We assessed for a variety of structural features, functions, subcellular locations and biophysical properties (Table 1).

From this analysis, some features were enriched in our positive cohort (Table 2) and some were depleted (Table 3). Most strikingly, both secretory pathway and signal peptide, and cell envelope and transmembrane helix features appeared enriched, identified by independent prediction methods. This gives power and confidence to the prediction software results and corroborates previous suggestions for associations between secreted proteins, from intracellular non-viral pathogens[28], and enhanced CD8+ T cell responses. However, other theories that had been proposed to improve immunogenicity of CD8+ T cell epitopes were not reflected in this study. In the context of *P. berghei*, we did not find a correlation to the position of immunogenic peptides within a protein and the magnitude of their response as previously described in *Toxoplasma gondii*[29].

We were also unable to find a correlation between antigen expression, using data from PlasmoDB[31], and immunogenicity.

Prediction tool	Classification	Feature
ProtFun-2.2[46, 47]	Functional category	Amino acid biosynthesis
		Biosynthesis of cofactors
		Cell envelope
		Cellular processes
		Central intermediary metabolism
		Energy metabolism
		Fatty acid metabolism
		Purines and pyrimidines
		Regulatory functions
		Replication and transcription
		Translation
		Transport and binding
	Enzyme/non-enzyme	Enzyme
		Non-enzyme
	Enzyme class	Oxidoreductase (EC 1)
		Transferase (EC 2)
		Hydrolase (EC 3)
		Isomerase (EC 4)
		Ligase (EC 5)
		Lyase (EC 6)
	Gene Ontology category	Signal transducer
		Receptor
		Hormone
		Structural protein

ProtFun-2.2[46, 47]	Gene Ontology category	Transporter
		Ion channel
		Voltage-gated ion channel
		Cation channel
		Transcription
		Transcription regulation
		Stress response
		Immune response
		Growth factor
		Metal ion transport
TargetP-1.1[48, 49]	Sub-cellular location	Secretory pathway
		Mitochondrial location
		Other location
TMHMM-2.0[50]	Transmembrane helices in proteins	Transmembrane helix
IUPred[51]	Disorder	Long disorder
		Short disorder
PlasmoDB[31]	Expression data	Expression
In-house script	Hydrophobicity	Hydrophobicity
SignalP-4.1[52]	Signal peptide and cleavage sites	Signal peptide
In-house script	Molecular weight	Molecular weight
In-house script	Isoelectric point	Isoelectric point
NetMHCpan-2.8[32]	MHC binders density	SIR score

Table 1 – Prediction of protein features

The prediction methods used to determine the enrichment and depletion of features in proteins that contain immunogenic CD8+ T cell epitopes.

Enriched features		p-value
Classification	**Feature**	
Functional category	Cell envelope	0.0042
	Transport and binding	0.0082
Sub-cellular location	Secretory pathway	0.0193
Signal peptide and cleavage sites	Signal peptide	0.0160
Transmembrane helices in proteins	Transmembrane helix	0.0474

Table 2 – The enrichment of features in immunogenic proteins

Features enriched in those 14 proteins harbouring the highest immunogenic epitopes from Figure 2E (9 novel peptides and 6 published peptides) compared to 10,000 random sets of 14 proteins from the remaining 2857 proteins in the dataset, including those containing peptides below the cut-off in Figure 2E.

Depleted features		p-value
Classification	**Feature**	
Functional category	Cellular processes	0.0369
	Replication and transcription	0.0424
Gene Ontology category	Voltage-gated ion channel	0.0241
	Immune response	0.0306
Disorder	Short disorder	0.0398

Table 3 - The depletion of features in immunogenic proteins

(A) Features depleted in those 14 proteins harbouring the highest immunogenic epitopes from Figure 2E (9 novel peptides and 6 published peptides) compared to 10,000 random sets of 14 proteins from the remaining 2857 proteins in the dataset, including those containing peptides below the cut-off in Figure 2E.

Accounting for more of the functional CD8+ T cell response

It has been noted that the majority of epitopes that make up the CD8+ T cell repertoire specific to *Plasmodium,* as well as other arms of the adaptive immune system, have yet to be discovered. Doll et al showed that 15% of the antigen-experienced ($CD11a^{hi}$) CD8+ T cell population induced after a single immunisation with radiation attenuated sporozoites produce IFN-γ with specificity for just four epitopes, determined by summing the four individual peptide restimulation responses together[53]. We asked, is it possible to account for more IFN-γ producing effector CD8+ T cells by restimulating with pooled peptides? Using this approach, we investigated whether we could account for antigen-specificity in a greater proportion of antigen-experienced CD8+ T cells induced following immunisation by restimulating with pools of peptides from our screens.,

We restimulated with a pool of 17 novel epitopes, 7 published peptides and a combined pool of 24 peptides. The 17 novel epitopes were chosen for pooling based on their high immunogenicity across several experiments in comparison to the irrelevant H-2-K^b restricted peptide, SIINFEKL. The 7 published peptides included the 6 highest responding published peptides used as controls in this study: $TRAP_{130\text{-}138}$, $S20_{318\text{-}326}$, $GAP50_{40\text{-}48}$, $RPA1_{227\text{-}234}$, $BLN_{592\text{-}599}$, $*NCY_{397\text{-}404}$ as well as the novel S20 8mer peptide identified in this study. A pool of 24 peptides combined these two groups. Splenocytes were restimulated with pools of a final concentration of 2μg/ml/peptide, in addition to restimulation with individual peptides at the same final concentration of 2μg/ml.

Compared to around 4% in naïve mice, around 25% of the total splenic CD8+ T cell population from mice immunised twice with sporozoites with azithromycin prophylaxis, exhibited a $CD11a^{hi}$ phenotype (Figure 4A-C). Comparing the responses to individual peptides, pooling the 7 published peptides restimulated around 20% of this compartment to produce IFN-γ, up from a maximum of nearly 4% IFN-γ production following restimulation with just VNYSFLYL (Figure 4B and 4D). Simply added, the combined

response to these 7 peptides should have been 30%. Pooling the 17 novel epitopes did not massively increase the proportion of IFN-γ+ producing cells accountable compared to individual peptide restimulation. When splenocytes were restimulated with the 24 peptide pool, the proportion of cells producing IFN-γ was reduced (~16%) compared to that seen when restimulating with the 7 published peptide pool. Simply added, the combined response to these 24 peptides should have been 35%.The same trend can be seen when the proportion of IFN-γ+ CD11a+ cells from the total CD8+ T cell population is gated for (Figure 4E and 4F) While, we have assigned antigen-specificity to a greater proportion of the sporozoite immunisation induced CD8+ T cell response than previously noted[53], our methodology suggests there may be a ceiling to the maximum proportion of IFN-γ producing cells that can be visualised using this approach. By pooling peptides in this system, we may be underestimating the proportion of antigen-specific CD8+ T cells that can be determined compared to simplistically summing individual responses.

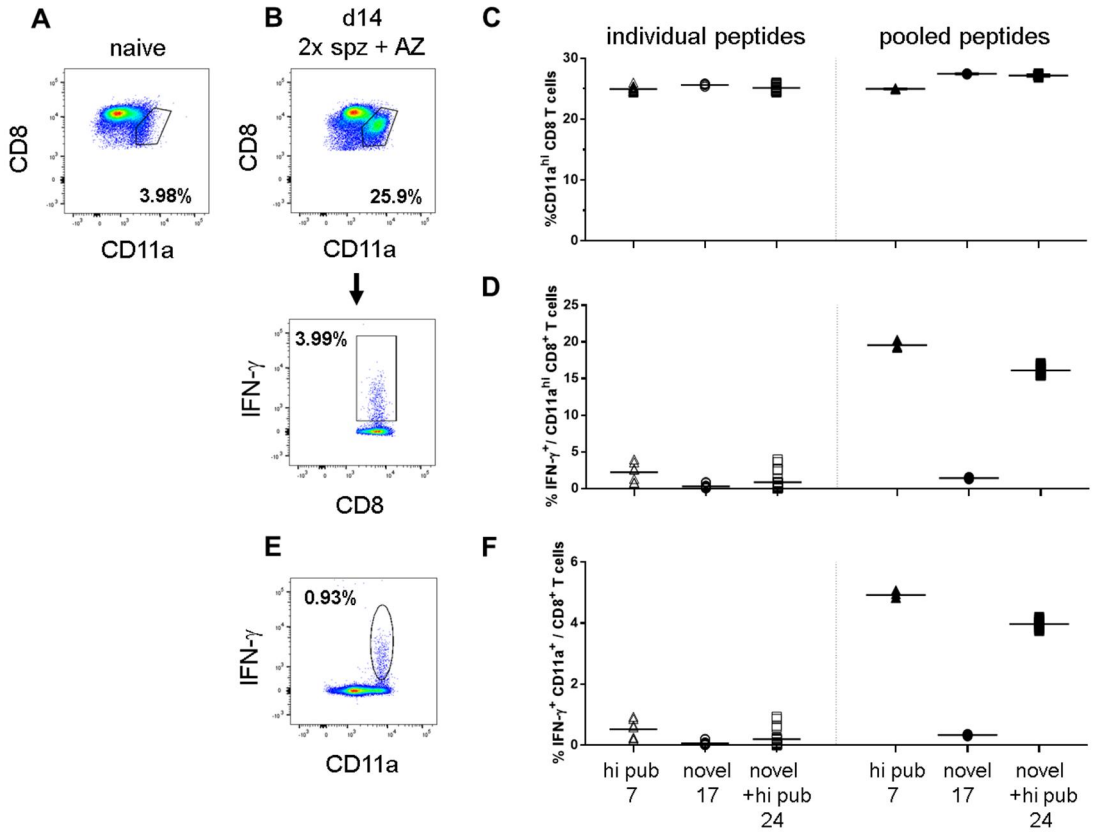
A
naive
CD8
3.98%
CD11a
B
d14
2x spz + AZ
CD8
25.9%
CD11a
3.99%
IFN-γ
CD8
C
individual peptides
pooled peptides
%CD11a^hi CD8 T cells
D
% IFN-γ+ / CD11a^hi CD8+ T cells
E
0.93%
IFN-γ
CD11a
F
% IFN-γ+ CD11a+ / CD8+ T cells
hi pub 7
novel 17
novel +hi pub 24
hi pub 7
novel 17
novel +hi pub 24

Figure 4 – Restimulating splenocytes with a pool of peptides can increase the production of IFN-γ by CD8+ T cells and allow more of the total CD11a^{hi} CD8+ T cell response to be accounted for

Mice were immunised twice with *P. berghei* ANKA sporozoites under azithromycin prophylaxis (n=3-4). 2x10^6 splenocytes harvested 14 days after the last immunisation were restimulated with a pool of 7 high responding published peptides (hi pub 7), 17 high responding novel peptides (novel 17) or a combined pool of 24 peptides (novel + hi pub 7) from both novel and published groups. Each peptide within the pool was at a final concentration of 2μg/ml. Restimulation with individual peptides was also at a concentration of 2μg/ml. (A) Flow cytometric example of the proportion of CD11a^{hi} CD8 T cells from naive mice splenocytes compared with (B) splenocytes from immunised mice and the proportion of IFN-γ producing CD8+ T cells from this population when restimulated with VNYSFLYL. (C, D) The left hand panel of the graphs show the proportion of (C) CD11a^{hi} CD8+ T cells and (D) IFN-γ producing CD11a^{hi} CD8+ T cells induced by individual peptide restimulation, with the right hand panel of the graphs showing the proportions of the same groups of cells following pooled peptide restimulation. (E) Flow cytometric example of the proportion of IFN-γ+ CD11a+ cells gated from total CD8+ T cells. (F) The left hand panel of the graph show the proportion of IFN-γ+ CD11a+ cells gated from CD8+ T cells induced by individual peptide restimulation and the right hand panel of the graphs shows the proportions of the same groups of cells following peptide pool restimulation. (C, D, F) Individual wells stimulated with individual peptides (left panel) or pooled peptides (right panel) are shown as single points as well as mean result. This figure shows results from one representative experiment from three independent experiments.

DISCUSSION

In our study, we have identified nine novel immunogenic CD8+ T cell epitopes, against pre-erythrocytic *P. berghei* proteins, using MHC-I epitope prediction methods. Using rodent models only epitopes from three sporozoite stage antigens (in *P. berghei*[22, 54] and *P. yoelii*[55]) and two liver stage antigens (in *P. yoelii*[56] and very recently in *P. berghei*[57]) have previously been published. Additionally several other CD8+ T cell epitopes had been discovered in the blood stage[37-40], with some being found to have cross-stage reactivity in the pre-erythrocytic stages[36, 40]. Four of the novel epitopes give responses stronger than the recently published $S20_{318-326}$ epitope[22] and $GAP50_{40-48}$ epitope[37]. The epitope with the highest IFN-γ response, even greater than our controls, was a refinement of that same $S20_{318-326}$ epitope[22], with a loss of a phenylalanine from the C-terminus likely allowing more favourable binding to the H-2-K^b MHC-I molecule[58, 59] which may have improved responses. All nine epitopes had an IC50 binding affinity of less than 50nM and a %Rank score of 0.15 or lower, which strengthens the notion that strong binding peptide-MHC interactions produce strong immunogenic responses[19-21]. This further highlights the value of the epitope prediction software used[33, 34] as effective methods of identifying novel CD8+ T cell epitopes. Most pertinently, all nine antigens, which harbour the novel epitopes discovered here, have orthologous genes in nearly all the five human infective species of *Plasmodium* highlighting their relevance in human malaria vaccine research.

In parallel, within our workflow, we also looked into the anomalous immunogenic $*NCY_{397-404}$ peptide. In our initial screenings, we found that this peptide gave a very high IC50 value and %Rank score, suggesting it binds much more weakly than other epitopes we had predicted. However, in the initial paper it was discovered in, this peptide gave strong immunogenic responses against the blood stages of *P. berghei* infection[40] and exhibits cross-stage reactivity with sporozoites[36, 40]. Given the lack of experimental data containing asparagine in position 1 and cysteine in position 2 for MHC-I restricted peptides, NetMHCcons may be biased towards considering these configurations as

deleterious. To address this bias, modified versions of the NC peptides were introduced to predict binding affinity, mutating asparagine to valine and cysteine to serine in positions 1 and 2 respectively. High scoring mutants were selected and their original versions synthesised and assayed for immunogenicity as before. Nonetheless, we were unable to enrich our panel of high responding peptides with NC or C2 peptides. It may not be a true anomaly as six of the other nine published epitopes also exhibit high affinity scores[38, 39], which would have excluded them from our selection criteria of an IC50 of below 50nM and/or %Rank of below 0.5. However, as we progressed in this project the predicted scores for these peptides decreased as the algorithms were retrained with more data from more diverse datasets. Nonetheless, the predicted scores for these peptides remain higher than we would expect for an immunogenic peptide which suggests that this may be a *Plasmodium* specific phenomenon, particularly for cross-stage reactive peptides.

With this in mind, could there be a defining feature of the derivative proteins from which these epitopes arise, which induce such strong CD8+ T cell responses? It became apparent that signal peptides and transmembrane regions were enriched in the positive cohort, further suggesting that secreted proteins are associated with increased antigen presentation and CD8+ T cell responses as previously proposed[28] and corroborating with results from tumour vaccination studies[60, 61] for an association between transmembrane domains and heightened CD8+ T cell responses. For peptides to be loaded onto MHC-I molecules, it is generally considered that antigens must be exposed to the cytosol to be processed by the proteasome. Thus, in the context of malaria liver stages, it is feasible that a parasite protein could be directed away by a signal peptide, transported across the parasitophorous vacuole membrane (PVM) and into the cytosol of the hepatocyte to be presented on an infected hepatocyte. Equally, if the protein were associated with the PVM, a portion of the transmembrane region would be exposed to the hepatocyte cytoplasm, providing an avenue for degradation by the proteasome. However, as can be seen by the large number of negative results compared to our comparatively small

immunogenic cohort, the strength of the suggestions we can make is limited by the size of the dataset.

Nonetheless, processing of pre-erythrocytic proteins is likely very complicated and the role of signal peptides, transmembrane domains and cell envelope association in relation to immunogenicity has not fully been realised, with their presence unlikely to be an absolute property that will result in immunogenic peptides. The mechanism for presentation of *Plasmodium* pre-erythrocytic antigens on infected hepatocytes has still yet to be fully characterised. Equally, we have not determined here whether non-responding peptides are not being presented on the surface of infected hepatocytes following MHC loading or if the derivative proteins fail to reach the cytosol and MHC loading machinery in the first instance. Cockburn et al. elegantly showed that CSP must arrive to the hepatocyte cytosol, and like dendritic cells, TAP1 is required for peptides to be loaded onto MHC molecules[62]. However, they also show that CSP does not have to arrive in the cytosol as a result of its PEXEL motif, a *Plasmodium* specific export signal discovered in blood stages[63, 64], nor does it traffic in an endosome-cytosol translocation manner as was shown to be possible for presentation on dendritic cells[62]. While this suggests a role for secretion in antigen presentation, more work is required to assess how peptides from antigens in the liver stages are presented and how this relates to CD8+ T cell responses. Are antigens from certain subcellular locations in the parasite more favourably presented? This will be important for focused vaccine target discovery to find protective antigens. It has already been shown in *Toxoplasma gondii* that targeting proteins with immunogenic epitopes to dense granules (facilitating increased protein secretion into the parasitophorous vacuole increases the protective cognate CD8+ T cell response[65]. It has also been shown that signal peptide regions from *M. tuberculosis* proteins contain a greater abundance of epitopes making them more inherently immunogenic[66], but we have not tested this concept in the context of *P. berghei*.

Another notion that has been shown in *Toxoplasma gondii* is that making a subdominant CD8+ T cell epitope more C-terminal enhances immunodominance. Feliu et al. showed that the presence of the HF10 epitope from the immunodominant GRA6 protein at the C-terminus of the protein was critical for its protective qualities[29]. Furthermore, appending the subdominant SM9 epitope from GRA4 at this same C-terminal position in GRA6 increased processing, presentation and ultimately protection. They hypothesise this may be due to GRA6 being a vacuolar protein with the C-terminus exposed to the cytosol[29]. Whilst we find an association for cell membrane and transmembrane regions, we however find no correlation with the position of an epitope in the protein and the IFN-γ response it induces in the context of *Plasmodium*. Equally, given the lack of annotation for many *Plasmodium* genes and proteins we cannot further strengthen arguments that vacuolar or vacuolar membrane protein are more immunogenic.

The expression data we have used to identify whether a protein is expressed in the sporozoite, liver stage or both is sparse compared to that available for blood stages of *Plasmodium*. While it may be difficult to draw correlations between immunogenicity and specific life stages, we report that there seems to be no correlation between the level of proteins expressed in the pre-erythrocytic stages and immunogenicity. This, however agrees with previous reports using other systems that also fail to find a correlation between antigen expression levels and CD8+ T cell responses[67], (Chapter 2: Müller and Gibbins et al, paper in preparation).

Part of this study included trying to account for the total IFN-γ producing compartment of the CD8+ T cell population induced in response to *Plasmodium berghei* sporozoite immunisation. Multiple immunisations of C57BL/6 mice with attenuated sporozoites can protect them from subsequent challenge[8] in a CD8+ T cell manner[14] however the entire epitope repertoire with which CD8+ T cells respond to *Plasmodium* is not known. Hence, we attempted to account for an increased proportion of CD8+ T cells capable of

producing IFN-γ by pooling our novel epitopes with previously published epitopes. Around a quarter of the total CD8+ T cell population exhibit a CD11a^{hi} antigen-experienced phenotype following a double homologous immunisation with sporozoites attenuated by azithromycin prophylaxis. We were able to account for 20% of the IFN-γ producing CD8+ T cells of the CD11a^{hi} compartment by restimulating with 6 published peptides and the novel S20 8mer VNYSFLYL. The proportion of cells producing IFN-γ was not increased when we combined 17 novel epitopes into this pool, in fact the proportion decreased. This may be because there are not enough antigen presenting cells in the system, with competition for presentation of peptides and a potential dominance for certain epitopes. Nonetheless, we have been able to show that an increased proportion of *Plasmodium berghei* antigen-specific CD8+ T cells producing IFN-γ can be accounted for by pooling peptides, in excess of that shown previously even without considering the strong responses to CSP[53]. With more antigen presenting cells in the assay, such as a dendritic cell line capable of superior antigen presentation to splenic cells, this may prove a useful method to determine the full repertoire of antigens that induce CD8+ T cell responses following sporozoite immunisation.

Despite the obvious benefit that immunogenic CD8+ T cell epitopes can give as read outs of correlates of protection, the importance of antigen immunogenicity may be overstated, misleading the efforts to characterise vaccine candidates. We showed recently, that SIINFEKL expressed as part of the UIS4 protein, a vacuolar membrane protein expressed soon after sporozoites invasion of hepatocytes[68, 69], is poorly immunogenic compared to SIINFEKL expressed in the context of sporozoite surface protein CSP. Despite this, when mice vaccinated with adenovirus expressing ovalbumin are challenged with sporozoites expressing SIINFEKL in the context of CSP or UIS4, both mice are protected with lower parasite liver loads and comparable high levels of sterile protection (Chapter 2: Müller & Gibbins et al, paper in preparation).

Thus, continued identification of *Plasmodium* antigens expressed in the pre-erythrocytic stages and dissection of the immune responses they induce is required to further inform vaccine research and aid development of an efficacious vaccine against malaria.

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ADDENDUM TO CHAPTER 6: Identification of non-CSP targets of CD8+ T cell responses to malaria pre-erythrocytic stages in a BALB/c model

INTRODUCTION

CSP-specific responses have been the standard in measuring cellular responses to malaria pre-erythrocytic stages in fundamental immunological studies in mice. The responses to the immunodominant CD8+ T cell epitope of *P. berghei* CSP (Chapter 5: Gibbins et al., paper in preparation) have been widely assessed using BALB/c mice as a model. This is because, in contrast to C57BL/6 that cannot present the SYIPSAEKI epitope due to its MHC restriction, BALB/c mice can present SYIPSAEKI on MHC class I H-2-K^d molecules. Here we attempted to uncover CD8+ T cell epitopes from non-CSP pre-erythrocytic antigens from *P. berghei* and *P. yoelii*, presented in the same mouse model where CD8+ T cell responses to SYIPSAEKI are immunodominant. We used an MHC-I prediction algorithm [70, 71] to identify potential epitopes from published and unpublished *P. berghei* and *P. yoelii* sporozoite and liver stage transcriptomic and proteomic data sets [23, 24, 72, 73] and *ex vivo* restimulation of splenocytes from immunised mice to screen the candidate epitopes.

RESULTS AND DISCUSSION

Identification of a novel epitope that induces reproducible CD8+ T cell responses and exhibits *Plasmodium* cross-species reactivity. Using *ex vivo* peptide restimulation of splenocytes from *P. berghei* RAS immunised mice, ICS and flow cytometry (Figure 4A), we identified a novel CD8+ T cell epitope exhibiting subdominant IFN-γ responses above the cut-off (Figure 4B). This epitope, LYIKSINNI, also exhibited cross-reactivity between species with consistent stimulation of IFN-γ production from CD8+ T cells when mice were immunised with *Pb*WT or *Py*WT under azithromycin prophylaxis[30] (Figure 4C). Immunisation with RAS and sporozoites under azithromycin

prophylaxis have been shown to give comparable proportions of antigen experienced CD8+ T cells[74], (Chapter 3: Gibbins et al., paper in preparation). Our novel epitope, LYIKSINNI, derives from the S14 protein, originally identified in *P. yoelii* [23] (PY17X_0608400 aa286-294) with total epitope sequence equivalence to *P. berghei* PBANKA_0605900 (aa247-255). At present this protein is a conserved uncharacterised antigen expressed in the sporozoite[23, 26] and liver stage[27] with an unknown function. Our results independently confirm IFN-γ production by S14-specific CD8+ T cells, which have recently been shown to be induced in BALB/c mice following multiple immunisations with a DNA vaccine based on *P. yoelii* S14 (PY17X_0608400)[75]. DNA vaccination with *P. yoelii* S14 led to a reduction in parasite load in the liver when mice were challenged with *P. yoelii*, with the protection only partially reliant on CD8+ T cells[75]. Additionally, the S14 epitope showed stronger responses compared to another recently published liver-stage antigen, ribosomal protein L3 epitope, discovered in *P. yoelii* [56] (Figure 4C) though we were unable to replicate the strong responses recorded by the authors using ELiSPOT[56] in our assay. In addition, our novel epitope induces responses in mice immunised with *Pb*CSP^{SIINFEKL} (Figure 4C) in the absence of CSP responses. Comparable CD8+ T cell responses to S14 presented here can also be seen when mice are immunised with one RAS inoculum of *Pb*WT or *Pb*CSP^{SIINFEKL} or two RAS immunisations with *Pb*WT, *Pb*CSP^{SIINFEKL} or another transgenic parasite also lacking the SYIPSAEKI epitope of CSP (unpublished and data not shown).

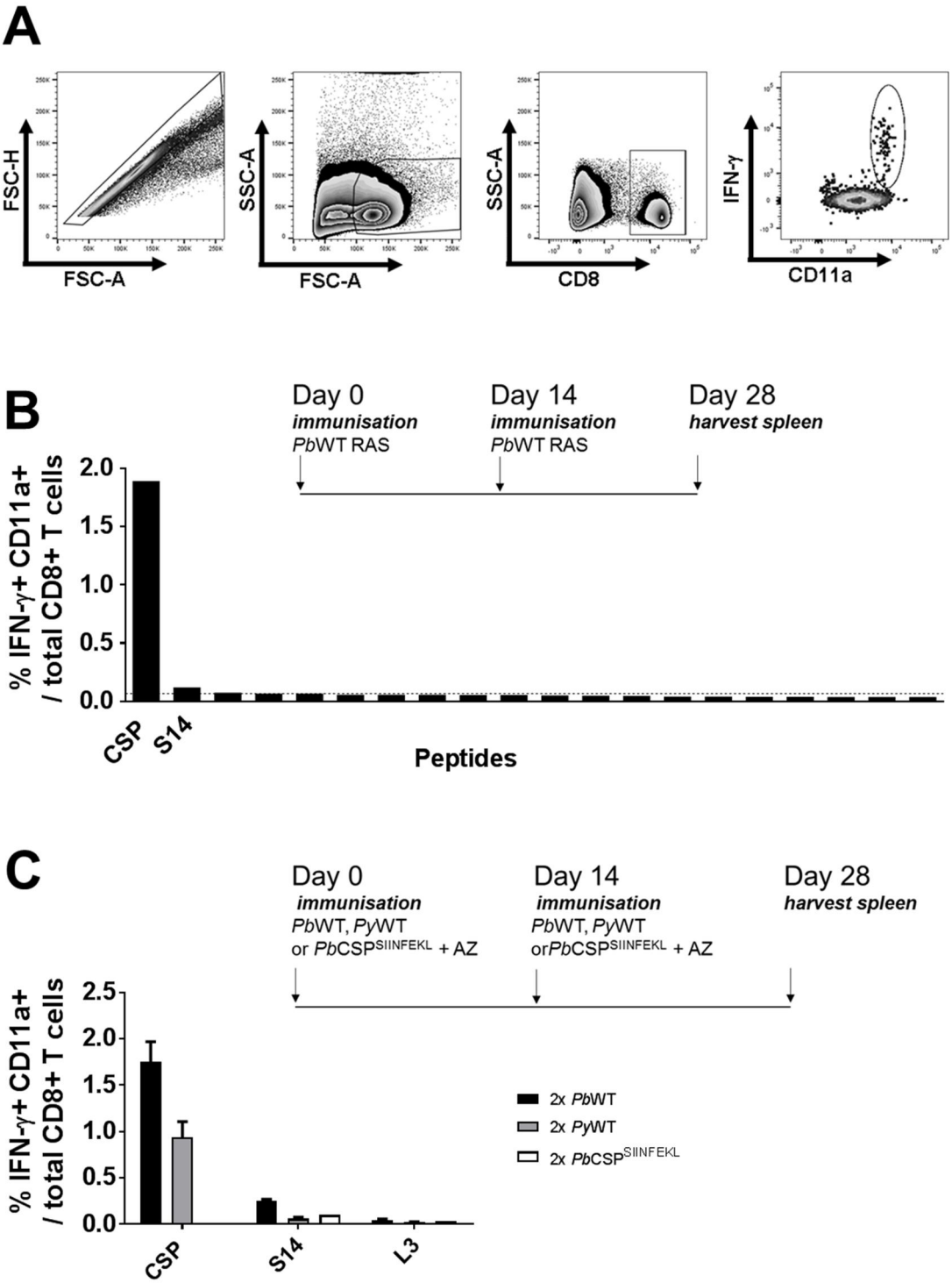
A
FSC-H
FSC-A
SSC-A
FSC-A
SSC-A
CD8
IFN-γ
CD11a
B
Day 0
immunisation
PbWT RAS
Day 14
immunisation
PbWT RAS
Day 28
harvest spleen
% IFN-γ+ CD11a+
/ total CD8+ T cells
2.0
1.5
1.0
0.5
0.0
CSP
S14
Peptides
C
Day 0
immunisation
PbWT, PyWT
or PbCSPSIINFEKL + AZ
Day 14
immunisation
PbWT, PyWT
orPbCSPSIINFEKL + AZ
Day 28
harvest spleen
% IFN-γ+ CD11a+
/ total CD8+ T cells
2.5
2.0
1.5
1.0
0.5
0.0
2x PbWT
2x PyWT
2x PbCSPSIINFEKL
CSP
S14
L3

FIGURE 5

Identification of a novel non-CSP CD8+ T cell epitope. (A) The flow cytometry gating strategy used to determine proportions of IFN-γ+ CD11a+ CD8+ T cells from splenocytes from immunised mice. (B) BALB/c mice (n=4) were twice immunised with *Pb*WT RAS. Spleens were harvested and pooled splenocytes restimulated with a panel of 144 predicted CD8+ T cell epitope peptides. The bar chart shows the magnitude of the responses to the top 20 most responsive peptides, in terms of percentage of IFN-γ+ CD11a+ CD8+ T cells. CSP: SYIPSAEKI; S14: LYIKSINNI. The cut-off is mean + 3 S.D. calculated by a finite mixture model. The data is representative of three separate experiments. (C) BALB/c mice (n=3-5) were immunised twice with *Pb*WT (black bars), *Py*WT (grey bars) or *Pb*CSP^{SIINFEKL} sporozoites (white bars) under azithromycin cover. Spleens were harvested and pooled splenocytes were restimulated with SYIPSAEKI from *Pb*CSP[54], SYVPSAEQI from *Py*CSP[55], LYIKSINNI from S14 and GYKSGMSHI from L3[56]. Data shown is the mean ± SEM from four separate experiments (only one result shown for *Pb*CSP^{SIINFEKL}).

This highlights the identification of a subdominant CD8+ T cell response in a mouse model, where the majority of the response seen is against CSP (Figure 5B-C). These results also highlight the use of transgenic parasites which lack the immunodominant epitope of CSP we describe in this paper. While $CSP^{SIINFEKL}$, which lacks the immunodominant epitope of CSP, has helped confirm the validity of the novel S14 epitope, the immunodominant nature of CSP in this mouse model, may be precluding the ability to identify further epitopes as the response to S14 was not increased in the absence of SYIPSAEKI presentation. The profile of CD8+ T cell responses observed from BALB/c mice certainly contrast with the profile of responses to novel CD8+ T cell epitopes assayed using C57BL/6 mice (Chapter 6: Gibbins et al., paper in preparation). It is possible that the CD8+ T cell response to SYIPSAEKI is swamping the CD8+ T cell response to other antigens. Alternatively, the host genetics of these two mice may play a role in the epitopes that are presented. The use of an appropriate animal models and discovery strategies are crucial to finding more targets against the *Plasmodium* parasite, to best inform vaccine research and push development towards an efficacious malaria vaccine.

ADDITIONAL MATERIALS AND METHODS

Mosquitoes were infected with *Pb*WT, *Pb*$CSP^{SIINFEKL}$ and *P. yoelii* XNL (*Py*WT) parasites and kept in 80% humidity incubators (Panasonic) at 20°C (*Pb*) or 26°C (*Py*). Proteins and genes shown to be upregulated in the pre-erythrocytic stages of malaria from published *P. berghei* and *P. yoelii* sporozoite and liver stage transcriptomic and proteomic datasets [23, 24, 27, 72, 73], orthologs of LSA-1[31, 76] and unpublished data (Alyssa Ingmundson, personal communication) were used to identify novel CD8+ T cell epitopes with sequences acquired from PlasmoDB[31]. 9-mer peptide sequences with a H-2-K^d and H-2-L^d MHC-I restriction were predicted *in silico* using NetMHC[70] and IEDB[71]. 144 peptides with strong predicted binding were synthesised and purchased from Peptides and Elephants (Potsdam, Germany). For peptide screening, mice were immunised twice

intravenously with 10^4 *Pb*WT RAS, two weeks apart. For comparing CD8+ T cell responses between *P. berghei* and *P. yoelii* immunised mice, 10^4 sporozoites were administered intravenously twice (two weeks apart) with azithromycin (Pfizer) given at a dose of 240 mg/kg intraperitoneally on the same day as immunisation and another dose the day after immunisation[30]. Two weeks after the last immunisation, spleens were harvested from immunised mice and 2-4x10^6 splenocytes were restimulated with peptides and assayed for flow cytometry as before.

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CHAPTER 7

Discussion

DISCUSSION

SUMMARY OF FINDINGS

The data presented in this thesis has furthered our understanding of the CD8+ T cell immune responses that are induced against the pre-erythrocytic stages of malaria, unveiling antigen-specific and protective responses, with particular focus on antigens expressed in the EEF. I have shown that an EEF vacuolar membrane protein, UIS4, can protect mice from challenge, following vaccination, despite the low magnitude of antigen-specific CD8+ T cell responses induced following RAS immunisation (Chapter 2). The protection induced is comparable to that targeted against the major sporozoite surface protein, CSP, which has been extensively investigated over the years, in part due to its high immunogenicity. This shows that poorly immunogenic antigens do not necessarily make poor vaccine targets. Additionally, I showed that attenuation of sporozoites using radiation or azithromycin drug prophylaxis did not affect CD8+ T cell responses seen against UIS4 (Chapter 3). Extending the expression of UIS4 prior to arrest did not enhance antigen-specific CD8+ T cell responses. To further probe responses to EEF antigens, I showed that mid-late expressed EEF antigens could offer some protection, following vaccination, despite no observable antigen expression in the first 12 hours of EEF development, and induction of low CD8+ T cell responses following sporozoite immunisation (Chapter 4). This, in conjunction with Chapter 2, highlights the potentiality for use of EEF antigens in pre-erythrocytic vaccines, where, if a large proportion of antigen-specific CD8+ T cells could be induced, then a degree of protection can be achieved. Furthermore, with reference to the protection offered by CSP, I have demonstrated that a single CD8+ T cell epitope of CSP is crucial for this protection (Chapter 5). Despite this, in the absence of this epitope, multiple immunisations with a parasite lacking this CSP epitope can provide complete sterile protection indicating that other antigens contribute to the induction of immune responses. From this, I have established novel pre-erythrocytic antigens that induce CD8+ T cell responses following sporozoite immunisation (Chapter 5 and 6). They were discovered in the context of two

different mouse models, BALB/c and C57BL/6, which, can or cannot, also present the immunodominant epitope of CSP respectively. Using bioinformatics analysis to predict epitopes, I have identified a novel CD8+ T cell epitope presented in BALB/c mice (Chapter 5) and nine CD8+ T cell epitopes presented in C57BL/6 mice (Chapter 6). These will be useful in providing readouts of immunisation status in addition to the small number of currently known CD8+ T cell epitopes, with a forward view to assessing their role in protection.

POTENTIAL FUTURE RESEARCH

Further probing of CD8+ T cell responses to EEF antigens

Shifts in *Plasmodium* gene expression lead to a distinct repertoire of antigens being expressed only during intra-hepatocyte development[1]. Infected hepatocytes have also been shown to be eliminated in a cognate manner; only parasite specific peptides presented on infected hepatocytes by host MHC molecules[2-4] will be destroyed by the corresponding CD8+ T cells. In the context of UIS4, LISP1 and LISP2, I have shown that CD8+ T cell responses to EEF antigens following sporozoite immunisation are poor. The identification of EEF targets has been slow, possibly due to their low immunogenicity. However, we provide a proof of principle that despite their poor CD8+ T cell immunogenicity, EEF antigens can elicit protection, following vaccination and the induction of a large population of antigen-specific CD8+ T cells. This suggests that the induction of protective responses against EEF antigens is no different to that against CSP- a 'numbers game'. It has been shown that induction of a memory anti-CSP CD8+ T cell response, above a threshold of 1% of total peripheral blood lymphocytes, provides long-term protection to mice against sporozoite challenge[5]. The reason for the poor immunogenicity of EEF antigens may revolve around the fact that EEF antigens are only presented on hepatocytes, which have been shown to be poor at priming T cell responses in the tolerogenic liver environment[6, 7]. In contrast, sporozoite surface antigen CSP is presented on hepatocytes as well on dendritic cells in the skin draining lymph nodes, an immune site responsible for CD8+ T cell priming[2].

Nonetheless, as effective presentation can occur, what remains to be determined is the mechanism for EEF antigen processing and presentation on MHC class I molecules. Cockburn et al showed that peptides from CSP are presented in different pathways depending on whether presentation occurs on dendritic cells or hepatocytes[8]. Cockburn et al also showed that presentation on hepatocytes occurs with CSP epitopes bring loaded onto MHC molecules in an endosomal independent, TAP dependent manner; which indicates that the protein must have made it to the hepatocyte cytosol and been processed by the proteasome[8]. In addition, parasites expressing ovalbumin, containing a PEXEL motif and localising at the PVM, has been shown to be presented better than cytosolic residing ovalbumin indicating a link between export signals and presentation[9]. The presentation of endogenous EEF proteins, associated and not associated with the PVM remains to be fully determined, including the mechanisms used for protein translocation across the PVM into the cytosol. By resolving these mechanisms, we will have a better understanding of the processes involved in the presentation of different parasite antigens, which may uncover subsets of antigens that are preferentially presented but, given the tolerogenic nature of the liver, manage to subvert cellular immune responses. If this was the case, as shown in Chapters 2 and 4, then large population of antigen-specific CD8+ T cells could be induced by vaccination to eliminate developing EEFs, with a combination of antigens likely to be necessary to achieve sterile protection. It is important to remember also that the development of human *Plasmodium* in the liver is longer than that of rodent *Plasmodium* and so the induction and contribution of different immune responses will likely be different. In addition, the presentation of proteins in humans will vary depending on MHC allele genotype, which will have an obvious effect on vaccine efficacy.

Research into the mode of parasite killing in the liver has also gained momentum, with the rise of intravital imaging and more advanced microscopy techniques. Several groups have visualised CD8+ T cells clustering around infected hepatocytes[3, 10], with IFN-γ generally considered the central mediator of protection[11] though other effector molecules

may act in concert[12, 13]. With respect to the results I have presented in Chapter 4, one consideration that remains to be resolved is that following vaccination, upon challenge, LISP1 and LISP2 induce a minimal reduction in parasite liver load, yet half of vaccinated mice in a parallel experiment go on to develop sterile protection. As discussed in the chapter, this suggests that the critical window of killing may occur in the final hours of EEF development, possibly because of the delayed activation of memory antigen-specific CD8+ T cells. The delay in parasitaemia in those mice that are not protected also suggests that there is considerable killing of EEFs, but there may just not be enough time to kill all the infected hepatocytes. This may be overcome by inducing even greater numbers of antigen-specific CD8+ T cells. Nonetheless, visualisation of EEF killing inside the livers of sporozoite challenged vaccinated mice, at several time points up to merozoite release, would be pertinent to fully determine the time at which antigen presentation on infected hepatocytes must occur for sufficient memory CD8+ T cells to be activated and protect. The later protein expression is turned on in the parasite, the later the protein could possibly be processed in the hepatocyte cytosol, leading to later MHC-peptide presentation and signalling to CD8+ T cells to mount a liver-wide response. In addition, it would be useful to generate a parasite from the same parent line as LISP1^{SIINFEKL} and LISP2^{SIINFEKL}; which expresses mCherry and SIINFEKL tagged UIS4 for better comparison of the degree of killing targeted against UIS4, LISP1 and LISP2 as UIS4^{SIINFEKL} described in Chapter 2 does not express GFP. By intravital imaging, targeted EEFs were shown to exhibit blebbing and a loss of GFP signal[10] with parasite death occurring after around four hours of interaction with CD8+ T cells[10]. Generating a timeline of EEF killing in response to early and later expressed EEF antigens could probably also be determined from stained liver slices from challenged vaccinated mice. However, using intravital imaging would reduce the number of mice required, whilst providing opportunities for more advanced *in vivo* analysis of *Plasmodium* infection.

Intravital microscopy has not been completely developed in humans yet[14], but if possible, ethical considerations for assessing EEF development in human challenge studies would

still need to be resolved. Yet developments in chimeric mouse models have started to allow the determination of the immune responses that occur during *P. falciparum* infection[15]. In terms of assessing the immune responses to *P. falciparum* in the liver, chimeric mice engrafted with human hepatocytes[16, 17] or hepatocytes and erythrocytes[18] have been shown to allow complete *P. falciparum* EEF development. Chimeric mouse models, which have an engrafted human immune system[19, 20], have allowed physiologically relevant antibody and CD8+ T cell responses to *P. falciparum* CSP to be determined when mice were infected with rodent *P. berghei* which had its orthologous CSP partially or fully replaced with *P. falciparum* CSP[20, 21]. Whilst a humanised mouse model which contains both human immune system and liver tissues exists[22], the contribution of mouse immune cells of the myeloid lineage cannot be discounted as they are not fully replaced in this model[15]. Alas, with contribution from hepatic dendritic cells[23, 24] and type I IFN effects on myeloid cells[25] during *Plasmodium* infection, a single one-stop-shop chimeric mouse that can fully capture representative human immune responses to *P. falciparum* in the liver has yet to be developed but would certainly be of benefit.

Further CD8+ T cell epitope discovery

The identification of novel CD8+ T cell epitopes seems to be skewed by host MHC class I (MHC-I) restriction. Two bioinformatics analyses were performed, predicting *P. berghei* peptides presented in two different mouse strains. The addendum of Chapter 5 predicted and assayed peptides with H-2-^{d} restriction as encoded by BALB/c mice, while Chapter 6 predicted and assessed peptides with H-2-^{b} restriction as encoded in C57BL/6 mice. Only one novel epitope was discovered with an MHC-I H-2-^{d} restriction, while nine novel epitopes were discovered with an MHC-I H-2-^{b} restriction. While the size of the datasets varied in terms of peptides assayed experimentally (144 vs 586), the profile of responses induced by *P. berghei* epitopes with a H-2-^{d} restriction compared to MHC-I H-2-^{b} restriction was striking. In BALB/c mice, there is a large response to the immunodominant epitope of CSP, with only a few, much weaker responses to other antigens seen. In

C57BL/6 mice, CD8+ T cell responses to the immunodominant epitope of CSP cannot be determined due to its H-2-^{b} genotype. In contrast, CD8+ T cell responses observed in C57BL/6 do not exhibit such an obvious immunodominance against one epitope. It would be interesting to know if this immunodominance is purely directed by the MHC-I molecules BALB/c express or whether host genetics play a role in anti-*Plasmodium* CD8+ T cell responses. To test this, responses in mice with a C57BL/6 background but H-2-^{d} haplotype could be assessed. If a difference in the protection mediated by SYIPSAEKI-specific CD8+ T cells is observed, then this suggests that host factors besides MHC haplotype affect immune responses against Plasmodium infection. It has been noted that different mice exhibit different protective immune responses following sporozoite immunisation[12] and that the effector mechanisms employed by memory CD8+ T cells differs depending on the parasite species and background strains of the immunised mice[13]. In addition, host genetic factors relating to susceptibility to liver infection, have been characterised though quantitative trait loci studies using different mouse strains[26, 27]. Host cell factors such as heme oxygenase[28, 29], SR-BI[28, 30] and CD81[31] have also been proposed to be involved in resistance of mice to *Plasmodium* liver infection. Nonetheless more research is required to deconvolute how host genetic factors contribute to resistance and immunity to *Plasmodium* infection[32].

With respect to identifying more EEF-specific CD8+ T cell responses, moving forward an alternative approach may be required. In principle we have shown that with UIS4, LISP1 and LISP2, poorly immunogenic antigens can offer protective immunity if the antigen is presented and the cognate CD8 T cell response is large enough. However, not using immunogenicity as an indicator for protective capability does not make it easier to identify potentially protective EEF antigens. In fact, our analysis of the protein features contained in the derivative proteins that contain the most highly immunogenic peptides did not return any striking results (Chapter 6). Apart from an enrichment for signal peptide regions and transmembrane structures, suggesting secreted and surface bound proteins are more immunogenic, we could not deduce any significant characteristic that made a

pre-erythrocytic *Plasmodium* protein particularly good at expanding antigen-specific CD8+ T cells following processing and presentation.

As we were concerned with identifying novel CD8+ T cell epitopes that could be used as an indicator of immunised state with the forward view to identifying systemic correlates of protection in murine and human infections, we focused on identifying those antigens and epitopes that induced large expansions of CD8+ T cells. However, it has been shown that liver tissue-resident CD8+ T cells (T_{RM}) are crucial cells for mediating protection from sporozoite challenge[33-37]. Whilst, screening of peptides by *ex vivo* restimulation of liver infiltrating lymphocytes would have reduced the number of peptides that could have been tested, it may have identified prominent antigen-specific CD8+ T cell populations in the liver. An alternative approach to CD8+ T cell epitope discovery thus could involve the isolation of CD8+ T cells from livers of immunised mice, particularly those that exhibit protection from sporozoite challenge. Dissociation of peptides from MHC molecules presented on CD8+ T_{RM} cells may shed some light on those antigens that can induce effective intra-hepatic CD8+ T cell responses. Generation of transgenic parasites, including chimeric rodent parasites expressing human *Plasmodium* proteins[38] for testing in human immune system engrafted mice as seen already[20, 21], would allow the degree of protection that these proteins can induce to be assessed. This information would be useful in informing subunit vaccine design to develop vaccines that can improve these populations of CD8+ T cells.

IMPACT ON ERADICATION OF MALARIA

The research I have presented here has clear implications for the development of next generation malaria vaccines. The majority of *P. falciparum* subunit malaria vaccines, that are currently in clinical trials target sporozoite antigens[39] with only one vaccine targeting EEF antigens, liver stage antigen 1(LSA1) and liver stage associated protein 2 (LSAP2)[40]. This may be due to the relative ease of characterising responses to

sporozoite antigens, as they are not restricted to presentation on hepatocytes, which we know exhibit poor T cell priming, likely not aided by the tolerogenic nature of the liver[6, 7]. In addition, the amount of transcriptomic and proteomic data available detailing the genetic expression repertoire during EEF development is poor compared to other stages of the life cycle[1, 41-43]. Thus, this has resulted in a distinct lack of data detailing antigen-specific immune responses targeting the EEF compared to those targeting the sporozoite or blood stages. However, data is starting to accumulate to determine expression differences between replicating EEFs and dormant hypnozoites in *P vivax* and other relapsing malarias[44-47], with the dawn of *in vivo* and *in vitro* systems[48] which may herald the onset of more human *Plasmodium* EEF expression datasets. Yet, here I have presented data that indicates that EEF antigens can offer protection against the pre-erythrocytic stages of malaria and that poor immunogenicity does not necessarily negate an antigen from being a poor vaccine target.

Vaccines that induce antigen-specific antibodies have been shown to protect individuals against many diseases[49] and the vast majority of vaccines currently licensed, mediate protection by inducing high titres of pathogen-specific antibody titres[50]. More recently, it has now been shown that memory CD8+ T cells above a certain threshold can protect against the pre-erythrocytic stages of malaria[5, 51]. Research has now started to focus on the development of vaccines that induce strong cellular immune responses[52] as the induction of effective memory CD8+ T cell responses will be a crucial arm in next generation malaria vaccines. The notion of hepatic CD8+ T_{RM} cells and protection is also really gaining traction with many papers released since the original indication showing that they are crucial for protection against sporozoite challenge in animal models[33-37]. While not investigated here, memory CD8+ T cell responses and protection against all new antigens should be considered in the context of these cells[53]. In addition, development of vaccines that induce hepatic CD8+ T_{RM} responses by liver centric vaccination regime[33, 37] are a very exciting, promising concept in the onward movement to generating a truly efficacious human malaria vaccine.

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APPENDIX 1

Inventory of novel H-2-^{b} restricted CD8+ T cell epitopes and supplementary information

Novel Peptide number	Peptide sequence	Novel peptides carried forward from batch purity -> high purity (black=top43, grey=other52)	Published control peptides	Top NetMHCpan peptides <22nM and <0.05 Rank %	NetMHCpan other peptides 22.381-388.44nM but always %Rank of 0.5 or below	Top NetMHCcons NC and C2	Top NetMHCcons peptides <22nM and <0.05 Rank %	NetMHCcons other peptides 23.05nM-531.38nM but always %Rank of 0.5 or below	Gene ID	Protein	Batch purity restimulation % IFN-γ+ CD11a+ / total CD8+ T cells	High purity restimulation % IFN-γ+ CD11a+ / total CD8+ T cells
	VNYSFLYL								PBANKA_1429200	conserved Plasmodium protein, unknown function	0.3725	0.795625
	NCYDFNNI								PBANKA_0714500	conserved Plasmodium protein, unknown function		0.67625
	SALLNVDNL								PBANKA_1349800	thrombospondin-related anonymous protein		0.57436
	IITDFENL								PBANKA_1137000	bergheilysin		0.370833333
	VNYSFLYLF								PBANKA_1429200	conserved Plasmodium protein, unknown function		0.212555556
1	FSIFNEFEI										0.046333333	0.16025
	EIYIFTNI								PBANKA_0416600	replication protein A1, small fragment		0.157
2	YSLANMIDTI										0.0625	0.140625
3	ISPDFYNNL										0.053	0.138125
	SQLLNAKYL								PBANKA_0819000	glideosome-associated protein 50, putative		0.121923077
4	ITFHWYPSYL										0.053	0.111625
5	YAYYNTYVL										0.0655	0.1085
6	VNYDFTYINLL										0.089	0.098375
7	YALKNVSYL										0.0805	0.0925
8	YSFLNVDNI										0.1	0.090205
9	TSMSNNIYI										0.1255	0.067375
10	FALNNFNYF										0.10525	0.06625
11	FAIYNLNNL										0.06875	0.066125
12	FSISNMDDF										0.14	0.060625
13	FSLTNNEVFL										0.11275	0.0475
14	STVSNYDVI										0.06425	0.04475
15	YALSNISAI										0.10275	0.044
16	VSYYFEYL										0.037	0.0425
17	YIIMNWTTI										0.0415	0.0415
18	DNYNFVGL										0.017	0.03975
19	VAYAFEII										0.022	0.03775
20	MAYVNSKYI										0.041	0.03307125
21	NSINNLDFI										0.10925	0.0325
22	YMHTNIYTI										0.13675	0.032
23	ANYFHFFQNYL										0.049	0.03183125
24	SNYSYIYFVFL										0.039	0.03025
25	IVYVFLHI										0.024	0.029
26	FAASNFNLDLL										0.1015	0.0275
27	VSFNYNNL										0.02	0.0270025
28	ISYSYYYL										0.021165	0.026
29	FAIYNLNNLSM										0.0735	0.0238775
30	RSIINNVAL										0.096	0.02275
31	IILYFFQL										0.024	0.0225
32	VAYKYYTYL										0.047	0.022
33	VSYDYYLAL										0.029	0.021805
34	MSFMNLKYLLL										0.03275	0.02075
35	SSYIFSIL										0.00952	0.0205
36	FIYFKYNYL										0.0405	0.01975
37	QNYYSFTNL										0.015	0.01975

Novel Peptide number	Peptide sequence	Signal peptide predicted	No. of transmembrane domains predicted	Protein length	Peptide length	Peptide start position	Percentile of protein	NetMHCcons 1.1 data				NetMHCpan 4.0 data				Expression data				
								MHC allele restriction	1-log50k	nM	%Rank score	MHC allele restriction	1-log50k	nM	%Rank score	**TARUN 2008 EEF** Mass Spec (LS40-50h)	**LINDNER 2013 Spz** Mass Spec	**LASONDER 2008 spz** Mass Spec	**LASONDER 2008 Spz** (only day 18-22) Mass Spec	**LINDNER 2013 Spz** Mass Spec
																+/-	+/-	+/-	+/-	+/-
																orthologs of *P. yoelii*		orthologs of *P. falciparum*		
	VNYSFLYL	no	0	583	8	317	54.37393	Kb	0.854	4.88	0.01	Kb	0.9211	2.3472	0.0044	-	+	+	+	+
	NCYDFNNI	no	1	745	8	396	53.15436	Kb	0.238	3786.7	9	Kb	0.3864	764.103	1.4628	-	-	-	-	-
	SALLNVDNL	yes	1	606	9	129	21.28713	Db	0.882	3.58	0.01	Db	0.8572	4.6876	0.0043	-	+	+	+	+
	IITDFENL	no	0	1149	8	591	51.43603	Kb	0.531	159.89	0.8	Kb	0.5658	109.749	0.2824	+	+	+	+	-
	VNYSFLYLF	no	0	583	9	317	54.37393	Kb	0.746	15.53	0.03	Kb	0.7121	22.5433	0.0548	-	+	+	+	+
1	FSIFNEFEI	no	3	4291	9	2850	66.41808	Db	0.821	6.97	0.01	Db	0.8256	6.602	0.0053	-	-	-	-	-
	EIYIFTNI	no	0	486	8	198	40.74074	Kb	0.568	107.14	0.4	Kb	0.6389	49.7716	0.1214	+	+	+	+	+
2	YSLANMIDTI	no	0	348	10	210	60.34483	Db	0.828	6.47	0.01	Db	0.8284	6.4043	0.0052	-	-	-	-	-
3	ISPDFYNNL	yes	0	1406	9	1121	79.72973	Kb	0.766	12.51	0.03	Kb	0.8378	5.7846	0.009	+	-	+	-	-
	SQLLNAKYL	yes	2	395	9	39	9.873418	Db	0.704	24.47	0.05	Db	0.6878	29.3083	0.0094	+	+	+	+	+
4	ITFHWYPSYL	yes	2	1349	10	627	46.47887	Kb	0.711	22.93	0.05	Kb	0.7652	12.6835	0.0256	+	-	-	-	+
5	YAYYNTYVL	yes	2	246	9	233	94.71545	Db	0.849	5.15	0.01	Db	0.841	5.586	0.0048	-	-	-	-	-
6	VNYDFTYINLL	no	11	500	11	455	91	Kb	0.654	42.25	0.15	Kb	0.6943	27.3265	0.0695	+	+	+	-	+
7	YALKNVSYL	no	0	1524	9	795	52.16535	Db	0.913	2.58	0.01	Db	0.8942	3.1415	0.0032	-	-	-	-	-
8	YSFLNVDNI	no	4	3245	9	448	13.80586	Db	0.871	4.06	0.01	Db	0.846	5.2896	0.0046	-	-	-	-	+
9	TSMSNNIYI	yes	0	721	9	65	9.015257	Db	0.815	7.4	0.01	Db	0.8033	8.3973	0.0059	-	-	+	+	-
10	FALNNFNYF	no	0	656	9	56	8.536585	Db	0.77	12.04	0.03	Db	0.7757	11.3217	0.0068	-	+	-	-	+
11	FAIYNLNNL	yes	2	1349	9	1037	76.87176	Db	0.807	8.03	0.01	Db	0.8065	8.1158	0.0058	+	-	-	-	+
12	FSISNMDDF	no	0	971	9	815	83.93409	Db	0.774	11.6	0.03	Db	0.6421	48.0466	0.013	-	+	-	-	+
13	FSLTNNEVFL	no	8	4154	10	3491	84.03948	Db	0.784	10.41	0.03	Db	0.6982	26.1897	0.0091	+	-	-	-	+
14	STVSNYDVI	no	0	950	9	923	97.15789	Db	0.83	6.29	0.01	Db	0.7974	8.9549	0.0061	+	+	-	-	+
15	YALSNISAI	no	0	441	9	184	41.72336	Db	0.914	2.54	0.01	Db	0.8694	4.1067	0.0039	+	-	-	-	-
16	VSYYFEYL	yes	11	1245	8	1199	96.30522	Kb	0.852	4.96	0.01	Kb	0.9226	2.3096	0.0043	-	-	-	-	+
17	YIIMNWTTI	no	6	2724	9	882	32.37885	Db	0.857	4.72	0.01	Db	0.8024	8.4835	0.006	-	-	-	-	-
18	DNYNFVGL	no	0	192	8	50	26.04167	Kb	0.624	58.45	0.2	Kb	0.5833	90.7555	0.2347	+	+	+	-	+
19	VAYAFEII	no	0	194	8	82	42.26804	Kb	0.668	36.12	0.1	Kb	0.7365	17.3072	0.0381	+	+	+	+	+
20	MAYVNSKYI	yes	0	1406	9	1010	71.83499	Db	0.714	22.19	0.05	Db	0.7217	20.3183	0.0084	+	-	+	-	-
21	NSINNLDFI	no	1	865	9	676	78.15029	Db	0.869	4.13	0.01	Db	0.8411	5.5782	0.0048	+	-	-	-	-
22	YMHTNIYTI	yes	0	721	9	295	40.9154	Db	0.875	3.85	0.01	Db	0.7794	10.8791	0.0067	-	-	+	+	-
23	ANYFHFFQNYL	no	4	4204	11	3921	93.26832	Kb	0.73	18.67	0.05	Kb	0.7097	23.1316	0.0563	-	+	-	-	+
24	SNYSYIYFVFL	no	0	147	11	33	22.44898	Kb	0.677	32.77	0.1	Kb	0.7119	22.5826	0.0549	-	-	-	-	-
25	IVYVFLHI	yes	1	2096	8	11	0.524809	Kb	0.752	14.55	0.03	Kb	0.8092	7.8778	0.0122	-	-	-	-	+
26	FAASNFNLDLL	no	0	5176	11	3749	72.43045	Db	0.656	41.12	0.07	Db	0.5839	90.2414	0.0265	+	+	-	-	+
27	VSFNYNNL	no	0	2966	8	422	14.22792	Kb	0.826	6.57	0.01	Kb	0.9185	2.4149	0.0045	-	-	-	-	+
28	ISYSYYYL	no	0	1203	8	746	62.01164	Kb	0.875	3.85	0.01	Kb	0.937	1.9772	0.0035	-	-	-	-	-
29	FAIYNLNNLSM	yes	2	1349	11	1037	76.87176	Db	0.733	17.97	0.05	Db	0.6473	45.4509	0.0124	+	-	-	-	+
30	RSIINNVAL	no	0	993	9	865	87.10977	Db	0.772	11.72	0.03	Db	0.7356	17.4782	0.008	+	+	+	+	-
31	IILYFFQL	yes	0	1796	8	1788	99.55457	Kb	0.726	19.39	0.05	Kb	0.7591	13.5475	0.0279	-	+	-	-	+
32	VAYKYYTYL	no	0	1285	9	407	31.67315	Kb	0.872	3.99	0.01	Kb	0.9314	2.0996	0.0038	-	-	-	-	-
33	VSYDYYLAL	no	0	486	9	475	97.73663	Kb	0.863	4.43	0.01	Kb	0.919	2.4011	0.0045	-	-	-	-	-
34	MSFMNLKYLLL	yes	0	3254	11	1671	51.35218	Kb	0.625	57.51	0.2	Kb	0.5717	102.973	0.2662	+	-	-	-	-
35	SSYIFSIL	no	9	736	8	464	63.04348	Kb	0.835	5.99	0.01	Kb	0.9057	2.7727	0.0052	-	+	+	-	+
36	FIYFKYNYL	yes	1	1096	9	494	45.07299	Kb	0.785	10.24	0.01	Kb	0.7347	17.6521	0.0389	-	-	-	-	-
37	QNYYSFTNL	yes	0	1864	9	419	22.47854	Kb	0.723	20.03	0.05	Kb	0.7922	9.4727	0.0163	-	+	+	+	+

Novel Peptide number	Peptide sequence	Expression data					
		Microarray		SSH		Microarray	
		TARUN 2008 spz	**Tarun 2008 EEF**	**UIS genes Matuschewski 2002** (oocyst spz v sg spz)	**S genes Kaiser 2004** (sg spz v merozoites)	**DSK hi** (highly downregulated in SLARP KO) **Silvie, unpublished**	**DSK lo** (lowly downregulated in SLARP KO) **Silvie, unpublished**
		+/-	+/-	+/-	+/-	+/-	+/-
		orthologs of *P. yoelii*				*P. berghei* origin	
	VNYSFLYL	-	-	-	+	-	-
	NCYDFNNI	-	-	-	-	-	-
	SALLNVDNL	+	-	-	+	-	-
	IITDFENL	+	+	-	-	-	-
	VNYSFLYLF	-	-	-	+	-	-
1	FSIFNEFEI	-	-	-	-	+	+
	EIYIFTNI	-	-	-	-	+	-
2	YSLANMIDTI	+	+	-	-	-	-
3	ISPDFYNNL	-	-	-	-	-	-
	SQLLNAKYL	-	-	-	-	-	-
4	ITFHWYPSYL	-	-	-	-	-	-
5	YAYYNTYVL	-	+	-	-	-	-
6	VNYDFTYINLL	-	-	-	-	+	-
7	YALKNVSYL	-	+	-	-	-	-
8	YSFLNVDNI	-	-	-	-	-	-
9	TSMSNNIYI	-	-	-	-	+	+
10	FALNNFNYF	+	-	-	-	-	-
11	FAIYNLNNL	-	-	-	-	-	-
12	FSISNMDDF	-	+	-	-	-	-
13	FSLTNNEVFL	-	-	-	-	-	-
14	STVSNYDVI	-	-	-	-	-	-
15	YALSNISAI	-	-	-	-	-	-
16	VSYYFEYL	-	-	-	-	-	-
17	YIIMNWTTI	+	-	+	-	-	-
18	DNYNFVGL	-	-	-	-	-	-
19	VAYAFEII	-	+	-	-	-	-
20	MAYVNSKYI	-	-	-	-	-	-
21	NSINNLDFI	-	-	-	-	-	-
22	YMHTNIYTI	-	-	-	-	+	+
23	ANYFHFFQNYL	-	+	-	-	-	-
24	SNYSYIYFVFL	-	+	-	-	-	+
25	IVYVFLHI	-	-	-	-	-	-
26	FAASNFNLDLL	-	-	-	-	-	-
27	VSFNYNNL	-	-	-	-	-	-
28	ISYSYYYL	-	-	-	-	-	+
29	FAIYNLNNLSM	-	-	-	-	-	-
30	RSIINNVAL	-	-	-	-	-	-
31	IILYFFQL	+	-	-	-	-	-
32	VAYKYYTYL	+	+	-	-	-	-
33	VSYDYYLAL	-	+	-	-	-	-
34	MSFMNLKYLLL	-	-	-	-	-	-
35	SSYIFSIL	-	-	-	-	-	-
36	FIYFKYNYL	-	+	-	-	-	-
37	QNYYSFTNL	+	-	-	-	-	-

Novel Peptide number	Peptide sequence	Novel peptides carried forward from batch purity -> high purity (black=top43, grey=other52)	Published control peptides	Top NetMHCpan peptides <22nM and <0.05 Rank %	NetMHCpan other peptides 22.381-388.44nM but always %Rank of 0.5 or below	Top NetMHCcons NC and C2	Top NetMHCcons peptides <22nM and <0.05 Rank %	NetMHCcons other peptides 23.05nM-531.38nM but always %Rank of 0.5 or below	Gene ID	Protein	Batch purity restimulation % IFN-γ+ CD11a+ / total CD8+ T cells	High purity restimulation % IFN-γ+ CD11a+ / total CD8+ T cells
38	VNYFLHSGHL										0.033	0.01957
39	ASFHNQTYI										0.1035	0.019
40	VSYFYFLDL										0.0825	0.0183075
41	TNYAHYLSI										0.025	0.01769
42	LSLVNETTI										0.076666667	0.0173725
43	FKNMNILEL										0.026	0.01725
44	YALMNDNNSVL										0.1525	0.017
	LSGRYNDL								PBANKA_1337300	conserved Plasmodium protein, unknown function		0.016828
45	KSMSNGSFL										0.0675	0.0165
46	LSYNNTLDYF										0.014	0.0159225
47	YAIKKKDEI										0.00715	0.01578
48	ICFEHYQLF										0.065	0.0155
49	SSISQNEIVI										0.00978	0.0155
50	IVYRFRKL										0.01	0.01525
51	FAINNNEHL										0.151	0.0151775
52	FSSCNDTLEL										0.012	0.0149575
53	ISFAGFNAL										0.036	0.01481
54	ASLENVETI										0.13525	0.0146675
55	HAHANYAFL										0.0745	0.01425
56	KTMNFYGM										0.015	0.01425
57	NSVNNINYI										0.021	0.0142325
	YYYDYDKI								PBANKA_0521700	transcription factor with AP2 domain(s), putative		0.014082
58	VCIYYFDLL										0.019	0.0139175
	WGDEFEKL								PBANKA_0611600	ribonucleoside-diphosphate reductase large subunit, putative		0.013147
59	KNYFHFFNM										0.01879	0.01308
60	YSLNNTHVL										0.1035	0.013
61	STFLYYLL										0.02	0.01279
62	YAYRNGLYF										0.1085	0.0127725
63	RTFYYFHGLL										0.031	0.0127225
64	NCYIYNYV										0.035	0.01256
65	YALRNFTLF										0.14425	0.01256
66	FSLINHSVI										0.019	0.01212
67	IAVLNSLYL										0.09275	0.0117975
68	ITYLNSINI										0.00733	0.0117675
69	AAINNIEFV										0.1095	0.0115325
70	AAVNNLFTI										0.081	0.01148
	LLPHFSIL								PBANKA_0316000	replication factor C subunit 1, putative		0.011395
71	VGMRHLNL										0.016	0.0111075
72	VSYFYFLDLL										0.0505	0.0110425
73	YAVRNTRYL										0.019	0.01075
74	YALFNGNLI										0.12825	0.010235
75	YNMFYYTIL										0.02	0.0097425
76	STYSFMSL										0.0316	0.00962
77	YAIGNNDIAL										0.1495	0.00947

Novel Peptide number	Peptide sequence	Signal peptide predicted	No. of transmembrane domains predicted	Protein length	Peptide length	Peptide start position	Percentile of protein	NetMHCcons 1.1 data				NetMHCpan 4.0 data				Expression data				
								MHC allele restriction	1-log50k	nM	%Rank score	MHC allele restriction	1-log50k	nM	%Rank score	**TARUN 2008 EEF** Mass Spec (LS40-50h)	**LINDNER 2013 Spz** Mass Spec	**LASONDER 2008 spz** Mass Spec	**LASONDER 2008 Spz** (only day 18-22) Mass Spec	**LINDNER 2013 Spz** Mass Spec
																+/-	+/-	+/-	+/-	+/-
																orthologs of *P. yoelii*		orthologs of *P. falciparum*		
38	VNYFLHSGHL	yes	0	668	10	369	55.23952	Kb	0.707	23.81	0.05	Kb	0.7319	18.1791	0.0404	+	+	+	+	+
39	ASFHNQTYI	yes	0	2997	9	1060	35.3687	Db	0.824	6.71	0.01	Db	0.7936	9.3281	0.0062	+	+	-	-	+
40	VSYFYFLDL	no	2	7480	9	2002	26.76471	Kb	0.818	7.2	0.01	Kb	0.8928	3.1888	0.0059	-	+	-	-	+
41	TNYAHYLSI	yes	2	1671	9	315	18.85099	Kb	0.716	21.6	0.05	Kb	0.8283	6.41	0.0095	-	-	-	-	+
42	LSLVNETTI	no	0	1757	9	1720	97.89414	Db	0.855	4.83	0.01	Db	0.7878	9.9291	0.0064	+	-	-	-	-
43	FKNMNILEL	no	0	255	9	70	27.45098	Db	0.429	482.07	0.4	Db	0.3628	987.103	0.2364	-	+	-	-	-
44	YALMNDNNSVL	no	2	2599	11	1783	68.60331	Db	0.657	40.9	0.07	Db	0.7014	25.311	0.009	-	-	-	-	+
	LSGRYNDL	yes	1	329	8	118	35.86626	Kb	0.333	1369.5	4	Kb	0.3226	1524.09	2.5409	-	+	-	-	-
45	KSMSNGSFL	no	0	5309	9	4271	80.4483	Db	0.782	10.52	0.03	Db	0.7949	9.1969	0.0062	+	+	-	-	+
46	LSYNNTLDYF	no	0	174	10	149	85.63218	Db	0.425	500.68	0.4	Db	0.3977	676.649	0.1709	-	+	-	-	-
47	YAIKKKDEI	no	0	194	9	177	91.23711	Db	0.488	255.99	0.25	Db	0.5549	123.457	0.035	+	+	+	+	+
48	ICFEHYQLF	no	0	3183	9	1805	56.70751	Kb	0.639	49.43	0.17	Kb	0.6407	48.7704	0.1196	+	+	-	-	+
49	SSISQNEIVI	no	0	104	10	12	11.53846	Db	0.495	234.76	0.2	Db	0.5955	79.5671	0.0234	+	+	+	+	+
50	IVYRFRKL	no	9	543	8	175	32.22836	Kb	0.83	6.33	0.01	Kb	0.898	3.0161	0.0056	-	+	-	-	+
51	FAINNNEHL	no	0	1613	9	434	26.90639	Db	0.807	8.07	0.01	Db	0.8087	7.9231	0.0058	+	-	-	-	-
52	FSSCNDTLEL	no	0	130	10	11	8.461538	Db	0.424	508.87	0.4	Db	0.4073	609.8	0.1534	+	+	+	+	+
53	ISFAGFNAL	yes	0	473	9	254	53.69979	Kb	0.695	27.26	0.07	Kb	0.7839	10.3658	0.0193	+	+	-	-	+
54	ASLENVETI	yes	0	554	9	195	35.19856	Db	0.889	3.32	0.01	Db	0.8595	4.5711	0.0042	-	-	-	-	-
55	HAHANYAFL	yes	0	1303	9	156	11.97237	Db	0.845	5.38	0.01	Db	0.7825	10.525	0.0066	+	+	-	-	+
56	KTMNFYGM	no	0	288	8	71	24.65278	Kb	0.614	65.13	0.25	Kb	0.7061	24.0399	0.0586	+	+	+	+	+
57	NSVNNINYI	yes	4	1785	9	687	38.48739	Db	0.722	20.24	0.05	Db	0.7233	19.9651	0.0083	-	-	-	-	+
	YYYDYDKI	no	0	2775	8	1956	70.48649	Kb	0.264	2858.2	7	Kb	0.3161	1634.72	2.7053	-	+	+	+	+
58	VCIYYFDLL	no	3	4291	9	1096	25.54183	Kb	0.603	73.76	0.3	Kb	0.6025	73.7649	0.187	-	-	-	-	-
	WGDEFEKL	no	0	847	8	401	47.34357	Kb	0.145	10471	32	Kb	0.1564	9206.28	12.8923	+	-	-	-	+
59	KNYFHFFNM	no	2	1585	9	935	58.99054	Kb	0.781	10.69	0.01	Kb	0.8497	5.0865	0.0083	-	-	-	-	-
60	YSLNNTHVL	no	0	2225	9	1829	82.20225	Db	0.831	6.26	0.01	Db	0.8156	7.3513	0.0056	-	+	-	-	+
61	STFLYYLL	no	2	957	8	549	57.36677	Kb	0.83	6.33	0.01	Kb	0.8686	4.1441	0.0073	-	-	-	-	-
62	YAYRNGLYF	yes	2	991	9	171	17.2553	Db	0.744	16.04	0.05	Db	0.7612	13.243	0.0072	+	-	+	+	-
63	RTFYYFHGLL	yes	2	1349	10	832	61.67532	Kb	0.676	33.12	0.1	Kb	0.7185	21.0266	0.0507	+	-	-	-	+
64	NCYIYNYV	no	2	1530	8	115	7.51634	Kb	0.311	1718.9	5	Kb	0.3701	911.712	1.6804	-	+	-	-	-
65	YALRNFTLF	no	11	1935	9	1497	77.36434	Db	0.842	5.53	0.01	Db	0.7852	10.2158	0.0065	+	-	-	-	-
66	FSLINHSVI	no	0	7263	9	3236	44.55459	Db	0.879	3.68	0.01	Db	0.8487	5.1418	0.0046	-	-	-	-	+
67	IAVLNSLYL	no	0	4287	9	3519	82.08537	Db	0.808	7.94	0.01	Db	0.7972	8.9735	0.0061	-	-	+	-	-
68	ITYLNSINI	no	0	261	9	229	87.73946	Db	0.477	288.34	0.25	Db	0.409	598.77	0.1503	+	-	-	-	-
69	AAINNIEFV	no	0	1149	9	479	41.68842	Db	0.867	4.24	0.01	Db	0.8333	6.0707	0.005	+	+	+	+	-
70	AAVNNLFTI	no	12	491	9	88	17.92261	Db	0.85	5.07	0.01	Db	0.8294	6.335	0.0051	+	+	+	-	+
	LLPHFSIL	no	0	861	8	650	75.49361	Kb	0.386	771.82	3	Kb	0.4523	374.756	0.8159	-	+	-	-	+
71	VGMRHLNL	no	0	148	8	55	37.16216	Kb	0.684	30.38	0.07	Kb	0.7246	19.6741	0.0461	+	+	+	+	+
72	VSYFYFLDLL	no	2	7480	10	2002	26.76471	Kb	0.76	13.35	0.03	Kb	0.786	10.1258	0.0185	-	+	-	-	+
73	YAVRNTRYL	yes	1	2096	9	1326	63.26336	Db	0.839	5.71	0.01	Db	0.8206	6.9694	0.0054	-	-	-	-	+
74	YALFNGNLI	yes	0	604	9	100	16.55629	Db	0.85	5.07	0.01	Db	0.8469	5.2433	0.0046	+	+	-	-	+
75	YNMFYYTIL	yes	1	2096	9	898	42.84351	Kb	0.721	20.47	0.05	Kb	0.7958	9.1127	0.0151	-	-	-	-	+
76	STYSFMSL	yes	2	175	8	91	52	Kb	0.855	4.8	0.01	Kb	0.9077	2.7136	0.0051	-	-	-	-	+
77	YAIGNNDIAL	no	0	8895	10	448	5.036537	Db	0.746	15.61	0.05	Db	0.6766	33.0909	0.0098	-	+	-	-	+

Novel Peptide number	Peptide sequence	Expression data					
		Microarray		SSH		Microarray	
		TARUN 2008 spz	**Tarun 2008 EEF**	**UIS genes Matuschewski 2002** (oocyst spz v sg spz)	**S genes Kaiser 2004** (sg spz v merozoites)	**DSK hi** (highly downregulated in SLARP KO) **Silvie, unpublished**	**DSK lo** (lowly downregulated in SLARP KO) **Silvie, unpublished**
		+/-	+/-	+/-	+/-	+/-	+/-
		orthologs of *P. yoelii*				*P. berghei* origin	
38	VNYFLHSGHL	-	-	-	-	-	-
39	ASFHNQTYI	-	+	-	-	-	-
40	VSYFYFLDL	-	-	-	-	-	+
41	TNYAHYLSI	-	-	-	-	-	-
42	LSLVNETTI	-	-	-	-	-	-
43	FKNMNILEL	-	+	-	-	-	-
44	YALMNDNNSVL	+	-	-	-	-	-
	LSGRYNDL	-	-	-	-	-	-
45	KSMSNGSFL	-	-	-	-	-	-
46	LSYNNTLDYF	-	-	-	-	-	+
47	YAIKKKDEI	-	+	-	-	-	-
48	ICFEHYQLF	-	-	-	-	-	-
49	SSISQNEIVI	-	+	-	-	-	-
50	IVYRFRKL	-	-	-	-	-	-
51	FAINNNEHL	-	-	-	-	-	-
52	FSSCNDTLEL	+	-	-	-	-	-
53	ISFAGFNAL	-	+	-	-	-	-
54	ASLENVETI	-	+	-	-	-	-
55	HAHANYAFL	-	-	-	-	-	-
56	KTMNFYGM	-	+	-	-	-	-
57	NSVNNINYI	-	-	-	-	-	-
	YYYDYDKI	-	-	-	-	-	-
58	VCIYYFDLL	-	-	-	-	+	+
	WGDEFEKL	-	+	-	-	-	-
59	KNYFHFFNM	-	+	-	-	-	-
60	YSLNNTHVL	-	-	-	-	-	+
61	STFLYYLL	-	-	-	-	-	+
62	YAYRNGLYF	-	-	-	-	-	-
63	RTFYYFHGLL	-	-	-	-	-	-
64	NCYIYNYV	+	+	-	-	-	-
65	YALRNFTLF	-	-	-	-	-	-
66	FSLINHSVI	-	-	-	-	-	-
67	IAVLNSLYL	-	-	-	-	-	-
68	ITYLNSINI	-	-	-	-	-	-
69	AAINNIEFV	+	+	-	-	-	-
70	AAVNNLFTI	-	+	-	-	-	-
	LLPHFSIL	-	-	-	-	-	-
71	VGMRHLNL	-	+	-	-	-	-
72	VSYFYFLDLL	-	-	-	-	-	+
73	YAVRNTRYL	-	-	-	-	-	-
74	YALFNGNLI	-	-	-	-	-	-
75	YNMFYYTIL	-	-	-	-	-	-
76	STYSFMSL	-	-	-	-	-	+
77	YAIGNNDIAL	-	-	-	-	-	+

Novel Peptide number	Peptide sequence	Novel peptides carried forward from batch purity -> high purity (black=top43, grey=other52)	Published control peptides	Top NetMHCpan peptides <22nM and <0.05 Rank %	NetMHCpan other peptides 22.381-388.44nM but always %Rank of 0.5 or below	Top NetMHCcons NC and C2	Top NetMHCcons peptides <22nM and <0.05 Rank %	NetMHCcons other peptides 23.05nM-531.38nM but always %Rank of 0.5 or below	Gene ID	Protein	Batch purity restimulation % IFN-γ+ CD11a+ / total CD8+ T cells	High purity restimulation % IFN-γ+ CD11a+ / total CD8+ T cells
78	NCLNYYPL										0.017	0.0090075
79	RIYNFYHKL										0.032	0.0090025
80	NCYKYMLL										0.011	0.0089375
81	VSMYFTMHI										0.033	0.0087525
82	NCYDYGTL										0.022	0.0086875
83	FAHKNNVPI										0.0775	0.00829
84	INYNFYSM										0.0385	0.00733
85	SNFKYSFV										0.015	0.00733
86	NSIKNVLPI										0.088666667	0.007205
87	HSMENVDTM										0.035	0.007155
88	ISYYLYINL										0.042	0.0070475
89	ISFNCFLSTL										0.0335	0.0067025
90	VVIKFLQYM										0.019	0.006655
91	ISYLFHYIHF										0.036	0.0065
92	YSYKYYNYF										0.03925	0.0063025
93	SSYSYSNPL										0.012	0.00551
94	NCFYFKNV										0.01	0.0032725
	EIYIFTNI (batch purity)								PBANKA_0416600	replication protein A1, small fragment	0.27	
	NCYDFNNI (batch purity)								PBANKA_0714500	conserved Plasmodium protein, unknown function	0.16	
	SQLLNAKYL (batch purity)								PBANKA_0819000	glideosome-associated protein 50, putative	0.0945	
	LSGRYNDL (batch purity)								PBANKA_1337300	conserved Plasmodium protein, unknown function	0.0895	
	WGDEFEKL (batch purity)								PBANKA_0611600	ribonucleoside-diphosphate reductase large subunit, putative	0.089	
	LLPHFSIL (batch purity)								PBANKA_0316000	replication factor C subunit 1, putative	0.0655	
	YYYDYDKI (batch purity)								PBANKA_0521700	transcription factor with AP2 domain(s), putative	0.061	
95	HSYPYYTNL										0.042	
96	YSLSNRLQL										0.041	
97	KSIQNMNCTEI										0.041	
98	YSYNRFLTI										0.039	
99	YIYRFFRSL										0.0385	
100	VTYENLDPL										0.0375	
101	SIFNFIYLL										0.037095	
102	VSYLKHFAMEM										0.0365	
103	ISFLHYYKL										0.036	
104	IAYYFMFL										0.035	
105	ISFYMFYHKM										0.035	
106	HSFCRYILL										0.035	
107	FTYLYYYYYL										0.035	
108	VVYFFIMPV										0.034	
109	FSIANVVYV										0.0335	
110	MSWANNTTFL										0.0335	
111	INYKFFKSI										0.032	
112	NCIEFYEL										0.031	
113	VCYKYMPLI										0.031	
114	INYEYYNL										0.03	

Novel Peptide number	Peptide sequence	Signal peptide predicted	No. of transmembrane domains predicted	Protein length	Peptide length	Peptide start position	Percentile of protein	NetMHCcons 1.1 data				NetMHCpan 4.0 data				Expression data				
								MHC allele restriction	1-log50k	nM	%Rank score	MHC allele restriction	1-log50k	nM	%Rank score	**TARUN 2008 EEF** Mass Spec (LS40-50h)	**LINDNER 2013 Spz** Mass Spec	**LASONDER 2008 spz** Mass Spec	**LASONDER 2008 Spz** (only day 18-22) Mass Spec	**LINDNER 2013 Spz** Mass Spec
																+/-	+/-	+/-	+/-	+/-
																orthologs of *P. yoelii*		orthologs of *P. falciparum*		
78	NCLNYYPL	no	0	347	8	72	20.74928	Kb	0.367	942.86	3	Kb	0.3645	968.567	1.7654	-	+	-	-	+
79	RIYNFYHKL	no	2	2042	9	1367	66.94417	Kb	0.773	11.66	0.03	Kb	0.8555	4.7742	0.008	-	+	-	-	+
80	NCYKYMLL	no	14	1881	8	1612	85.6991	Kb	0.503	216.47	0.8	Kb	0.5582	119.159	0.3035	-	-	-	-	+
81	VSMYFTMHI	yes	1	1296	9	1094	84.41358	Kb	0.733	17.97	0.05	Kb	0.787	10.0168	0.0181	+	-	-	-	+
82	NCYDYGTL	no	0	583	8	416	71.35506	Kb	0.34	1262.8	4	Kb	0.429	482.323	0.9797	-	+	+	+	+
83	FAHKNNVPI	no	0	5176	9	5025	97.08269	Db	0.891	3.27	0.01	Db	0.8486	5.1477	0.0046	+	+	-	-	+
84	INYNFYSM	no	3	1115	8	1012	90.76233	Kb	0.833	6.12	0.01	Kb	0.8875	3.3793	0.0062	-	-	-	-	-
85	SNFKYSFV	no	0	288	8	243	84.375	Kb	0.71	23.05	0.05	Kb	0.8209	6.9417	0.0099	+	+	+	+	+
86	NSIKNVLPI	no	0	5176	9	412	7.959815	Db	0.845	5.38	0.01	Db	0.8102	7.795	0.0057	+	+	-	-	+
87	HSMENVDTM	no	0	946	9	885	93.5518	Db	0.88	3.66	0.01	Db	0.867	4.2184	0.004	-	-	-	-	-
88	ISYYLYINL	no	5	1461	9	155	10.60917	Kb	0.808	7.94	0.01	Kb	0.8837	3.5207	0.0064	-	+	-	-	+
89	ISFNCFLSTL	no	2	1407	10	804	57.14286	Kb	0.658	40.46	0.12	Kb	0.7903	9.6689	0.017	-	-	-	-	-
90	VVIKFLQYM	no	0	130	9	32	24.61538	Kb	0.682	31.04	0.1	Kb	0.721	20.463	0.049	+	-	+	+	+
91	ISYLFHYIHF	yes	1	599	10	569	94.99165	Kb	0.702	25.14	0.07	Kb	0.6946	27.2438	0.0692	-	+	+	-	+
92	YSYKYYNYF	no	0	380	9	318	83.68421	Kb	0.759	13.57	0.03	Kb	0.7979	8.9018	0.0146	-	-	-	-	-
93	SSYSYSNPL	no	0	233	9	39	16.7382	Kb	0.834	6.03	0.01	Kb	0.8552	4.7934	0.008	-	-	-	-	-
94	NCFYFKNV	no	0	1113	8	763	68.55346	Kb	0.268	2737.1	7	Kb	0.3731	883.014	1.6428	-	+	-	-	+
	EIYIFTNI (batch purity)	no	0	486	8	198	40.74074	Kb	0.568	107.14	0.4	Kb	0.6389	49.7716	0.1214	+	+	+	+	+
	NCYDFNNI (batch purity)	no	1	745	8	396	53.15436	Kb	0.238	3786.7	9	Kb	0.3864	764.103	1.4628	-	-	-	-	-
	SQLLNAKYL (batch purity)	yes	2	395	9	39	9.873418	Db	0.704	24.47	0.05	Db	0.6878	29.3083	0.0094	+	+	+	+	+
	LSGRYNDL (batch purity)	yes	1	329	8	118	35.86626	Kb	0.333	1369.5	4	Kb	0.3226	1524.09	2.5409	-	+	-	-	-
	WGDEFEKL (batch purity)	no	0	847	8	401	47.34357	Kb	0.145	10471	32	Kb	0.1564	9206.28	12.8923	+	-	-	-	+
	LLPHFSIL (batch purity)	no	0	861	8	650	75.49361	Kb	0.386	771.82	3	Kb	0.4523	374.756	0.8159	-	+	-	-	+
	YYYDYDKI (batch purity)	no	0	2775	8	1956	70.48649	Kb	0.264	2858.2	7	Kb	0.3161	1634.72	2.7053	-	+	+	+	+
95	HSYPYYTNL	no	13	2715	9	1655	60.95764	Kb	0.792	9.49	0.01	Kb	0.8997	2.9593	0.0056	-	-	+	-	-
96	YSLSNRLQL	no	0	367	9	249	67.84741	Db	0.815	7.44	0.01	Db	0.7648	12.7404	0.0071	-	+	-	-	+
97	KSIQNMNCTEI	no	0	3018	11	1987	65.8383	Db	0.653	42.71	0.07	Db	0.5719	102.679	0.0302	-	-	-	-	-
98	YSYNRFLTI	no	6	2724	9	2053	75.36711	Kb	0.78	10.87	0.01	Kb	0.7778	11.0693	0.0213	-	-	-	-	-
99	YIYRFFRSL	no	0	1155	9	182	15.75758	Kb	0.79	9.65	0.01	Kb	0.8812	3.6165	0.0066	+	+	-	-	-
100	VTYENLDPL	no	1	366	9	224	61.20219	Db	0.75	14.87	0.05	Db	0.7369	17.2266	0.0079	-	+	+	+	+
101	SIFNFIYLL	yes	0	1218	9	118	9.688013	Kb	0.728	19.08	0.05	Kb	0.7615	13.2062	0.027	+	-	+	+	+
102	VSYLKHFAMEM	no	0	778	11	537	69.02314	Kb	0.639	49.43	0.17	Kb	0.6321	53.542	0.1321	-	+	-	-	+
103	ISFLHYYKL	no	0	1360	9	704	51.76471	Kb	0.795	9.19	0.01	Kb	0.8978	3.0219	0.0057	+	-	-	-	+
104	IAYYFMFL	no	1	151	8	142	94.03974	Kb	0.827	6.54	0.01	Kb	0.8968	3.0556	0.0057	+	-	-	-	+
105	ISFYMFYHKM	no	11	1935	10	359	18.55297	Kb	0.685	30.05	0.07	Kb	0.8144	7.4507	0.0111	+	-	-	-	-
106	HSFCRYILL	no	0	1090	9	159	14.58716	Kb	0.74	16.57	0.05	Kb	0.721	20.475	0.049	-	-	-	-	+
107	FTYLYYYYYL	yes	10	2361	10	2068	87.59	Kb	0.686	29.73	0.07	Kb	0.6247	58.0379	0.149	-	-	-	-	-
108	VVYFFIMPV	yes	1	1296	9	1174	90.58642	Kb	0.762	13.13	0.03	Kb	0.7299	18.5857	0.042	+	-	-	-	+
109	FSIANVVYV	no	0	566	9	4	0.706714	Db	0.878	3.74	0.01	Db	0.8372	5.8204	0.0049	+	-	-	-	-
110	MSWANNTTFL	no	0	513	10	481	93.76218	Db	0.687	29.56	0.07	Db	0.5471	134.311	0.0372	+	+	-	-	+
111	INYKFFKSI	yes	1	2096	9	2062	98.37786	Kb	0.729	18.77	0.05	Kb	0.8822	3.5774	0.0065	-	-	-	-	+
112	NCIEFYEL	no	0	439	8	394	89.74943	Kb	0.274	2579	7	Kb	0.3385	1283.42	2.212	+	+	-	-	+
113	VCYKYMPLI	yes	1	185	9	142	76.75676	Kb	0.584	90.6	0.4	Kb	0.6201	60.9461	0.1564	+	+	-	-	+
114	INYEYYNL	no	0	7263	8	5736	78.97563	Kb	0.845	5.38	0.01	Kb	0.9141	2.5318	0.0048	-	-	-	-	+

Novel Peptide number	Peptide sequence	Expression data					
		Microarray		SSH		Microarray	
		TARUN 2008 spz	**Tarun 2008 EEF**	**UIS genes Matuschewski 2002** (oocyst spz v sg spz)	**S genes Kaiser 2004** (sg spz v merozoites)	**DSK hi** (highly downregulated in SLARP KO) **Silvie, unpublished**	**DSK lo** (lowly downregulated in SLARP KO) **Silvie, unpublished**
		+/-	+/-	+/-	+/-	+/-	+/-
		orthologs of *P. yoelii*				*P. berghei* origin	
78	NCLNYYPL	-	-	-	-	-	-
79	RIYNFYHKL	-	-	-	-	-	+
80	NCYKYMLL	-	-	-	-	-	-
81	VSMYFTMHI	-	-	-	-	-	-
82	NCYDYGTL	-	-	-	+	-	-
83	FAHKNNVPI	-	-	-	-	-	-
84	INYNFYSM	-	-	-	-	-	+
85	SNFKYSFV	-	+	-	-	-	-
86	NSIKNVLPI	-	-	-	-	-	-
87	HSMENVDTM	+	-	-	-	-	-
88	ISYYLYINL	-	-	-	-	-	+
89	ISFNCFLSTL	-	-	-	-	-	+
90	VVIKFLQYM	-	+	-	-	-	-
91	ISYLFHYIHF	-	-	-	-	-	-
92	YSYKYYNYF	-	+	-	-	-	-
93	SSYSYSNPL	+	-	-	-	-	-
94	NCFYFKNV	-	-	-	-	-	-
	EIYIFTNI (batch purity)	-	-	-	-	+	-
	NCYDFNNI (batch purity)	-	-	-	-	-	-
	SQLLNAKYL (batch purity)	-	-	-	-	-	-
	LSGRYNDL (batch purity)	-	-	-	-	-	-
	WGDEFEKL (batch purity)	-	+	-	-	-	-
	LLPHFSIL (batch purity)	-	-	-	-	-	-
	YYYDYDKI (batch purity)	-	-	-	-	-	-
95	HSYPYYTNL	-	-	-	-	-	-
96	YSLSNRLQL	-	-	-	-	-	-
97	KSIQNMNCTEI	+	-	-	-	-	-
98	YSYNRFLTI	+	-	+	-	-	-
99	YIYRFFRSL	-	-	-	-	-	+
100	VTYENLDPL	+	-	-	-	-	-
101	SIFNFIYLL	-	-	-	-	-	-
102	VSYLKHFAMEM	-	-	-	-	-	-
103	ISFLHYYKL	-	-	-	-	-	-
104	IAYYFMFL	-	+	-	-	-	-
105	ISFYMFYHKM	-	-	-	-	-	-
106	HSFCRYILL	-	-	+	-	-	-
107	FTYLYYYYYL	-	-	-	-	-	+
108	VVYFFIMPV	-	-	-	-	-	-
109	FSIANVVYV	-	-	-	-	-	-
110	MSWANNTTFL	-	-	-	-	-	-
111	INYKFFKSI	-	-	-	-	-	-
112	NCIEFYEL	-	-	-	-	-	-
113	VCYKYMPLI	-	+	-	-	-	-
114	INYEYYNL	-	-	-	-	-	-

Novel Peptide number	Peptide sequence	Novel peptides carried forward from batch purity -> high purity (black=top43, grey=other52)	Published control peptides	Top NetMHCpan peptides <22nM and <0.05 Rank %	NetMHCpan other peptides 22.381-388.44nM but always %Rank of 0.5 or below	Top NetMHCcons NC and C2	Top NetMHCcons peptides <22nM and <0.05 Rank %	NetMHCcons other peptides 23.05nM-531.38nM but always %Rank of 0.5 or below	Gene ID	Protein	Batch purity restimulation % IFN-γ+ CD11a+ / total CD8+ T cells	High purity restimulation % IFN-γ+ CD11a+ / total CD8+ T cells
115	KNYYFTLSL										0.03	
116	YSFFMYNEM										0.03	
117	SCFYRMQML										0.029	
118	YSYSYFINF										0.029	
119	YAFYFWFFALL										0.029	
120	SAINNCLI										0.0285	
121	SSYIFILL										0.028	
122	NCINFLLL										0.028	
123	NCMSFYHI										0.028	
124	MSILNEYNI										0.028	
125	VSPFYHAL										0.028	
126	WGYGFKYYPL										0.028	
127	SSYKKFILLL										0.028	
128	KSIINYNTI										0.027	
129	FAYFNFEEI										0.027	
130	YMHMNLSPL										0.027	
131	ICYFFFYNI										0.027	
132	SVYFSFRNL										0.027	
133	RSFNFILL										0.027	
134	ISFYRYFIM										0.027	
135	TSLRNGNTL										0.027	
136	IMYEFLLYGL										0.027	
137	MSYPFFPLLL										0.0265	
138	FALINFIAL										0.026	
139	INYNFNSL										0.026	
140	VSYRYREL										0.026	
141	ITFFYRNGL										0.026	
142	VNYHFSNYMNF										0.026	
143	AAILNHTNI										0.0255	
144	YSLNNANINIL										0.0255	
145	YTYRYTPL										0.0255	
146	ISFCFQAL										0.025	
147	YSFFFMHL										0.025	
148	CCYEYYCSL										0.025	
149	NCFHLINL										0.025	
150	IIYLFRETNL										0.025	
151	VNYTYLCSIEL										0.025	
152	INYNKYIHLL										0.02458	
153	SNYAYFTIL										0.0245	
154	AQYSNNFDYL										0.0245	
155	SMINNDIPL										0.02449	
156	ILYSFYNYL										0.024	
157	SSILNNELI										0.024	
158	VMYLFGRL										0.024	

Novel Peptide number	Peptide sequence	Signal peptide predicted	No. of transmembrane domains predicted	Protein length	Peptide length	Peptide start position	Percentile of protein	NetMHCcons 1.1 data				NetMHCpan 4.0 data				Expression data				
								MHC allele restriction	1-log50k	nM	%Rank score	MHC allele restriction	1-log50k	nM	%Rank score	**TARUN 2008 EEF** Mass Spec (LS40-50h)	**LINDNER 2013 Spz** Mass Spec	**LASONDER 2008 spz** Mass Spec	**LASONDER 2008 Spz** (only day 18-22) Mass Spec	**LINDNER 2013 Spz** Mass Spec
																+/-	+/-	+/-	+/-	+/-
																orthologs of *P. yoelii*		orthologs of *P. falciparum*		
115	KNYYFTLSL	yes	10	2361	9	2145	90.85133	Kb	0.758	13.64	0.03	Kb	0.8403	5.6278	0.0088	-	-	-	-	-
116	YSFFMYNEM	yes	1	2081	9	1043	50.12013	Kb	0.718	21.14	0.05	Kb	0.6962	26.7536	0.0676	-	+	+	+	+
117	SCFYRMQML	no	0	1474	9	446	30.2578	Kb	0.588	86.76	0.3	Kb	0.5514	128.178	0.3236	+	-	-	-	-
118	YSYSYFINF	yes	2	896	9	259	28.90625	Kb	0.762	13.2	0.03	Kb	0.7932	9.3706	0.016	-	+	+	-	+
119	YAFYFWFFALL	no	4	611	11	266	43.53519	Kb	0.698	26.39	0.07	Kb	0.6034	73.062	0.1851	-	+	-	-	+
120	SAINNCLI	yes	0	3254	8	539	16.56423	Db	0.399	666.93	0.5	Db	0.449	388.441	0.1045	+	-	-	-	-
121	SSYIFILL	no	6	705	8	529	75.03546	Kb	0.828	6.47	0.01	Kb	0.8744	3.8914	0.007	-	+	-	-	+
122	NCINFLLL	no	0	1471	8	988	67.16519	Kb	0.277	2496.6	7	Kb	0.3316	1382.3	2.3504	-	-	-	-	+
123	NCMSFYHI	no	0	228	8	200	87.7193	Kb	0.211	5099	15	Kb	0.3277	1443.1	2.4307	-	-	-	-	-
124	MSILNEYNI	yes	2	1671	9	860	51.46619	Db	0.743	16.13	0.05	Db	0.7429	16.1554	0.0078	-	-	-	-	+
125	VSPFYHAL	yes	2	1671	8	578	34.59007	Kb	0.759	13.57	0.03	Kb	0.8224	6.834	0.0098	-	-	-	-	+
126	WGYGFKYYPL	yes	1	1192	10	659	55.28523	Kb	0.663	38.12	0.12	Kb	0.645	46.564	0.1154	+	+	-	-	+
127	SSYKKFILLL	no	0	5309	10	1102	20.7572	Kb	0.742	16.39	0.03	Kb	0.7168	21.4241	0.0518	+	+	-	-	+
128	KSIINYNTI	no	0	5249	9	3330	63.44066	Db	0.854	4.88	0.01	Db	0.8345	5.9952	0.005	+	-	-	-	-
129	FAYFNFEEI	no	0	511	9	191	37.37769	Db	0.877	3.8	0.01	Db	0.8558	4.7581	0.0043	-	-	-	-	+
130	YMHMNLSPL	no	8	1272	9	1190	93.55346	Db	0.883	3.53	0.01	Db	0.8267	6.5242	0.0052	-	-	-	-	+
131	ICYFFFYNI	yes	11	732	9	681	93.03279	Kb	0.577	97.73	0.4	Kb	0.6893	28.8443	0.0732	-	-	+	+	-
132	SVYFSFRNL	yes	1	3204	9	1326	41.38577	Kb	0.728	18.87	0.05	Kb	0.8347	5.9831	0.0092	-	-	+	+	-
133	RSFNFILL	yes	0	824	8	133	16.14078	Kb	0.785	10.18	0.01	Kb	0.8282	6.4175	0.0095	-	+	-	-	+
134	ISFYRYFIM	no	4	3053	9	1107	36.25942	Kb	0.789	9.81	0.01	Kb	0.8136	7.5126	0.0112	-	+	-	-	+
135	TSLRNGNTL	yes	0	477	9	33	6.918239	Db	0.777	11.17	0.03	Db	0.7632	12.9621	0.0071	+	-	-	-	-
136	IMYEFLLYGL	yes	0	562	10	476	84.69751	Kb	0.738	17.12	0.05	Kb	0.6791	32.2151	0.0806	-	+	-	-	+
137	MSYPFFPLLL	no	0	4287	10	3924	91.53254	Kb	0.758	13.79	0.03	Kb	0.8018	8.5334	0.0138	-	-	+	-	-
138	FALINFIAL	no	2	190	9	143	75.26316	Db	0.881	3.62	0.01	Db	0.8083	7.9614	0.0058	-	+	-	-	+
139	INYNFNSL	no	4	3350	8	2661	79.43284	Kb	0.834	6.03	0.01	Kb	0.9018	2.8923	0.0054	-	+	-	-	+
140	VSYRYREL	no	4	1504	8	971	64.56117	Kb	0.837	5.8	0.01	Kb	0.9083	2.696	0.0051	+	-	-	-	-
141	ITFFYRNGL	no	2	2091	9	1831	87.56576	Kb	0.749	15.12	0.03	Kb	0.8193	7.0627	0.01	-	-	-	-	-
142	VNYHFSNYMNF	yes	1	2081	11	811	38.97165	Kb	0.639	49.7	0.17	Kb	0.6704	35.3906	0.089	-	+	+	+	+
143	AAILNHTNI	no	4	4204	9	2090	49.71456	Db	0.865	4.33	0.01	Db	0.8136	7.5134	0.0056	-	+	-	-	+
144	YSLNNANINIL	yes	0	1478	11	489	33.08525	Db	0.627	56.89	0.1	Db	0.6103	67.798	0.02	-	-	-	-	+
145	YTYRYTPL	no	10	452	8	83	18.36283	Kb	0.846	5.29	0.01	Kb	0.9014	2.9059	0.0055	-	+	-	-	-
146	ISFCFQAL	no	8	411	8	198	48.17518	Kb	0.842	5.53	0.01	Kb	0.906	2.7647	0.0052	-	+	+	-	+
147	YSFFFMHL	no	0	1653	8	946	57.22928	Kb	0.824	6.71	0.01	Kb	0.8964	3.069	0.0057	-	+	-	-	+
148	CCYEYYCSL	no	0	277	9	154	55.59567	Kb	0.637	50.78	0.17	Kb	0.6921	27.9884	0.0712	-	-	-	-	-
149	NCFHLINL	no	2	7126	8	2543	35.68622	Kb	0.265	2858.2	7	Kb	0.3237	1505.51	2.5121	-	-	-	-	+
150	IIYLFRETNL	no	13	585	10	472	80.68376	Kb	0.645	46.32	0.15	Kb	0.7392	16.8036	0.0368	-	+	+	-	+
151	VNYTYLCSIEL	yes	0	1218	11	972	79.80296	Kb	0.635	51.89	0.17	Kb	0.6976	26.3685	0.0662	+	-	+	+	+
152	INYNKYIHLL	no	0	5249	10	3477	66.24119	Kb	0.707	23.81	0.05	Kb	0.7852	10.2132	0.0188	+	-	-	-	-
153	SNYAYFTIL	yes	0	1354	9	691	51.03397	Kb	0.82	7.05	0.01	Kb	0.9054	2.7834	0.0052	+	+	-	-	+
154	AQYSNNFDYL	yes	0	999	10	122	12.21221	Db	0.681	31.38	0.07	Db	0.6399	49.2067	0.0133	-	+	+	+	+
155	SMINNDIPL	no	0	1766	9	4	0.226501	Db	0.809	7.9	0.01	Db	0.7709	11.933	0.0069	-	-	-	-	-
156	ILYSFYNYL	no	0	1310	9	906	69.16031	Kb	0.83	6.29	0.01	Kb	0.865	4.3095	0.0075	-	-	-	-	-
157	SSILNNELI	yes	0	764	9	297	38.87435	Db	0.865	4.29	0.01	Db	0.8373	5.8119	0.0049	-	-	-	-	+
158	VMYLFGRL	no	12	735	8	372	50.61224	Kb	0.834	6.03	0.01	Kb	0.8954	3.1013	0.0058	-	+	-	-	+

Novel Peptide number	Peptide sequence	Expression data					
		Microarray		SSH		Microarray	
		TARUN 2008 spz	**Tarun 2008 EEF**	**UIS genes Matuschewski 2002** (oocyst spz v sg spz)	**S genes Kaiser 2004** (sg spz v merozoites)	**DSK hi** (highly downregulated in SLARP KO) **Silvie, unpublished**	**DSK lo** (lowly downregulated in SLARP KO) **Silvie, unpublished**
		+/-	+/-	+/-	+/-	+/-	+/-
		orthologs of *P. yoelii*				*P. berghei* origin	
115	KNYYFTLSL	-	-	-	-	-	+
116	YSFFMYNEM	-	-	-	-	-	-
117	SCFYRMQML	-	-	-	-	-	-
118	YSYSYFINF	-	-	-	-	-	-
119	YAFYFWFFALL	-	-	-	-	-	-
120	SAINNCLI	-	-	-	-	-	-
121	SSYIFILL	-	-	-	-	-	-
122	NCINFLLL	+	-	-	-	-	-
123	NCMSFYHI	+	+	-	-	-	-
124	MSILNEYNI	-	-	-	-	-	-
125	VSPFYHAL	-	-	-	-	-	-
126	WGYGFKYYPL	-	-	-	-	-	-
127	SSYKKFILLL	-	-	-	-	-	-
128	KSIINYNTI	-	-	-	-	-	-
129	FAYFNFEEI	-	-	-	-	-	-
130	YMHMNLSPL	-	-	-	-	-	-
131	ICYFFFYNI	-	-	-	-	-	-
132	SVYFSFRNL	-	-	-	-	-	-
133	RSFNFILL	-	-	-	-	-	-
134	ISFYRYFIM	-	-	-	-	-	-
135	TSLRNGNTL	-	+	-	-	-	-
136	IMYEFLLYGL	-	-	-	-	-	-
137	MSYPFFPLLL	-	-	-	-	-	-
138	FALINFIAL	-	-	-	-	-	-
139	INYNFNSL	-	-	-	-	-	-
140	VSYRYREL	-	-	-	-	-	-
141	ITFFYRNGL	-	-	-	-	-	+
142	VNYHFSNYMNF	-	-	-	-	-	-
143	AAILNHTNI	-	+	-	-	-	-
144	YSLNNANINIL	-	-	-	-	-	-
145	YTYRYTPL	-	-	-	+	-	-
146	ISFCFQAL	-	-	-	-	-	-
147	YSFFFMHL	-	-	-	-	-	-
148	CCYEYYCSL	+	-	-	-	-	-
149	NCFHLINL	-	-	-	-	-	+
150	IIYLFRETNL	-	-	-	-	-	-
151	VNYTYLCSIEL	-	-	-	-	-	-
152	INYNKYIHLL	-	-	-	-	-	-
153	SNYAYFTIL	-	-	-	-	-	-
154	AQYSNNFDYL	+	-	-	+	-	-
155	SMINNDIPL	-	-	-	-	-	+
156	ILYSFYNYL	-	+	-	-	-	-
157	SSILNNELI	-	-	-	-	-	-
158	VMYLFGRL	-	+	-	-	-	-

Novel Peptide number	Peptide sequence	Top NetMHCpan peptides <22nM and <0.05 Rank %	NetMHCpan other peptides 22.381-388.44nM but always %Rank of 0.5 or below	Top NetMHCcons NC and C2	Top NetMHCcons peptides <22nM and <0.05 Rank %	NetMHCcons other peptides 23.05nM-531.38nM but always %Rank of 0.5 or below	Batch purity restimulation % IFN-γ+ CD11a+ / total CD8+ T cells	Signal peptide predicted	No. of transmembrane domains predicted	Protein length	Peptide length	Peptide start position	Percentile of protein	NetMHCcons 1.1 data			
														MHC allele restriction	1-log50k	nM	%Rank score
159	VVYRHFATL						**0.024**	no	0	157	9	62	39.4904459	Kb	0.857	4.67	0.01
160	YALSNGVNI						**0.024**	no	1	602	9	355	58.9700997	Db	0.885	3.45	0.01
161	VCYDYLYSL						**0.024**	no	0	2453	9	1920	78.2715043	Kb	0.633	52.74	0.17
162	AAFRNIKSI						**0.024**	no	0	194	9	150	77.3195876	Db	0.506	209.55	0.2
163	KTYPFFSNI						**0.024**	yes	0	2150	9	1952	90.7906977	Kb	0.728	18.87	0.05
164	VSYARHFL						**0.024**	yes	1	2081	8	1291	62.037482	Kb	0.729	18.77	0.05
165	ISYRHYSL						**0.023**	yes	7	628	8	328	52.2292994	Kb	0.851	5.01	0.01
166	KSLQNVDYI						**0.023**	no	0	647	9	33	5.10046368	Db	0.883	3.55	0.01
167	MIYYFQSL						**0.023**	no	2	1765	8	1446	81.9263456	Kb	0.823	6.79	0.01
168	SIYSYSYL						**0.023**	no	0	651	8	617	94.7772657	Kb	0.834	6.06	0.01
169	YSIMNINEI						**0.023**	no	0	1221	9	101	8.27190827	Db	0.917	2.47	0.01
170	KCISFFNTL						**0.023**	no	0	584	9	571	97.7739726	Kb	0.612	66.56	0.25
171	NCFNFNYI						**0.023**	no	2	1030	8	194	18.8349515	Kb	0.24	3705.68	9
172	NCIKYIKL						**0.023**	no	3	871	8	146	16.7623421	Kb	0.247	3435.38	9
173	INFSYNNM						**0.023**	yes	4	1785	8	1218	68.2352941	Kb	0.773	11.66	0.03
174	YSTWNLSFI						**0.023**	yes	1	1230	9	674	54.796748	Db	0.774	11.6	0.03
175	KAFDRHCNL						**0.023**	no	0	101	9	46	45.5445545	Kb	0.608	69.5	0.25
176	RCLKNNYTL						**0.023**	no	0	192	9	120	62.5	Db	0.42	531.38	0.4
177	VGYIFYNRL						**0.023**	yes	0	256	9	222	86.71875	Kb	0.841	5.62	0.01
178	FSYYKFSSL						**0.023**	no	0	1737	9	1140	65.6303972	Kb	0.829	6.4	0.01
179	TMLKFYNML						**0.023**	yes	1	633	9	536	84.6761453	Kb	0.778	10.99	0.01
180	SSMINNDIPL						**0.023**	no	0	1766	10	3	0.16987542	Db	0.776	11.29	0.03
181	YQLKNLETPI						**0.023**	yes	0	1406	10	571	40.6116643	Db	0.756	14.09	0.05
182	SNYYNHYFFL						**0.023**	yes	1	1192	10	701	58.8087248	Kb	0.714	22.19	0.05
183	MVYKKYIGL						**0.0225**	no	0	778	9	635	81.6195373	Kb	0.79	9.65	0.01
184	SVLSFFYKPL						**0.0225**	yes	0	2775	10	158	5.69369369	Kb	0.621	60.71	0.25
185	ISYTFLTM						**0.022**	no	2	177	8	163	92.0903955	Kb	0.832	6.19	0.01
186	RAIQNASTI						**0.022**	no	0	1331	9	178	13.3734035	Db	0.891	3.27	0.01
187	NCYVNLNL						**0.022**	no	2	2599	8	2430	93.497499	Kb	0.235	3911.67	9
188	TCFYYFILL						**0.022**	no	2	1618	9	300	18.5414091	Kb	0.653	42.71	0.15
189	SNYIFNFL						**0.022**	yes	2	1671	8	1522	91.0831837	Kb	0.805	8.2	0.01
190	KMFVNLSGFI						**0.022**	no	0	258	10	210	81.3953488	Db	0.507	207.3	0.2
191	MSYPFFPL						**0.022**	no	0	4287	8	3924	91.5325402	Kb	0.87	4.08	0.01
192	LNYYFYQEI						**0.022**	yes	3	1149	9	1085	94.4299391	Kb	0.768	12.24	0.03
193	CSMENSTYI						**0.022**	no	2	2017	9	514	25.4833912	Db	0.841	5.62	0.01
194	SSISFLSSL						**0.022**	yes	0	654	9	633	96.7889908	Kb	0.763	12.99	0.03
195	ISYKYKNYM						**0.022**	yes	0	1796	9	81	4.51002227	Kb	0.76	13.42	0.03
196	YTYPYYNLI						**0.022**	yes	8	5371	9	3996	74.3995532	Kb	0.732	18.17	0.05
197	IMVPFFSIM						**0.022**	no	13	585	9	121	72.8915663	Kb	0.736	17.49	0.05
198	VSYARHFLF						**0.022**	yes	1	2081	9	1291	62.037482	Kb	0.787	10.02	0.01
199	ISYFKYQPPV						**0.022**	yes	2	713	10	83	11.6409537	Kb	0.725	19.6	0.05
200	VNYFNQNLL						**0.022**	yes	0	1109	9	854	77.006312	Kb	0.734	17.88	0.05
201	FMYSRKLKL						**0.022**	yes	1	3204	9	1984	61.9225968	Kb	0.692	28.01	0.07

Novel Peptide number	Peptide sequence	NetMHCpan 4.0 data				Expression data											
											Microarray		SSH		Microarray	
		MHC allele restriction	1-log50k	nM	%Rank score	**TARUN 2008 EEF** Mass Spec (LS40-50h)	**LINDNER 2013 Spz** Mass Spec	**LASONDER 2008 spz** Mass Spec	**LASONDER 2008 Spz** (only day 18-22) Mass Spec	**LINDNER 2013 Spz** Mass Spec	**TARUN 2008 spz**	**Tarun 2008 EEF**	**UIS genes Matuschewski 2002** (oocyst spz v sg spz)	**S genes Kaiser 2004** (sg spz v merozoites)	**DSK hi** (highly downregulated in SLARP KO) **Silvie, unpublished**	**DSK lo** (lowly downregulated in SLARP KO) **Silvie, unpublished**
						+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-
						orthologs of *P. yoelii*		orthologs of *P. falciparum*			orthologs of *P. yoelii*				*P. berghei* origin	
159	VVYRHFATL	Kb	0.9077	2.7156	0.0051	-	-	-	-	+	-	+	-	-	-	-
160	YALSNGVNI	Db	0.8413	5.5693	0.0048	-	-	-	-	-	+	-	-	-	-	-
161	VCYDYLYSL	Kb	0.7114	22.7002	0.0552	-	+	-	-	+	-	-	-	-	-	-
162	AAFRNIKSI	Db	0.6274	56.3182	0.0147	+	+	+	+	+	-	+	-	-	-	-
163	KTYPFFSNI	Kb	0.8556	4.7694	0.008	-	+	-	-	-	-	-	-	-	-	-
164	VSYARHFL	Kb	0.7846	10.285	0.019	-	+	+	+	+	-	-	-	-	-	-
165	ISYRHYSL	Kb	0.9048	2.8009	0.0053	-	-	-	-	+	-	-	-	-	-	-
166	KSLQNVDYI	Db	0.8693	4.1111	0.0039	-	-	-	-	-	-	-	-	-	-	-
167	MIYYFQSL	Kb	0.877	3.7855	0.0068	-	-	-	-	+	-	-	-	-	-	-
168	SIYSYSYL	Kb	0.8895	3.3052	0.0061	-	+	-	-	+	-	-	-	-	-	+
169	YSIMNINEI	Db	0.8976	3.0276	0.0031	-	-	-	-	+	-	-	-	-	-	-
170	KCISFFNTL	Kb	0.6478	45.1686	0.1127	-	-	-	-	-	-	+	-	-	-	-
171	NCFNFNYI	Kb	0.3705	908.1188	1.6757	-	-	-	-	+	-	-	-	-	-	-
172	NCIKYIKL	Kb	0.287	2241.1646	3.5287	-	+	-	-	+	-	-	-	-	-	-
173	INFSYNNM	Kb	0.8695	4.1031	0.0072	-	-	-	-	+	-	-	-	-	-	-
174	YSTWNLSFI	Db	0.7163	21.5292	0.0086	-	+	-	-	+	+	-	-	-	-	-
175	KAFDRHCNL	Kb	0.7044	24.4841	0.0596	+	+	-	-	+	-	+	-	-	-	-
176	RCLKNNYTL	Db	0.4655	324.8614	0.0891	+	+	+	-	+	-	-	-	-	-	-
177	VGYIFYNRL	Kb	0.9212	2.3465	0.0044	-	+	-	-	+	-	+	-	-	-	-
178	FSYYKFSSL	Kb	0.8398	5.6598	0.0089	+	-	-	-	-	-	-	-	-	-	-
179	TMLKFYNML	Kb	0.7839	10.3599	0.0192	-	+	+	+	+	-	+	-	-	-	-
180	SSMINNDIPL	Db	0.7688	12.196	0.007	-	-	-	-	-	-	-	-	-	-	+
181	YQLKNLETPI	Db	0.7513	14.7429	0.0075	+	-	+	-	-	-	-	-	-	-	-
182	SNYYNHYFFL	Kb	0.745	15.7899	0.034	+	+	-	-	+	-	-	-	-	-	-
183	MVYKKYIGL	Kb	0.7895	9.757	0.0173	-	+	-	-	+	-	-	-	-	-	-
184	SVLSFFYKPL	Kb	0.6918	28.0645	0.0714	-	+	-	-	-	-	-	-	-	-	-
185	ISYTFLTM	Kb	0.8838	3.5167	0.0064	-	-	-	-	-	-	+	-	-	-	-
186	RAIQNASTI	Db	0.8624	4.4333	0.0042	-	-	-	-	+	-	-	-	-	-	-
187	NCYVNLNL	Kb	0.2696	2704.5781	4.1452	-	-	-	-	+	+	-	-	-	-	-
188	TCFYYFILL	Kb	0.6237	58.6502	0.1509	-	+	-	-	+	-	-	-	-	-	-
189	SNYIFNFL	Kb	0.8775	3.7634	0.0068	-	-	-	-	+	-	-	-	-	-	-
190	KMFVNLSGFI	Db	0.5454	136.8296	0.0377	+	-	-	-	-	-	-	-	-	-	-
191	MSYPFFPL	Kb	0.9114	2.6074	0.0049	-	-	+	-	-	-	-	-	-	-	-
192	LNYYFYQEI	Kb	0.8545	4.8261	0.0081	+	-	-	-	-	-	-	-	-	-	-
193	CSMENSTYI	Db	0.8429	5.4727	0.0047	+	+	-	-	-	-	-	-	-	-	-
194	SSISFLSSL	Kb	0.8177	7.1895	0.0104	-	+	-	-	+	-	-	-	-	-	-
195	ISYKYKNYM	Kb	0.8102	7.796	0.012	-	+	-	-	+	+	-	-	-	-	-
196	YTYPYYNLI	Kb	0.7879	9.9237	0.0178	-	-	+	-	+	+	-	-	-	-	-
197	IMVPFFSIM	Kb	0.7561	14.0025	0.029	-	+	+	-	+	-	-	-	-	-	-
198	VSYARHFLF	Kb	0.7464	15.5473	0.0334	-	+	+	+	+	-	-	-	-	-	-
199	ISYFKYQPPV	Kb	0.7167	21.4353	0.0519	-	-	+	-	-	-	-	-	-	-	-
200	VNYFNQNLL	Kb	0.6949	27.1484	0.0689	-	-	-	-	-	-	-	-	-	-	+
201	FMYSRKLKL	Kb	0.5764	97.8019	0.2532	-	-	+	+	-	-	-	-	-	-	-

Novel Peptide number	Peptide sequence	Top NetMHCpan peptides <22nM and <0.05 Rank %	NetMHCpan other peptides 22.381-388.44nM but always %Rank of 0.5 or below	Top NetMHCcons NC and C2	Top NetMHCcons peptides <22nM and <0.05 Rank %	NetMHCcons other peptides 23.05nM-531.38nM but always %Rank of 0.5 or below	Batch purity restimulation % IFN-γ+ CD11a+ / total CD8+ T cells	Signal peptide predicted	No. of transmembrane domains predicted	Protein length	Peptide length	Peptide start position	Percentile of protein	NetMHCcons 1.1 data			
														MHC allele restriction	1-log50k	nM	%Rank score
202	VNYLYSNL						**0.0215**	no	0	920	8	582	63.2608696	Kb	0.861	4.48	0.01
203	IMYTYYFFTSL						**0.021495**	no	7	288	11	203	70.4861111	Kb	0.695	27.11	0.07
204	LTYNYYHPL						**0.021006667**	no	0	257	9	8	3.11284047	Kb	0.829	6.36	0.01
205	HAINNIDEI						**0.021**	no	0	1240	9	459	37.016129	Db	0.865	4.29	0.01
206	TALRNYATL						**0.021**	no	0	278	9	142	51.0791367	Db	0.889	3.31	0.01
207	YSILNDIFL						**0.021**	no	0	1485	9	883	59.4612795	Db	0.876	3.83	0.01
208	FCIYRYNNL						**0.021**	no	4	1504	9	28	1.86170213	Kb	0.581	93.59	0.4
209	LCFTFFPIL						**0.021**	no	6	755	9	736	97.4834437	Kb	0.555	123.32	0.5
210	VCFFCFTSL						**0.021**	yes	0	649	9	522	80.431433	Kb	0.607	70.64	0.25
211	GSISFLDYI						**0.021**	no	0	255	9	211	82.745098	Db	0.467	321.29	0.25
212	SIIIRHNEL						**0.021**	no	0	288	9	151	52.4305556	Kb	0.581	93.08	0.4
213	VIFMIVFL						**0.021**	no	2	101	8	51	50.4950495	Kb	0.574	100.41	0.4
214	KIYAFYNNL						**0.021**	no	4	2077	9	282	13.5772749	Kb	0.818	7.2	0.01
215	INYNYYDML						**0.021**	no	0	525	9	390	74.2857143	Kb	0.82	7.01	0.01
216	VSYIRYYCSL						**0.021**	no	0	142	10	110	77.4647887	Kb	0.746	15.7	0.03
217	YSLSNNEYYL						**0.021**	no	0	5317	10	2243	42.1854429	Db	0.843	5.47	0.01
218	YSIENAVGI						**0.021**	yes	2	887	9	497	56.0315671	Db	0.766	12.51	0.03
219	YLYNYFYKPL						**0.021**	yes	0	1272	10	1133	89.072327	Kb	0.61	68.01	0.25
220	YSYKYYNYFKL						**0.021**	no	0	380	11	318	83.6842105	Kb	0.669	35.92	0.1
221	SLYNYFFNLL						**0.020865**	no	0	3018	10	1805	59.8078197	Kb	0.717	21.49	0.05
222	FNFLFSNPM						**0.0205**	yes	1	341	9	320	93.8416422	Kb	0.755	14.17	0.03
223	VTYNFSKL						**0.02**	no	4	3472	8	1548	44.5852535	Kb	0.837	5.83	0.01
224	ICYSKYIGI						**0.02**	no	0	849	9	91	10.7184923	Kb	0.594	81.31	0.3
225	RCFKFFTFL						**0.02**	no	0	1228	9	1187	96.6612378	Kb	0.571	104.28	0.4
226	IAYAKFNDF						**0.02**	yes	0	1122	9	783	69.7860963	Kb	0.72	20.69	0.05
227	VSLININEV						**0.02**	yes	1	2081	9	1099	52.8111485	Db	0.733	17.97	0.05
228	YGILNLNNM						**0.02**	yes	1	1296	9	1021	78.7808642	Db	0.773	11.6	0.03
229	NALQNKASVV						**0.02**	no	0	255	10	88	34.5098039	Db	0.461	340.99	0.25
230	TMVIMTSTM						**0.02**	no	2	101	9	81	80.1980198	Db	0.453	371.82	0.3
231	VGMRHLNLL						**0.02**	no	0	148	9	55	37.1621622	Kb	0.671	34.96	0.1
232	YNISNDQVL						**0.02**	no	0	288	9	222	77.0833333	Db	0.59	84.45	0.12
233	IAILNNFEYI						**0.02**	no	0	5317	10	181	3.40417529	Db	0.843	5.44	0.01
234	YTIINDNEI						**0.02**	yes	0	1030	9	664	64.4660194	Db	0.76	13.35	0.03
235	YNYSFFYLYL						**0.02**	no	0	871	10	198	22.7324914	Kb	0.76	13.35	0.03
236	VIFTFYHILL						**0.02**	no	13	585	10	202	34.5299145	Kb	0.679	32.06	0.1
237	FGSQNYDTI						**0.02**	yes	1	2731	9	2023	74.0754302	Db	0.758	13.64	0.03
238	VSFVRILLL						**0.0195**	no	11	500	9	441	88.2	Kb	0.782	10.63	0.01
239	MMYLYNRL						**0.019**	no	0	3018	8	2024	67.064281	Kb	0.823	6.82	0.01
240	SMYYFSGL						**0.019**	no	0	489	8	44	8.99795501	Kb	0.85	5.07	0.01
241	ICYKRTSSL						**0.019**	yes	1	381	9	21	5.51181102	Kb	0.573	101.5	0.4
242	LCIEYFANL						**0.019**	no	0	1240	9	934	75.3225806	Kb	0.568	107.72	0.4
243	NCYNYANV						**0.019**	yes	0	2997	8	487	16.2495829	Kb	0.437	442.1	2
244	YCFHYFALM						**0.019**	no	1	1051	9	8	0.76117983	Kb	0.648	45.09	0.15

Novel Peptide number	Peptide sequence	NetMHCpan 4.0 data				Expression data											
											Microarray		SSH		Microarray	
		MHC allele restriction	1-log50k	nM	%Rank score	**TARUN 2008 EEF** Mass Spec (LS40-50h)	**LINDNER 2013 Spz** Mass Spec	**LASONDER 2008 spz** Mass Spec	**LASONDER 2008 Spz** (only day 18-22) Mass Spec	**LINDNER 2013 Spz** Mass Spec	**TARUN 2008 spz**	**Tarun 2008 EEF**	**UIS genes Matuschewski 2002** (oocyst spz v sg spz)	**S genes Kaiser 2004** (sg spz v merozoites)	**DSK hi** (highly downregulated in SLARP KO) **Silvie, unpublished**	**DSK lo** (lowly downregulated in SLARP KO) **Silvie, unpublished**
						+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-
						orthologs of *P. yoelii*		orthologs of *P. falciparum*			orthologs of *P. yoelii*				*P. berghei* origin	
202	VNYLYSNL	Kb	0.9379	1.9576	0.0034	-	-	-	-	-	-	-	-	-	+	-
203	IMYTYYFFTSL	Kb	0.8173	7.2186	0.0104	-	-	+	+	+	-	+	-	-	-	-
204	LTYNYYHPL	Kb	0.8999	2.9544	0.0055	+	+	-	-	+	-	+	-	-	-	-
205	HAINNIDEI	Db	0.8351	5.9576	0.005	-	-	-	-	+	-	-	-	-	-	-
206	TALRNYATL	Db	0.8254	6.6131	0.0053	-	+	-	-	+	-	-	-	-	-	-
207	YSILNDIFL	Db	0.8429	5.4719	0.0047	-	-	-	-	+	-	-	-	-	-	-
208	FCIYRYNNL	Kb	0.5216	177.0402	0.4201	+	-	-	-	-	-	-	-	-	-	-
209	LCFTFFPIL	Kb	0.6516	43.3848	0.109	-	+	-	-	+	-	-	-	-	-	-
210	VCFFCFTSL	Kb	0.672	34.7588	0.0874	-	-	+	-	-	-	-	-	-	-	-
211	GSISFLDYI	Db	0.5271	166.7706	0.0436	-	+	-	-	-	-	+	-	-	-	-
212	SIIIRHNEL	Kb	0.5155	189.1068	0.4493	+	+	+	+	+	-	+	-	-	-	-
213	VIFMIVFL	Kb	0.5508	129.0767	0.3255	-	+	-	-	-	-	-	-	-	-	-
214	KIYAFYNNL	Kb	0.8881	3.3566	0.0062	-	+	-	-	+	-	-	-	-	-	-
215	INYNYYDML	Kb	0.8858	3.4422	0.0063	-	+	-	-	+	-	-	-	-	-	-
216	VSYIRYYCSL	Kb	0.8618	4.4605	0.0076	-	-	-	-	-	-	+	-	-	-	-
217	YSLSNNEYYL	Db	0.7605	13.3528	0.0072	-	+	+	-	-	-	-	-	-	-	-
218	YSIENAVGI	Db	0.753	14.4809	0.0075	-	+	+	-	+	-	-	-	-	-	-
219	YLYNYFYKPL	Kb	0.6418	48.1887	0.1185	+	+	-	-	+	-	-	-	-	-	-
220	YSYKYYNYFKL	Kb	0.728	18.9723	0.0435	-	-	-	-	-	-	+	-	-	-	-
221	SLYNYFFNLL	Kb	0.7122	22.5106	0.0547	-	-	-	-	-	+	-	-	-	-	-
222	FNFLFSNPM	Kb	0.7396	16.7381	0.0366	+	+	+	+	+	-	+	-	-	-	-
223	VTYNFSKL	Kb	0.9104	2.636	0.005	-	-	-	-	-	-	-	-	-	-	+
224	ICYSKYIGI	Kb	0.608	69.4875	0.1753	-	+	-	-	+	-	-	-	-	-	-
225	RCFKFFTFL	Kb	0.7104	22.9627	0.0559	-	+	-	-	+	-	-	-	-	-	-
226	IAYAKFNDF	Kb	0.676	33.3157	0.0836	+	-	+	-	-	-	-	-	-	-	-
227	VSLININEV	Db	0.7345	17.6785	0.008	-	+	+	+	+	-	-	-	-	-	-
228	YGILNLNNM	Db	0.7695	12.1048	0.007	+	-	-	-	+	-	-	-	-	-	-
229	NALQNKASVV	Db	0.3624	991.2014	0.2372	-	+	-	-	-	-	+	-	-	-	-
230	TMVIMTSTM	Db	0.4056	621.2883	0.1565	-	+	-	-	-	-	-	-	-	-	-
231	VGMRHLNLL	Kb	0.6509	43.6749	0.1096	+	+	+	+	+	-	+	-	-	-	-
232	YNISNDQVL	Db	0.6012	74.7904	0.0221	+	+	+	+	+	-	+	-	-	-	-
233	IAILNNFEYI	Db	0.7922	9.4699	0.0063	-	+	+	-	-	-	-	-	-	-	-
234	YTIINDNEI	Db	0.7805	10.7546	0.0066	-	+	-	-	-	-	-	-	-	-	-
235	YNYSFFYLYL	Kb	0.775	11.4078	0.0222	-	+	-	-	-	-	-	-	-	-	-
236	VIFTFYHILL	Kb	0.7467	15.5043	0.0332	-	+	+	-	+	-	-	-	-	-	-
237	FGSQNYDTI	Db	0.7127	22.3809	0.0087	-	+	-	-	-	-	-	-	-	-	-
238	VSFVRILLL	Kb	0.7761	11.276	0.0219	+	+	+	-	+	-	-	-	-	-	-
239	MMYLYNRL	Kb	0.8691	4.1212	0.0072	-	-	-	-	-	+	-	-	-	-	-
240	SMYYFSGL	Kb	0.8971	3.0443	0.0057	-	-	-	-	+	-	-	-	-	-	-
241	ICYKRTSSL	Kb	0.6038	72.7031	0.1842	-	-	-	-	+	-	+	-	-	-	-
242	LCIEYFANL	Kb	0.7308	18.4047	0.0413	-	-	-	-	+	-	-	-	-	-	-
243	NCYNYANV	Kb	0.4647	327.5546	0.7298	+	+	-	-	+	-	+	-	-	-	-
244	YCFHYFALM	Kb	0.6693	35.795	0.09	-	+	-	-	-	+	+	+	-	-	-

Novel Peptide number	Peptide sequence	Top NetMHCpan peptides <22nM and <0.05 Rank %	NetMHCpan other peptides 22.381-388.44nM but always %Rank of 0.5 or below	Top NetMHCcons NC and C2	Top NetMHCcons peptides <22nM and <0.05 Rank %	NetMHCcons other peptides 23.05nM-531.38nM but always %Rank of 0.5 or below	Batch purity restimulation % IFN-γ+ CD11a+ / total CD8+ T cells	Signal peptide predicted	No. of transmembrane domains predicted	Protein length	Peptide length	Peptide start position	Percentile of protein	NetMHCcons 1.1 data			
														MHC allele restriction	1-log50k	nM	%Rank score
245	IIIRHNEL						**0.019**	no	0	288	8	152	52.7777778	Kb	0.601	74.57	0.3
246	NALQNKASV						**0.019**	no	0	255	9	88	34.5098039	Db	0.586	88.66	0.12
247	KSYSYFSGL						**0.019**	no	0	812	9	430	52.955665	Kb	0.847	5.24	0.01
248	RSFFYYRL						**0.019**	yes	0	2997	8	2896	96.6299633	Kb	0.806	8.11	0.01
249	YTYPYYNL						**0.019**	yes	8	5371	8	3996	74.3995532	Kb	0.818	7.16	0.01
250	SSIINSITL						**0.019**	no	0	367	9	168	45.7765668	Db	0.867	4.24	0.01
251	STYLYWLYL						**0.019**	no	0	2623	9	1876	71.521159	Kb	0.781	10.69	0.01
252	SSYTHYISKL						**0.019**	no	3	646	10	469	72.6006192	Kb	0.725	19.7	0.05
253	ASINNTAFV						**0.019**	no	1	689	9	594	86.2119013	Db	0.816	7.36	0.01
254	FTLINIPYI						**0.019**	yes	10	2361	9	2191	92.7996612	Db	0.756	13.94	0.05
255	VSFMYSRKLKL						**0.019**	yes	1	3204	11	1982	61.8601748	Kb	0.633	53.32	0.17
256	YSYLYLPL						**0.01869**	no	10	376	8	98	26.0638298	Kb	0.843	5.47	0.01
257	TSISNDNVIYI						**0.01867**	yes	0	506	11	326	64.4268775	Db	0.646	46.07	0.07
258	IVYTHFYNL						**0.0185**	yes	1	1296	9	429	33.1018519	Kb	0.81	7.77	0.01
259	ITYYYKNL						**0.018**	no	0	647	8	495	76.5069552	Kb	0.823	6.79	0.01
260	SIYFFMAL						**0.018**	no	0	1194	8	453	37.9396985	Kb	0.828	6.47	0.01
261	YSFLNPNYI						**0.018**	no	3	1429	9	31	2.1693492	Db	0.875	3.89	0.01
262	ICYEFQQEL						**0.018**	no	0	2704	9	2563	94.785503	Kb	0.606	71.02	0.25
263	TCYLFFGGF						**0.018**	yes	4	262	9	237	90.4580153	Kb	0.609	68.38	0.25
264	IIYFFSKI						**0.018**	yes	0	1796	8	1705	94.9331849	Kb	0.75	14.87	0.03
265	ISILNDTFL						**0.018**	yes	2	1671	9	795	47.5763016	Db	0.853	4.91	0.01
266	YSFNFHNTF						**0.018**	yes	4	1785	9	521	29.1876751	Kb	0.742	16.22	0.03
267	VSFYHFSNL						**0.018**	no	0	3439	9	1342	39.0229718	Kb	0.863	4.38	0.01
268	VNYRHLSIL						**0.018**	no	0	2310	9	1856	80.3463203	Kb	0.788	9.91	0.01
269	YNYKFFLL						**0.018**	yes	4	277	8	150	54.1516245	Kb	0.813	7.56	0.01
270	SIFNFIYL						**0.018**	yes	0	1218	8	118	9.68801314	Kb	0.791	9.54	0.01
271	YMFKNINPCYL						**0.018**	yes	0	824	11	500	60.6796117	Db	0.685	30.05	0.07
272	SSYYYYDNM						**0.017895**	no	0	824	9	753	91.3834951	Kb	0.823	6.79	0.01
273	NSIFNFIYL						**0.0175**	yes	0	1218	9	117	9.60591133	Db	0.78	10.81	0.03
274	SGYNNFTYL						**0.0175**	yes	10	2361	9	2063	87.3782296	Db	0.73	18.67	0.05
275	INFYFSML						**0.017**	no	0	943	8	754	79.9575822	Kb	0.851	5.04	0.01
276	ISYRHYSLL						**0.017**	yes	7	628	9	328	52.2292994	Kb	0.881	3.6	0.01
277	SSLSNFNYL						**0.017**	no	0	3796	9	278	7.32349842	Db	0.875	3.85	0.01
278	YSFYFYTFL						**0.017**	no	1	1563	9	236	15.0991683	Kb	0.831	6.22	0.01
279	NCLNYSKL						**0.017**	no	0	349	8	325	93.1232092	Kb	0.232	4062.65	10
280	NCYHYFFHL						**0.017**	no	0	6471	9	3756	58.043579	Kb	0.577	97.73	0.4
281	SCYKYNNLL						**0.017**	no	0	1650	9	116	7.03030303	Kb	0.592	82.64	0.3
282	SVYDFYFNL						**0.017**	no	2	7480	9	1340	17.9144385	Kb	0.844	5.41	0.01
283	FSLKNLNTM						**0.017**	no	0	1519	9	1261	83.0151415	Db	0.862	4.45	0.01
284	IIFDHFMNM						**0.017**	no	0	607	9	248	40.8566722	Kb	0.78	10.87	0.01
285	FSFNFLNNL						**0.017**	yes	8	5371	9	1679	31.2604729	Kb	0.782	10.52	0.01
286	VSYLKHFAM						**0.017**	no	0	778	9	537	69.0231362	Kb	0.777	11.17	0.01
287	TAHLNDHYI						**0.017**	yes	1	472	9	310	65.6779661	Db	0.816	7.32	0.01

Novel Peptide number	Peptide sequence	NetMHCpan 4.0 data				Expression data											
											Microarray		SSH		Microarray	
		MHC allele restriction	1-log50k	nM	%Rank score	**TARUN 2008 EEF** Mass Spec (LS40-50h)	**LINDNER 2013 Spz** Mass Spec	**LASONDER 2008 spz** Mass Spec	**LASONDER 2008 Spz** (only day 18-22) Mass Spec	**LINDNER 2013 Spz** Mass Spec	**TARUN 2008 spz**	**Tarun 2008 EEF**	**UIS genes Matuschewski 2002** (oocyst spz v sg spz)	**S genes Kaiser 2004** (sg spz v merozoites)	**DSK hi** (highly downregulated in SLARP KO) **Silvie, unpublished**	**DSK lo** (lowly downregulated in SLARP KO) **Silvie, unpublished**
						+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-
						orthologs of *P. yoelii*		orthologs of *P. falciparum*			orthologs of *P. yoelii*				*P. berghei* origin	
245	IIIRHNEL	Kb	0.5877	86.6073	0.2232	+	+	+	+	+	-	+	-	-	-	-
246	NALQNKASV	Db	0.5356	152.1599	0.0405	-	+	-	-	-	-	+	-	-	-	-
247	KSYSYFSGL	Kb	0.9065	2.7494	0.0052	-	+	-	-	-	-	-	-	-	-	-
248	RSFFYYRL	Kb	0.8916	3.2321	0.006	+	+	-	-	+	-	+	-	-	-	-
249	YTYPYYNL	Kb	0.8729	3.957	0.007	-	-	+	-	+	+	-	-	-	-	-
250	SSIINSITL	Db	0.8463	5.2739	0.0046	-	+	-	-	+	-	+	-	-	-	-
251	STYLYWLYL	Kb	0.7966	9.0283	0.0149	+	+	-	-	+	+	-	-	-	-	-
252	SSYTHYISKL	Kb	0.7845	10.2937	0.019	-	+	+	+	+	-	+	-	-	-	-
253	ASINNTAFV	Db	0.7541	14.3099	0.0074	-	-	-	-	-	-	+	-	-	-	+
254	FTLINIPYI	Db	0.7385	16.9284	0.0079	-	-	-	-	-	-	-	-	-	-	+
255	VSFMYSRKLKL	Kb	0.6685	36.1093	0.091	-	-	+	+	-	-	-	-	-	-	-
256	YSYLYLPL	Kb	0.893	3.1827	0.0059	-	-	-	-	+	-	+	-	-	-	-
257	TSISNDNVIYI	Db	0.5971	78.2277	0.023	+	+	+	-	+	-	-	-	-	-	-
258	IVYTHFYNL	Kb	0.8936	3.1626	0.0059	+	-	-	-	+	-	-	-	-	-	-
259	ITYYYKNL	Kb	0.9127	2.5716	0.0048	-	-	-	-	-	-	-	-	-	-	-
260	SIYFFMAL	Kb	0.8691	4.122	0.0072	-	+	-	-	+	-	-	-	-	-	-
261	YSFLNPNYI	Db	0.8768	3.7904	0.0037	-	-	-	-	+	-	-	-	-	-	-
262	ICYEFQQEL	Kb	0.6772	32.8682	0.0824	-	+	-	-	+	-	-	-	-	-	-
263	TCYLFFGGF	Kb	0.5963	78.9018	0.2003	+	+	-	-	-	-	+	-	-	-	-
264	IIYFFSKI	Kb	0.8538	4.8627	0.0081	-	+	-	-	+	+	-	-	-	-	-
265	ISILNDTFL	Db	0.8062	8.1379	0.0058	-	-	-	-	+	-	-	-	-	-	-
266	YSFNFHNTF	Kb	0.7053	24.2505	0.0591	-	-	-	-	+	-	-	-	-	-	-
267	VSFYHFSNL	Kb	0.932	2.0877	0.0038	-	+	-	-	+	-	-	-	-	-	-
268	VNYRHLSIL	Kb	0.8427	5.4872	0.0087	-	+	-	-	+	-	-	-	-	-	-
269	YNYKFFLL	Kb	0.8416	5.5501	0.0088	-	+	-	-	+	-	-	-	-	-	-
270	SIFNFIYL	Kb	0.8371	5.8298	0.009	+	-	+	+	+	-	-	-	-	-	-
271	YMFKNINPCYL	Db	0.6573	40.7577	0.0113	-	+	-	-	+	-	-	-	-	-	-
272	SSYYYYDNM	Kb	0.8775	3.7637	0.0068	-	-	-	-	-	-	-	-	-	-	+
273	NSIFNFIYL	Db	0.7615	13.2012	0.0072	+	-	+	+	+	-	-	-	-	-	-
274	SGYNNFTYL	Db	0.7471	15.4323	0.0076	-	-	-	-	-	-	-	-	-	-	+
275	INFYFSML	Kb	0.9277	2.1861	0.004	-	-	-	-	+	-	-	-	-	-	-
276	ISYRHYSLL	Kb	0.9237	2.2824	0.0042	-	-	-	-	+	-	-	-	-	-	-
277	SSLSNFNYL	Db	0.873	3.9497	0.0038	-	-	-	-	+	-	-	-	-	-	-
278	YSFYFYTFL	Kb	0.8784	3.7267	0.0067	-	-	-	-	+	-	-	-	-	-	-
279	NCLNYSKL	Kb	0.2666	2792.7485	4.266	-	-	-	-	+	-	+	-	-	-	+
280	NCYHYFFHL	Kb	0.6713	35.0227	0.0881	-	-	+	-	-	-	-	-	-	-	+
281	SCYKYNNLL	Kb	0.5878	86.5034	0.2229	-	-	+	+	-	-	-	-	-	-	-
282	SVYDFYFNL	Kb	0.8987	2.9922	0.0056	-	+	-	-	+	-	-	-	-	-	+
283	FSLKNLNTM	Db	0.8406	5.609	0.0048	-	+	-	-	+	-	-	-	-	-	-
284	IIFDHFMNM	Kb	0.8321	6.148	0.0093	-	+	-	-	-	-	-	-	-	-	-
285	FSFNFLNNL	Kb	0.8247	6.6636	0.0097	-	-	+	-	+	+	-	-	-	-	-
286	VSYLKHFAM	Kb	0.7857	10.1572	0.0186	-	+	-	-	+	-	-	-	-	-	-
287	TAHLNDHYI	Db	0.7776	11.0946	0.0067	-	+	+	+	+	-	-	-	-	-	-

Novel Peptide number	Peptide sequence	Top NetMHCpan peptides <22nM and <0.05 Rank %	NetMHCpan other peptides 22.381-388.44nM but always %Rank of 0.5 or below	Top NetMHCcons NC and C2	Top NetMHCcons peptides <22nM and <0.05 Rank %	NetMHCcons other peptides 23.05nM-531.38nM but always %Rank of 0.5 or below	Batch purity restimulation % IFN-γ+ CD11a+ / total CD8+ T cells	High purity restimulation % IFN-γ+ CD11a+ / total CD8+ T cells	Signal peptide predicted	No. of transmembrane domains predicted	Protein length	Peptide length	Peptide start position	Percentile of protein
288	SQYHRFLKL						**0.017**		no	0	1246	9	650	52.1669342
289	VMYYTYEL						**0.017**		yes	1	3204	8	498	15.5430712
290	YGATNYDEM						**0.017**		yes	8	5371	9	3019	56.209272
291	ITILNYLPL						**0.017**		yes	0	1030	9	304	29.5145631
292	SVLWFFYKPL						**0.017**		yes	0	2773	10	158	5.69780022
293	VAYSHEYIGHM						**0.017**		yes	0	1272	11	1243	97.7201258
294	SNYAYFTILNL						**0.016605**		yes	0	1354	11	691	51.0339734
295	SSYLSYYLLPL						**0.0165**		yes	2	395	11	231	58.4810127
296	FSYSCHKYLLL						**0.0165**		yes	1	1791	11	845	47.1803462
297	INYLFGTL						**0.016**		no	2	2004	8	1126	56.1876248
298	SAVLNFTIL						**0.016**		no	6	230	9	201	87.3913043
299	SSLSFGNYI						**0.016**		no	0	91	9	24	26.3736264
300	INFSYFYSL						**0.016**		no	0	2038	9	833	40.8734053
301	VTYQMYYSRL						**0.016**		yes	0	248	10	210	84.6774194
302	SSFFFFSKF						**0.016**		no	11	948	9	425	44.8312236
303	YSFIRFSIL						**0.016**		no	0	3439	9	942	27.3916836
304	VIYKKFILL						**0.016**		no	0	2775	9	1374	49.5135135
305	YSFAKKYNYL						**0.016**		yes	0	335	10	299	89.2537313
306	FSPRNYFEI						**0.0155**		no	8	4154	9	2168	52.1906596
307	SGISNFFFI						**0.01517**		no	0	742	9	286	38.5444744
308	FAYNKYAPL						**0.015**		no	9	1936	9	422	21.7975207
309	MAMLNGFTL						**0.015**		no	0	438	9	4	0.91324201
310	TSYFFFPFL						**0.015**		yes	3	1058	9	886	83.7429112
311	LCLRYYALL						**0.015**		no	3	2151	9	383	17.8056718
312	SCYLFISLI						**0.015**		no	6	951	9	350	36.8033649
313	VNPFYHYL						**0.015**		yes	1	2081	8	1737	83.4694858
314	VNVHFYINL						**0.015**		yes	2	429	9	353	82.2843823
315	INYSFSIFL						**0.015**		yes	1	720	9	698	96.9444444
316	YSMSNYEDI						**0.015**		no	0	1828	9	10	0.54704595
317	VNYINFNYL						**0.015**		no	0	697	9	225	32.2812052
318	FNIYNLDFI						**0.015**		yes	0	2150	9	728	33.8604651
319	LSYTRFNNF						**0.015**		no	0	855	9	590	69.005848
320	STYFFRSIPL						**0.015**		yes	16	1292	10	870	67.3374613
321	SSYFNCAPI						**0.015**		no	0	1057	9	522	49.385052
322	SMFFYLSFNL						**0.015**		yes	0	2150	10	1580	73.4883721
323	YSYKYFYNFIL						**0.015**		yes	0	1796	11	211	11.7483296
324	VVMNFYFLL						**0.01468**		no	0	1155	9	34	2.94372294
	irrelevant negative peptide (SIINFEKL)							**0.01468**						
325	RSFFYYRLL						**0.01465**		yes	0	2997	9	2896	96.6299633
326	LNYIRYNML						**0.01462**		no	0	1069	9	1044	97.6613658
327	ISYLNYLNL						**0.01457**		no	0	441	9	199	45.1247166
328	YSYQNVNTM						**0.014545**		no	16	988	9	28	2.8340081
329	YTYYFFTSL						**0.0145**		no	7	288	9	205	71.1805556
330	STYYYSML						**0.014**		no	0	426	8	359	84.2723005

Novel Peptide number	Peptide sequence	NetMHCcons 1.1 data				NetMHCpan 4.0 data				Expression data						
															Microarray	
		MHC allele restriction	1-log50k	nM	%Rank score	MHC allele restriction	1-log50k	nM	%Rank score	**TARUN 2008 EEF** Mass Spec (LS40-50h)	**LINDNER 2013 Spz** Mass Spec	**LASONDER 2008 spz** Mass Spec	**LASONDER 2008 Spz** (only day 18-22) Mass Spec	**LINDNER 2013 Spz** Mass Spec	**TARUN 2008 spz**	**Tarun 2008 EEF**
										+/-	+/-	+/-	+/-	+/-	+/-	+/-
										orthologs of *P. yoelii*		orthologs of *P. falciparum*			orthologs of *P. yoelii*	
288	SQYHRFLKL	Kb	0.756	14.09	0.03	Kb	0.7662	12.5458	0.0252	+	+	+	+	+	-	-
289	VMYYTYEL	Kb	0.687	29.56	0.07	Kb	0.7616	13.1841	0.0269	-	-	+	+	-	-	-
290	YGATNYDEM	Db	0.802	8.47	0.03	Db	0.7419	16.3306	0.0078	-	-	+	-	+	+	-
291	ITILNYLPL	Db	0.775	11.41	0.03	Db	0.7213	20.4	0.0084	-	+	-	-	-	-	-
292	SVLWFFYKPL	Kb	0.599	76.2	0.3	Kb	0.6916	28.1152	0.0715	-	+	-	-	-	-	-
293	VAYSHEYIGHM	Kb	0.606	71.02	0.25	Kb	0.6039	72.6403	0.184	+	+	-	-	+	-	-
294	SNYAYFTILNL	Kb	0.668	36.31	0.1	Kb	0.8295	6.3243	0.0094	+	+	-	-	+	-	-
295	SSYLSYYLLPL	Kb	0.633	52.74	0.17	Kb	0.7	25.6876	0.0638	+	+	+	+	+	-	-
296	FSYSCHKYLLL	Kb	0.591	83.99	0.3	Kb	0.5404	144.409	0.3564	+	-	-	-	+	-	-
297	INYLFGTL	Kb	0.833	6.12	0.01	Kb	0.914	2.5364	0.0048	+	+	-	-	+	-	-
298	SAVLNFTIL	Db	0.866	4.29	0.01	Db	0.8265	6.5339	0.0052	-	-	-	-	+	-	-
299	SSLSFGNYI	Db	0.461	340.99	0.25	Db	0.487	257.467	0.0673	-	-	-	-	+	-	-
300	INFSYFYSL	Kb	0.802	8.52	0.01	Kb	0.907	2.7351	0.0051	-	+	-	-	+	-	-
301	VTYQMYYSRL	Kb	0.685	30.21	0.07	Kb	0.8584	4.6299	0.0078	-	+	+	+	-	-	-
302	SSFFFFSKF	Kb	0.796	9.04	0.01	Kb	0.8279	6.4396	0.0095	-	+	-	-	-	-	-
303	YSFIRFSIL	Kb	0.776	11.35	0.01	Kb	0.8059	8.1645	0.0129	-	+	-	-	+	-	-
304	VIYKKFILL	Kb	0.792	9.49	0.01	Kb	0.8058	8.1774	0.0129	-	+	+	+	+	-	-
305	YSFAKKYNYL	Kb	0.595	80	0.3	Kb	0.6087	69.0043	0.1742	+	-	+	-	-	-	-
306	FSPRNYFEI	Db	0.837	5.83	0.01	Db	0.7631	12.9736	0.0071	+	-	-	-	+	-	-
307	SGISNFFFI	Db	0.844	5.41	0.01	Db	0.8438	5.4172	0.0047	+	-	-	-	-	-	-
308	FAYNKYAPL	Kb	0.85	5.07	0.01	Kb	0.8417	5.5457	0.0088	-	-	-	-	+	-	-
309	MAMLNGFTL	Db	0.868	4.17	0.01	Db	0.8755	3.8457	0.0038	-	-	-	-	+	-	-
310	TSYFFFPFL	Kb	0.831	6.26	0.01	Kb	0.8971	3.043	0.0057	-	-	-	-	-	+	-
311	LCLRYYALL	Kb	0.596	79.14	0.3	Kb	0.6616	38.8957	0.099	-	+	-	-	+	-	-
312	SCYLFISLI	Kb	0.557	120.03	0.5	Kb	0.5682	106.856	0.2756	-	+	-	-	+	-	-
313	VNPFYHYL	Kb	0.736	17.49	0.05	Kb	0.7967	9.0203	0.0149	-	+	+	+	+	-	-
314	VNVHFYINL	Kb	0.786	10.07	0.01	Kb	0.8703	4.0673	0.0072	-	-	+	-	-	-	-
315	INYSFSIFL	Kb	0.796	9.09	0.01	Kb	0.863	4.4023	0.0076	-	+	+	-	+	-	-
316	YSMSNYEDI	Db	0.883	3.55	0.01	Db	0.8535	4.8784	0.0044	-	+	-	-	+	-	-
317	VNYINFNYL	Kb	0.797	8.94	0.01	Kb	0.8284	6.4057	0.0095	-	+	-	-	+	-	-
318	FNIYNLDFI	Db	0.774	11.6	0.03	Db	0.7868	10.046	0.0064	-	+	-	-	-	-	-
319	LSYTRFNNF	Kb	0.774	11.53	0.03	Kb	0.7517	14.6821	0.0308	-	+	-	-	+	-	-
320	STYFFRSIPL	Kb	0.755	14.17	0.03	Kb	0.7367	17.271	0.038	-	+	-	-	+	-	-
321	SSYFNCAPI	Db	0.76	13.42	0.03	Db	0.7352	17.6	0.008	-	+	-	-	+	-	-
322	SMFFYLSFNL	Kb	0.698	26.39	0.07	Kb	0.7237	19.8754	0.0468	-	+	-	-	-	-	-
323	YSYKYFYNFIL	Kb	0.631	53.9	0.2	Kb	0.7186	21.0134	0.0507	-	+	-	-	+	+	-
324	VVMNFYFLL	Kb	0.801	8.61	0.01	Kb	0.8091	7.8923	0.0122	+	+	-	-	-	-	-
	irrelevant negative peptide (SIINFEKL)															
325	RSFFYYRLL	Kb	0.796	9.09	0.01	Kb	0.8802	3.6565	0.0066	+	+	-	-	+	-	+
326	LNYIRYNML	Kb	0.801	8.57	0.01	Kb	0.8212	6.9238	0.0099	+	+	-	-	+	-	-
327	ISYLNYLNL	Kb	0.777	11.17	0.01	Kb	0.8721	3.9901	0.0071	+	-	-	-	-	-	-
328	YSYQNVNTM	Db	0.837	5.8	0.01	Db	0.8333	6.0702	0.005	+	+	-	-	+	-	-
329	YTYYFFTSL	Kb	0.833	6.09	0.01	Kb	0.8841	3.5032	0.0064	-	-	+	+	+	-	+
330	STYYYSML	Kb	0.853	4.93	0.01	Kb	0.8983	3.0057	0.0056	-	+	-	-	+	-	-

Novel Peptide number	Peptide sequence	Expression data			
		SSH		Microarray	
		UIS genes Matuschewski 2002 (oocyst spz v sg spz)	**S genes Kaiser 2004** (sg spz v merozoites)	**DSK hi** (highly downregulated in SLARP KO) **Silvie, unpublished**	**DSK lo** (lowly downregulated in SLARP KO) **Silvie, unpublished**
		+/-	+/-	+/-	+/-
		orthologs of *P. yoelii*		*P. berghei* origin	
288	SQYHRFLKL	-	-	-	-
289	VMYYTYEL	-	-	-	-
290	YGATNYDEM	-	-	-	-
291	ITILNYLPL	-	-	-	-
292	SVLWFFYKPL	-	-	-	-
293	VAYSHEYIGHM	-	-	-	-
294	SNYAYFTILNL	-	-	-	-
295	SSYLSYYLLPL	-	-	-	-
296	FSYSCHKYLLL	-	-	-	-
297	INYLFGTL	-	-	-	-
298	SAVLNFTIL	-	-	-	-
299	SSLSFGNYI	-	-	-	-
300	INFSYFYSL	-	-	-	-
301	VTYQMYYSRL	-	-	-	-
302	SSFFFFSKF	-	-	-	-
303	YSFIRFSIL	-	-	-	-
304	VIYKKFILL	-	-	-	-
305	YSFAKKYNYL	-	-	-	-
306	FSPRNYFEI	-	-	-	-
307	SGISNFFFI	-	-	-	-
308	FAYNKYAPL	-	-	-	-
309	MAMLNGFTL	-	-	-	-
310	TSYFFFPFL	-	-	-	-
311	LCLRYYALL	-	-	-	+
312	SCYLFISLI	-	-	-	-
313	VNPFYHYL	-	-	-	-
314	VNVHFYINL	-	-	-	-
315	INYSFSIFL	-	-	-	-
316	YSMSNYEDI	-	-	-	-
317	VNYINFNYL	-	-	-	-
318	FNIYNLDFI	-	-	-	-
319	LSYTRFNNF	-	-	-	-
320	STYFFRSIPL	-	-	-	-
321	SSYFNCAPI	-	-	-	+
322	SMFFYLSFNL	-	-	-	-
323	YSYKYFYNFIL	-	-	-	-
324	VVMNFYFLL	-	-	-	+
	irrelevant negative peptide (SIINFEKL)				
325	RSFFYYRLL	-	-	-	-
326	LNYIRYNML	-	-	-	+
327	ISYLNYLNL	-	-	-	-
328	YSYQNVNTM	-	-	-	-
329	YTYYFFTSL	-	-	-	-
330	STYYYSML	-	-	-	-

Novel Peptide number	Peptide sequence	Top NetMHCpan peptides <22nM and <0.05 Rank %	NetMHCpan other peptides 22.381-388.44nM but always %Rank of 0.5 or below	Top NetMHCcons NC and C2	Top NetMHCcons peptides <22nM and <0.05 Rank %	NetMHCcons other peptides 23.05nM-531.38nM but always %Rank of 0.5 or below	Batch purity restimulation % IFN-γ+ CD11a+ / total CD8+ T cells	Signal peptide predicted	No. of transmembrane domains predicted	Protein length	Peptide length	Peptide start position	Percentile of protein	NetMHCcons 1.1 data			
														MHC allele restriction	1-log50k	nM	%Rank score
331	ICFPFFNRL						**0.014**	no	0	2038	9	408	20.01962709	Kb	0.717	21.37	0.05
332	ICYKYFDVL						**0.014**	no	0	447	9	236	52.79642058	Kb	0.577	97.73	0.4
333	MCYDHCSAL						**0.014**	yes	1	2081	9	1757	84.43056223	Kb	0.545	136.67	0.5
334	SCYSYINTM						**0.014**	no	1	940	9	590	62.76595745	Kb	0.616	64.08	0.25
335	SVFDYFTSF						**0.014**	yes	1	2096	9	199	9.494274809	Kb	0.752	14.63	0.03
336	KMFVNLSGF						**0.014**	no	0	258	9	210	81.39534884	Kb	0.568	107.72	0.4
337	VLYLKFCNF						**0.014**	no	0	174	9	160	91.95402299	Kb	0.649	44.36	0.15
338	INYNFYSML						**0.014**	no	3	1115	9	1012	90.76233184	Kb	0.877	3.76	0.01
339	MAITNILTI						**0.014**	no	0	1153	9	968	83.95490026	Db	0.89	3.29	0.01
340	YNYTFQAL						**0.014**	yes	0	1272	8	525	41.27358491	Kb	0.804	8.29	0.01
341	ISLVNNYVYI						**0.014**	no	4	2562	10	1330	51.91256831	Db	0.821	6.9	0.01
342	VIYAHILNL						**0.0135**	yes	2	1349	9	446	33.06152706	Kb	0.75	15.03	0.03
343	LAYAYYSSL						**0.0135**	no	0	655	9	582	88.85496183	Kb	0.814	7.48	0.01
344	VSYTRYASEM						**0.0135**	no	0	70	10	6	8.571428571	Kb	0.679	32.24	0.1
345	INYNFINL						**0.013485**	yes	0	495	8	320	64.64646465	Kb	0.843	5.5	0.01
346	STYVFFPPI						**0.01324**	yes	5	420	9	406	96.66666667	Kb	0.818	7.16	0.01
347	SVYFFYAYL						**0.013**	no	4	3976	9	3108	78.16901408	Kb	0.863	4.38	0.01
348	MCYLYTLLL						**0.013**	no	4	1504	9	953	63.3643617	Kb	0.573	102.05	0.4
349	VCWRHFLAM						**0.013**	no	0	478	9	415	86.82008368	Kb	0.569	105.99	0.4
350	RGYDTFMNL						**0.013**	no	0	83	9	36	43.37349398	Kb	0.598	77.44	0.3
351	LSYKFFPEL						**0.013**	no	0	572	9	245	42.83216783	Kb	0.819	7.13	0.01
352	KAINNFEFM						**0.013**	no	12	1088	9	641	58.91544118	Db	0.862	4.45	0.01
353	ISIFHYPYL						**0.013**	no	0	915	9	675	73.7704918	Kb	0.805	8.2	0.01
354	AALCNQWYI						**0.013**	no	0	1249	9	623	49.87990392	Db	0.875	3.85	0.01
355	MSLVNNAYI						**0.013**	no	0	1801	9	462	25.65241532	Db	0.885	3.45	0.01
356	SSYFHFSFI						**0.013**	no	0	631	9	13	2.06022187	Kb	0.791	9.54	0.01
357	IAYFRSSNL						**0.013**	no	0	2840	9	770	27.11267606	Kb	0.797	8.94	0.01
358	VSFYKYNSM						**0.013**	no	0	811	9	381	46.97903822	Kb	0.82	7.01	0.01
359	IAFMFFLNSL						**0.013**	no	3	341	10	51	14.95601173	Kb	0.713	22.32	0.05
360	TSVINTDLL						**0.013**	yes	0	989	9	486	49.14054601	Db	0.803	8.38	0.03
361	VAYYFTYHSYM						**0.013**	no	0	1073	11	262	24.41752097	Kb	0.726	19.39	0.05
362	SSMFFYLSF						**0.013**	yes	0	2150	9	1579	73.44186047	Kb	0.75	15.03	0.03
363	FSFQFYHFTSF						**0.013**	yes	2	896	11	194	21.65178571	Kb	0.613	65.84	0.25
364	YANKNNNLQFL						**0.013**	no	0	343	11	87	25.36443149	Db	0.648	45.09	0.07
365	YSYFYFQNNL						**0.012895**	no	7	5611	10	3535	63.00124755	Kb	0.724	19.92	0.05
366	ITYSRQPHL						**0.012665**	no	0	167	9	103	61.67664671	Kb	0.786	10.18	0.01
367	RTLSNFTFI						**0.01265**	yes	0	1304	9	875	67.10122699	Db	0.816	7.32	0.01
368	SAIVNISLV						**0.012525**	no	16	988	9	362	36.63967611	Db	0.858	4.65	0.01
369	HSMSNHVPM						**0.01219**	no	0	3296	9	1074	32.58495146	Db	0.861	4.5	0.01
370	LAYAYYSSLL						**0.012135**	no	0	655	10	582	88.85496183	Kb	0.701	25.41	0.07
371	RALENYTNI						**0.012105**	no	0	1986	9	731	36.80765358	Db	0.875	3.89	0.01
372	CMFSFFSYL						**0.012**	yes	2	259	9	239	92.27799228	Kb	0.838	5.77	0.01
373	SSLVNREFI						**0.012**	no	0	1005	9	985	98.00995025	Db	0.915	2.51	0.01

Novel Peptide number	Peptide sequence	NetMHCpan 4.0 data				Expression data											
											Microarray		SSH		Microarray	
		MHC allele restriction	1-log50k	nM	%Rank score	**TARUN 2008 EEF** Mass Spec (LS40-50h)	**LINDNER 2013 Spz** Mass Spec	**LASONDER 2008 spz** Mass Spec	**LASONDER 2008 Spz** (only day 18-22) Mass Spec	**LINDNER 2013 Spz** Mass Spec	**TARUN 2008 spz**	**Tarun 2008 EEF**	**UIS genes Matuschewski 2002** (oocyst spz v sg spz)	**S genes Kaiser 2004** (sg spz v merozoites)	**DSK hi** (highly downregulated in SLARP KO) **Silvie, unpublished**	**DSK lo** (lowly downregulated in SLARP KO) **Silvie, unpublished**
						+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-
						orthologs of *P. yoelii*		orthologs of *P. falciparum*			orthologs of *P. yoelii*				*P. berghei* origin	
331	ICFPFFNRL	Kb	0.8342	6.015	0.0092	-	+	-	-	+	-	-	-	-	-	-
332	ICYKYFDVL	Kb	0.7062	24.008	0.0585	-	-	-	-	+	-	+	-	-	-	-
333	MCYDHCSAL	Kb	0.5326	157.0695	0.3795	-	+	+	+	+	-	-	-	-	-	-
334	SCYSYINTM	Kb	0.649	44.5869	0.1115	-	-	-	-	+	-	-	-	-	-	-
335	SVFDYFTSF	Kb	0.7994	8.7614	0.0143	-	-	-	-	+	-	-	-	-	-	-
336	KMFVNLSGF	Kb	0.4877	255.3201	0.5884	+	-	-	-	-	-	-	-	-	-	-
337	VLYLKFCNF	Kb	0.6757	33.3991	0.0838	-	+	-	-	-	-	-	-	-	-	+
338	INYNFYSML	Kb	0.9404	1.9053	0.0033	-	-	-	-	-	-	-	-	-	-	+
339	MAITNILTI	Db	0.8657	4.2761	0.004	-	+	-	-	+	-	-	-	-	-	-
340	YNYTFQAL	Kb	0.8442	5.3939	0.0086	+	+	-	-	+	-	-	-	-	-	-
341	ISLVNNYVYI	Db	0.6688	35.9876	0.01	-	-	-	-	+	-	-	-	-	-	+
342	VIYAHILNL	Kb	0.8192	7.0698	0.01	+	-	-	-	+	-	-	-	-	-	-
343	LAYAYYSSL	Kb	0.9052	2.7894	0.0052	-	-	-	-	-	-	+	-	-	-	-
344	VSYTRYASEM	Kb	0.7769	11.1745	0.0216	-	+	+	-	+	-	+	-	-	-	-
345	INYNFINL	Kb	0.9222	2.3202	0.0043	-	-	-	-	-	+	-	-	-	-	-
346	STYVFFPPI	Kb	0.8783	3.7304	0.0067	-	-	-	-	-	-	+	-	-	-	+
347	SVYFFYAYL	Kb	0.89	3.2885	0.0061	-	-	-	-	+	-	-	-	-	-	-
348	MCYLYTLLL	Kb	0.5592	117.9035	0.3006	+	-	-	-	-	-	-	-	-	-	-
349	VCWRHFLAM	Kb	0.5924	82.3221	0.2107	-	-	-	-	-	-	+	-	-	-	-
350	RGYDTFMNL	Kb	0.7266	19.2606	0.0446	-	+	+	-	+	-	+	-	-	-	-
351	LSYKFFPEL	Kb	0.8919	3.2218	0.006	-	+	-	-	+	-	-	-	-	-	-
352	KAINNFEFM	Db	0.877	3.7845	0.0037	-	+	+	+	+	-	-	-	-	-	-
353	ISIFHYPYL	Kb	0.8625	4.4255	0.0076	-	+	-	-	+	-	-	-	-	-	-
354	AALCNQWYI	Db	0.856	4.7513	0.0043	-	+	+	+	+	-	-	-	-	-	-
355	MSLVNNAYI	Db	0.8559	4.7562	0.0043	-	-	+	-	+	-	-	-	-	-	-
356	SSYFHFSFI	Kb	0.8495	5.0967	0.0083	-	+	+	+	+	-	-	-	-	-	-
357	IAYFRSSNL	Kb	0.8399	5.6536	0.0089	-	+	-	-	+	-	-	-	-	-	-
358	VSFYKYNSM	Kb	0.8219	6.8697	0.0099	-	+	-	-	+	-	-	-	-	-	-
359	IAFMFFLNSL	Kb	0.8147	7.4251	0.011	-	-	+	-	+	-	-	-	-	-	-
360	TSVINTDLL	Db	0.7496	15.0122	0.0076	-	+	-	-	+	-	-	-	-	-	-
361	VAYYFTYHSYM	Kb	0.7322	18.1357	0.0402	-	+	-	-	+	-	-	-	-	-	-
362	SSMFFYLSF	Kb	0.7221	20.2177	0.0481	-	+	-	-	-	-	-	-	-	-	-
363	FSFQFYHFTSF	Kb	0.5516	127.9255	0.3231	-	+	+	-	+	-	-	-	-	-	-
364	YANKNNNLQFL	Db	0.5123	195.8389	0.0489	-	+	-	-	+	-	-	-	-	-	-
365	YSYFYFQNNL	Kb	0.8214	6.9052	0.0099	-	-	-	-	-	-	-	-	-	-	+
366	ITYSRQPHL	Kb	0.776	11.2833	0.0219	+	+	-	-	+	-	+	-	-	-	-
367	RTLSNFTFI	Db	0.7682	12.2865	0.007	+	-	-	-	-	+	-	-	-	-	-
368	SAIVNISLV	Db	0.8354	5.9341	0.005	+	+	-	-	+	-	-	-	-	-	-
369	HSMSNHVPM	Db	0.8386	5.7361	0.0049	-	+	+	+	+	-	-	-	-	-	-
370	LAYAYYSSLL	Kb	0.7903	9.6666	0.017	-	-	-	-	-	-	+	-	-	-	-
371	RALENYTNI	Db	0.7955	9.1365	0.0062	+	+	+	+	+	-	-	-	-	-	-
372	CMFSFFSYL	Kb	0.8312	6.2133	0.0093	-	-	-	-	+	-	-	-	-	-	-
373	SSLVNREFI	Db	0.8791	3.6988	0.0036	-	-	-	-	+	-	-	-	-	-	-

Novel Peptide number	Peptide sequence	Top NetMHCpan peptides <22nM and <0.05 Rank %	NetMHCpan other peptides 22.381-388.44nM but always %Rank of 0.5 or below	Top NetMHCcons NC and C2	Top NetMHCcons peptides <22nM and <0.05 Rank %	NetMHCcons other peptides 23.05nM-531.38nM but always %Rank of 0.5 or below	Batch purity restimulation % IFN-γ+ CD11a+ / total CD8+ T cells	Signal peptide predicted	No. of transmembrane domains predicted	Protein length	Peptide length	Peptide start position	Percentile of protein	NetMHCcons 1.1 data			
														MHC allele restriction	1-log50k	nM	%Rank score
374	VSYKYYDL						**0.012**	no	0	596	8	146	24.4966443	Kb	0.825	6.64	0.01
375	NCYYFYEI						**0.012**	no	4	608	8	137	22.53289474	Kb	0.319	1593.49	5
376	VCYIFFISF						**0.012**	no	22	3004	9	1391	46.30492676	Kb	0.583	91.09	0.4
377	IMYYFSIL						**0.012**	no	6	4789	8	296	6.180831071	Kb	0.83	6.33	0.01
378	YSIKNSYSL						**0.012**	no	2	519	9	479	92.29287091	Db	0.84	5.65	0.01
379	ANFNRYSFL						**0.012**	yes	0	255	9	1	0.392156863	Kb	0.782	10.52	0.01
380	YSIINNRFI						**0.012**	no	2	2434	9	1634	67.13229252	Db	0.841	5.56	0.01
381	NSMRNSETI						**0.012**	yes	0	786	9	367	46.69211196	Db	0.815	7.4	0.01
382	SSFLRLGLL						**0.012**	no	10	554	9	529	95.48736462	Kb	0.756	14.01	0.03
383	YAIKNSNYEIV						**0.012**	no	4	611	11	88	14.40261866	Db	0.713	22.32	0.05
384	YSIFNNDNEI						**0.012**	no	4	3053	10	2608	85.42417294	Db	0.801	8.61	0.03
385	VSYARHFLFM						**0.012**	yes	1	2081	10	1291	62.03748198	Kb	0.742	16.31	0.03
386	SSFLFLSNL						**0.011905**	no	0	1411	9	505	35.7902197	Kb	0.815	7.4	0.01
387	INYFAYYISYL						**0.011585**	yes	1	453	11	311	68.65342163	Kb	0.651	43.88	0.15
388	QTYPYYSTL						**0.0115**	no	0	243	9	174	71.60493827	Kb	0.806	8.11	0.01
389	ASYEFTTL						**0.011**	no	0	365	8	89	24.38356164	Kb	0.829	6.4	0.01
390	IIYRRYASL						**0.011**	no	0	146	9	56	38.35616438	Kb	0.832	6.19	0.01
391	SAMENYFVL						**0.011**	no	0	797	9	752	94.35382685	Db	0.884	3.51	0.01
392	SIYTFMRL						**0.011**	no	0	542	8	276	50.92250923	Kb	0.828	6.43	0.01
393	NCLIYSLL						**0.011**	no	0	349	8	232	66.4756447	Kb	0.27	2678.54	7
394	NCYNFGLV						**0.011**	no	0	501	8	219	43.71257485	Kb	0.304	1854.1	5
395	RCYSKYIYL						**0.011**	no	0	2418	9	728	30.10752688	Kb	0.622	59.73	0.25
396	SCFFFFYEM						**0.011**	no	0	1114	9	153	13.73429084	Kb	0.618	62.37	0.25
397	SCYSYSNLF						**0.011**	no	0	129	9	41	31.78294574	Kb	0.586	88.18	0.4
398	VNFFFMYL						**0.011**	no	1	918	8	119	12.96296296	Kb	0.838	5.77	0.01
399	KSIVNKDFI						**0.011**	no	0	649	9	242	37.28813559	Db	0.873	3.97	0.01
400	VMYFFGSSL						**0.011**	no	12	505	9	153	30.2970297	Kb	0.81	7.77	0.01
401	SSLQNVSFL						**0.011**	yes	3	628	9	75	11.94267516	Db	0.868	4.17	0.01
402	YSIPNSYSI						**0.011**	yes	0	1049	9	849	80.93422307	Db	0.824	6.75	0.01
403	STICNTDSI						**0.011**	no	0	2518	9	934	37.0929309	Db	0.788	9.91	0.03
404	FSFMNGVLI						**0.011**	no	13	585	9	222	37.94871795	Db	0.828	6.43	0.01
405	YSINNNEQL						**0.011**	yes	1	473	9	404	85.41226216	Db	0.767	12.44	0.03
406	YMYVNIFEI						**0.011**	no	10	2269	9	1278	56.32437197	Db	0.871	4.06	0.01
407	AAIHNANDLAL						**0.011**	yes	0	917	11	241	26.28135224	Db	0.648	45.09	0.07
408	FAFSFFNGL						**0.01069**	no	11	500	9	107	21.4	Kb	0.824	6.71	0.01
	no peptide restimulation						**0.010650367**										
409	YANKNYSSI						**0.01064**	yes	2	679	9	339	49.9263623	Db	0.786	10.07	0.03
410	VGYESFSPL						**0.010625**	no	0	131	9	15	11.45038168	Kb	0.782	10.58	0.01
411	FSYINYSNL						**0.0103375**	yes	1	1192	9	556	46.6442953	Kb	0.77	12.04	0.03
412	ICPSYYLKL						**0.01**	yes	0	908	9	229	25.22026432	Kb	0.556	122	0.5
413	NCIFYFLL						**0.01**	no	4	3976	8	176	4.426559356	Kb	0.296	2021.74	6
414	KSVENPTEI						**0.01**	yes	0	1406	9	94	6.685633001	Db	0.75	14.95	0.05
415	SSYIYFLL						**0.01**	yes	11	1035	8	899	86.85990338	Kb	0.846	5.29	0.01

Novel Peptide number	Peptide sequence	NetMHCpan 4.0 data				Expression data											
											Microarray		SSH		Microarray	
		MHC allele restriction	1-log50k	nM	%Rank score	**TARUN 2008 EEF** Mass Spec (LS40-50h)	**LINDNER 2013 Spz** Mass Spec	**LASONDER 2008 spz** Mass Spec	**LASONDER 2008 Spz** (only day 18-22) Mass Spec	**LINDNER 2013 Spz** Mass Spec	**TARUN 2008 spz**	**Tarun 2008 EEF**	**UIS genes Matuschewski 2002** (oocyst spz v sg spz)	**S genes Kaiser 2004** (sg spz v merozoites)	**DSK hi** (highly downregulated in SLARP KO) **Silvie, unpublished**	**DSK lo** (lowly downregulated in SLARP KO) **Silvie, unpublished**
						+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-
						orthologs of *P. yoelii*		orthologs of *P. falciparum*			orthologs of *P. yoelii*				*P. berghei* origin	
374	VSYKYYDL	Kb	0.8961	3.0762	0.0057	-	-	-	-	+	-	-	-	-	-	-
375	NCYYFYEI	Kb	0.4334	459.5107	0.9427	-	+	-	-	+	-	-	-	-	-	-
376	VCYIFFISF	Kb	0.4978	229.0651	0.5367	-	-	+	+	-	-	-	-	-	-	-
377	IMYYFSIL	Kb	0.8813	3.6112	0.0066	-	-	+	+	+	-	-	-	-	-	-
378	YSIKNSYSL	Db	0.8388	5.7221	0.0049	-	+	-	-	+	-	-	-	-	-	-
379	ANFNRYSFL	Kb	0.8264	6.5408	0.0096	-	+	+	+	+	-	-	-	-	-	-
380	YSIINNRFI	Db	0.8134	7.5271	0.0056	-	+	-	-	+	-	-	-	-	-	-
381	NSMRNSETI	Db	0.8123	7.6178	0.0057	+	+	-	-	+	-	-	-	-	-	-
382	SSFLRLGLL	Kb	0.7581	13.7059	0.0283	-	+	-	-	-	-	-	-	-	-	-
383	YAIKNSNYEIV	Db	0.7283	18.9165	0.0082	-	+	-	-	+	-	-	-	-	-	-
384	YSIFNNDNEI	Db	0.7228	20.0629	0.0084	-	+	-	-	+	-	-	-	-	-	-
385	VSYARHFLFM	Kb	0.7167	21.448	0.0519	-	+	+	+	+	-	-	-	-	-	-
386	SSFLFLSNL	Kb	0.904	2.8252	0.0053	-	-	-	-	-	-	-	-	-	-	+
387	INYFAYYISYL	Kb	0.7772	11.1472	0.0215	+	+	+	-	+	-	-	-	-	-	-
388	QTYPYYSTL	Kb	0.8685	4.1505	0.0073	+	+	+	+	+	-	-	-	-	-	-
389	ASYEFTTL	Kb	0.9015	2.9032	0.0055	-	+	-	-	+	-	-	-	-	-	-
390	IIYRRYASL	Kb	0.8753	3.8552	0.0069	-	-	-	-	+	-	-	-	-	-	-
391	SAMENYFVL	Db	0.8911	3.2477	0.0033	-	-	-	-	+	-	-	-	-	-	-
392	SIYTFMRL	Kb	0.9059	2.7677	0.0052	-	+	-	-	+	+	+	-	-	-	-
393	NCLIYSLL	Kb	0.3542	1082.694	1.9291	+	+	+	-	+	-	-	-	-	-	-
394	NCYNFGLV	Kb	0.3572	1047.956	1.8854	+	+	+	+	+	-	-	-	-	-	-
395	RCYSKYIYL	Kb	0.6131	65.7385	0.1672	-	+	-	-	+	-	-	-	-	-	-
396	SCFFFFYEM	Kb	0.6484	44.8802	0.1121	-	-	-	-	+	-	-	-	-	-	-
397	SCYSYSNLF	Kb	0.5492	131.3236	0.3303	-	-	-	-	-	-	+	-	-	-	-
398	VNFFFMYL	Kb	0.9108	2.6254	0.0049	-	+	-	-	+	-	-	-	-	-	-
399	KSIVNKDFI	Db	0.8611	4.4935	0.0042	-	-	-	-	-	-	-	-	-	-	+
400	VMYFFGSSL	Kb	0.8493	5.1044	0.0083	-	+	-	-	+	-	+	-	-	-	-
401	SSLQNVSFL	Db	0.8457	5.3078	0.0047	-	+	+	-	+	-	+	-	-	-	-
402	YSIPNSYSI	Db	0.8223	6.8395	0.0054	-	-	+	+	-	-	-	-	-	-	-
403	STICNTDSI	Db	0.8183	7.1399	0.0055	-	-	-	-	-	-	-	-	-	-	+
404	FSFMNGVLI	Db	0.7852	10.2136	0.0065	-	+	+	-	+	-	-	-	-	-	-
405	YSINNNEQL	Db	0.7673	12.406	0.007	-	+	-	-	+	-	-	-	-	-	-
406	YMYVNIFEI	Db	0.7601	13.4036	0.0072	-	+	-	-	+	-	-	-	-	-	+
407	AAIHNANDLAL	Db	0.5566	121.2569	0.0345	+	+	-	-	+	-	-	-	-	-	-
408	FAFSFFNGL	Kb	0.8464	5.2669	0.0085	+	+	+	-	+	-	-	-	-	-	-
	no peptide restimula															
409	YANKNYSSI	Db	0.7631	12.9754	0.0071	+	-	-	-	-	-	+	-	-	-	-
410	VGYESFSPL	Kb	0.8498	5.081	0.0083	-	+	-	-	+	+	+	-	-	-	-
411	FSYINYSNL	Kb	0.8319	6.2	0.0093	+	+	-	-	+	-	-	-	-	-	-
412	ICPSYYLKL	Kb	0.6753	33.5571	0.0842	+	+	+	-	+	-	-	-	-	-	-
413	NCIFYFLL	Kb	0.3662	951.0274	1.7381	-	-	-	-	+	-	-	-	-	-	-
414	KSVENPTEI	Db	0.7052	24.2862	0.0089	+	-	+	-	-	-	-	-	-	-	-
415	SSYIYFLL	Kb	0.8986	2.994	0.0056	-	+	-	-	+	-	-	-	-	-	-

Novel Peptide number	Peptide sequence	Top NetMHCpan peptides <22nM and <0.05 Rank %	NetMHCpan other peptides 22.381-388.44nM but always %Rank of 0.5 or below	Top NetMHCcons NC and C2	Top NetMHCcons peptides <22nM and <0.05 Rank %	NetMHCcons other peptides 23.05nM-531.38nM but always %Rank of 0.5 or below	Batch purity restimulation % IFN-γ+ CD11a+ / total CD8+ T cells	Signal peptide predicted	No. of transmembrane domains predicted	Protein length	Peptide length	Peptide start position	Percentile of protein	NetMHCcons 1.1 data			
														MHC allele restriction	1-log50k	nM	%Rank score
416	VSYLFTPNM						**0.01**	no	5	1902	9	1744	91.69295478	Kb	0.81	7.77	0.01
417	TTFSFFFTL						**0.01**	no	5	399	9	379	94.98746867	Kb	0.794	9.24	0.01
418	TTYYFMLHL						**0.01**	no	0	3368	9	3183	94.50712589	Kb	0.806	8.16	0.01
419	SNYFRFRLAL						**0.01**	no	0	494	10	5	1.012145749	Kb	0.75	14.95	0.03
420	SAPNNNINPL						**0.01**	no	0	607	10	190	31.3014827	Db	0.77	11.98	0.03
421	KAMINDITI						**0.00998**	no	0	1192	9	703	58.97651007	Db	0.844	5.41	0.01
422	SSYKKFILL						**0.009976667**	no	0	5309	9	1102	20.75720475	Kb	0.823	6.79	0.01
423	YTILNDENM						**0.00995**	yes	1	2096	9	903	43.08206107	Db	0.736	17.49	0.05
424	SSYGKLMYFLM						**0.00995**	no	0	609	11	223	36.61740558	Kb	0.679	32.06	0.1
425	ISVENYPVI						**0.00993**	yes	1	1809	9	1280	70.75732449	Db	0.747	15.45	0.05
426	INYNYYLM						**0.0099**	no	0	1370	8	294	21.45985401	Kb	0.828	6.47	0.01
427	SNYRNFFLL						**0.00986**	no	0	4800	9	415	8.645833333	Kb	0.774	11.47	0.03
428	KNYNFIFL						**0.00979**	yes	4	1785	8	621	34.78991597	Kb	0.772	11.79	0.03
429	ITFLFYNIL						**0.00979**	yes	0	2150	9	1910	88.8372093	Kb	0.793	9.34	0.01
430	VNYHFSNYM						**0.00978**	yes	1	2081	9	811	38.97164825	Kb	0.826	6.61	0.01
431	YIYERYIRL						**0.00974**	no	0	2412	9	381	15.7960199	Kb	0.799	8.8	0.01
432	KIYGYFTLL						**0.00972**	no	0	1250	9	39	3.12	Kb	0.81	7.77	0.01
433	ASVVFQEL						**0.00969**	no	0	255	8	94	36.8627451	Kb	0.601	74.97	0.3
434	YGFKYYPL						**0.00962**	yes	1	1192	8	661	55.45302013	Kb	0.812	7.65	0.01
435	IGFNRFTTF						**0.009615**	no	0	541	9	490	90.57301294	Kb	0.76	13.35	0.03
436	VIFTFYHIL						**0.00961**	no	13	585	9	202	34.52991453	Kb	0.764	12.92	0.03
437	IVYYFYARM						**0.00955**	no	4	675	9	306	45.33333333	Kb	0.865	4.29	0.01
438	SSFYFFFNSL						**0.00953**	no	0	1415	10	472	33.35689046	Kb	0.79	9.7	0.01
439	VSFEFNNL						**0.0095**	no	6	4789	8	2356	49.19607434	Kb	0.836	5.93	0.01
440	HAIENIPAI						**0.00945**	no	0	349	9	155	44.41260745	Db	0.797	8.94	0.03
441	ISMSHYLYSTL						**0.00943**	no	12	1210	11	1119	92.47933884	Kb	0.629	55.08	0.2
442	INFNYFSLL						**0.00938**	no	2	524	9	441	84.16030534	Kb	0.864	4.36	0.01
443	ITYLYFNL						**0.00934**	no	0	2337	8	398	17.03038083	Kb	0.849	5.15	0.01
444	VSLSNLFYL						**0.00932**	no	0	1228	9	304	24.75570033	Db	0.827	6.5	0.01
445	IMFAFAGL						**0.00927**	no	6	283	8	199	70.3180212	Kb	0.833	6.09	0.01
446	YALENKSLL						**0.00927**	yes	0	479	9	257	53.65344468	Db	0.83	6.33	0.01
447	KTYLYYHTLL						**0.00925**	no	0	663	10	59	8.898944193	Kb	0.688	29.41	0.07
448	INYIHMCLFLL						**0.00922**	yes	0	748	11	544	72.72727273	Kb	0.617	63.05	0.25
449	KSYYFYISL						**0.00918**	no	0	1059	9	366	34.56090652	Kb	0.842	5.53	0.01
450	ITMSNIDYI						**0.00915**	no	0	410	9	387	94.3902439	Db	0.813	7.56	0.01
451	FAMKNNVDCI						**0.00914**	no	0	255	10	98	38.43137255	Db	0.779	10.93	0.03
452	FSLENNITEL						**0.00907**	yes	0	879	10	626	71.21729238	Db	0.792	9.44	0.03
453	MSYPFFPLL						**0.00902**	no	0	4287	9	3924	91.53254024	Kb	0.865	4.31	0.01
454	IMFERWNQL						**0.00895**	no	6	4789	9	3151	65.79661725	Kb	0.816	7.36	0.01
455	YSIFNVNAEII						**0.00887**	no	2	7126	11	1166	16.36261577	Db	0.657	40.68	0.07
456	KAVKNYVEI						**0.008725**	yes	0	776	9	472	60.82474227	Db	0.823	6.82	0.01
457	STYYYEYAM						**0.00869**	yes	0	3254	9	1912	58.75845114	Kb	0.737	17.21	0.05
458	VIFSRLSNF						**0.008675**	no	0	1384	9	843	60.91040462	Kb	0.748	15.28	0.03

Novel Peptide number	Peptide sequence	NetMHCpan 4.0 data				Expression data											
											Microarray		SSH		Microarray	
		MHC allele restriction	1-log50k	nM	%Rank score	**TARUN 2008 EEF** Mass Spec (LS40-50h)	**LINDNER 2013 Spz** Mass Spec	**LASONDER 2008 spz** Mass Spec	**LASONDER 2008 Spz** (only day 18-22) Mass Spec	**LINDNER 2013 Spz** Mass Spec	**TARUN 2008 spz**	**Tarun 2008 EEF**	**UIS genes Matuschewski 2002** (oocyst spz v sg spz)	**S genes Kaiser 2004** (sg spz v merozoites)	**DSK hi** (highly downregulated in SLARP KO) **Silvie, unpublished**	**DSK lo** (lowly downregulated in SLARP KO) **Silvie, unpublished**
						+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-
						orthologs of *P. yoelii*		orthologs of *P. falciparum*			orthologs of *P. yoelii*				*P. berghei* origin	
416	VSYLFTPNM	Kb	0.8832	3.5393	0.0065	-	+	-	-	+	-	-	-	-	-	-
417	TTFSFFFTL	Kb	0.86	4.5485	0.0077	-	+	+	-	+	+	-	-	-	-	-
418	TTYYFMLHL	Kb	0.8293	6.3383	0.0094	-	+	-	-	+	-	-	-	-	-	-
419	SNYFRFRLAL	Kb	0.8169	7.2514	0.0105	-	+	-	-	-	-	-	-	-	-	-
420	SAPNNNINPL	Db	0.6424	47.8919	0.013	-	-	-	-	+	-	-	-	-	-	-
421	KAMINDITI	Db	0.8371	5.8243	0.0049	-	+	-	-	-	-	-	-	-	-	-
422	SSYKKFILL	Kb	0.8357	5.9148	0.0091	+	+	-	-	+	-	-	-	-	-	-
423	YTILNDENM	Db	0.6846	30.3523	0.0095	-	-	-	-	+	-	-	-	-	-	-
424	SSYGKLMYFLM	Kb	0.5482	132.7579	0.3333	-	+	-	-	+	-	-	-	-	-	-
425	ISVENYPVI	Db	0.7045	24.4579	0.0089	-	-	-	-	+	-	-	-	-	-	+
426	INYNYYLM	Kb	0.8719	3.9999	0.0071	-	+	-	-	+	-	-	-	-	-	-
427	SNYRNFFLL	Kb	0.8136	7.5158	0.0112	-	+	-	-	+	-	-	-	-	-	-
428	KNYNFIFL	Kb	0.8367	5.8507	0.009	-	-	-	-	+	-	-	-	-	-	-
429	ITFLFYNIL	Kb	0.8518	4.9718	0.0082	-	+	-	-	-	-	-	-	-	-	-
430	VNYHFSNYM	Kb	0.8392	5.6943	0.0089	-	+	+	+	+	-	-	-	-	-	-
431	YIYERYIRL	Kb	0.781	10.6944	0.0203	-	+	-	-	+	-	-	-	-	-	-
432	KIYGYFTLL	Kb	0.8425	5.4975	0.0087	-	+	+	+	+	-	-	-	-	-	-
433	ASVVFQEL	Kb	0.702	25.123	0.0618	-	+	-	-	-	-	+	-	-	-	-
434	YGFKYYPL	Kb	0.8332	6.0781	0.0092	+	+	-	-	+	-	-	-	-	-	-
435	IGFNRFTTF	Kb	0.7512	14.7576	0.0311	-	+	-	-	+	-	-	+	-	-	-
436	VIFTFYHIL	Kb	0.8715	4.0152	0.0071	-	+	+	-	+	-	-	-	-	-	-
437	IVYYFYARM	Kb	0.899	2.983	0.0056	-	+	-	-	-	-	-	-	-	-	-
438	SSFYFFFNSL	Kb	0.8426	5.4902	0.0087	-	-	-	-	-	-	-	+	-	-	-
439	VSFEFNNL	Kb	0.9238	2.2811	0.0042	-	-	+	+	+	-	-	-	-	-	-
440	HAIENIPAI	Db	0.7467	15.4925	0.0076	+	+	+	-	+	-	-	-	-	-	-
441	ISMSHYLYSTL	Kb	0.6648	37.5724	0.0952	-	-	+	-	-	-	-	-	-	-	-
442	INFNYFSLL	Kb	0.9277	2.1872	0.004	-	-	+	-	-	-	-	-	-	-	-
443	ITYLYFNL	Kb	0.9194	2.3906	0.0045	-	-	+	+	-	-	-	-	-	-	-
444	VSLSNLFYL	Db	0.8209	6.9409	0.0054	-	+	-	-	+	-	-	-	-	-	-
445	IMFAFAGL	Kb	0.905	2.7937	0.0053	-	+	+	+	+	-	-	-	-	-	-
446	YALENKSLL	Db	0.809	7.8936	0.0058	-	-	+	+	+	-	-	-	-	-	-
447	KTYLYYHTLL	Kb	0.7478	15.316	0.0327	-	-	+	+	-	-	-	-	-	-	-
448	INYIHMCLFLL	Kb	0.5888	85.5336	0.2201	+	+	-	-	+	-	-	-	-	-	-
449	KSYYFYISL	Kb	0.8912	3.2467	0.006	-	+	-	-	+	-	-	-	-	-	-
450	ITMSNIDYI	Db	0.8368	5.8443	0.0049	-	+	+	+	+	-	-	-	-	-	-
451	FAMKNNVDCI	Db	0.723	20.0252	0.0084	-	+	-	-	-	-	+	-	-	-	-
452	FSLENNITEL	Db	0.6862	29.8297	0.0095	-	-	+	+	-	-	-	-	-	-	-
453	MSYPFFPLL	Kb	0.904	2.8252	0.0053	-	-	+	-	-	-	-	-	-	-	-
454	IMFERWNQL	Kb	0.7578	13.7455	0.0284	-	-	+	+	+	-	-	-	-	-	-
455	YSIFNVNAEII	Db	0.7047	24.4137	0.0089	-	-	-	-	+	-	-	-	-	-	+
456	KAVKNYVEI	Db	0.7909	9.6097	0.0063	+	+	+	+	+	-	-	-	-	-	+
457	STYYYEYAM	Kb	0.7242	19.7647	0.0464	+	-	-	-	-	-	-	-	-	-	-
458	VIFSRLSNF	Kb	0.711	22.7966	0.0555	+	+	-	-	+	+	-	-	-	-	-

Novel Peptide number	Peptide sequence	Top NetMHCpan peptides <22nM and <0.05 Rank %	NetMHCpan other peptides 22.381-388.44nM but always %Rank of 0.5 or below	Top NetMHCcons NC and C2	Top NetMHCcons peptides <22nM and <0.05 Rank %	NetMHCcons other peptides 23.05nM-531.38nM but always %Rank of 0.5 or below	Batch purity restimulation % IFN-γ+ CD11a+ / total CD8+ T cells	Signal peptide predicted	No. of transmembrane domains predicted	Protein length	Peptide length	Peptide start position	Percentile of protein	NetMHCcons 1.1 data			
														MHC allele restriction	1-log50k	nM	%Rank score
459	KSIKNNDFI						**0.00864**	no	2	7480	9	3533	47.23262032	Db	0.85	5.1	0.01
460	SSYLSYYLL						**0.008565**	yes	2	395	9	231	58.48101266	Kb	0.768	12.24	0.03
461	NSLLNVDEI						**0.00853**	no	0	622	9	336	54.0192926	Db	0.863	4.43	0.01
462	ISYNFYRIF						**0.008525**	no	0	787	9	75	9.529860229	Kb	0.796	9.09	0.01
463	RTFYYFHGL						**0.0085**	yes	2	1349	9	832	61.67531505	Kb	0.761	13.28	0.03
464	YANYNNTYI						**0.00842**	no	2	3724	9	311	8.351235231	Db	0.869	4.1	0.01
465	INYNKYIHL						**0.00839**	no	0	5249	9	3477	66.2411888	Kb	0.828	6.47	0.01
466	VSIENYHLI						**0.00838**	no	0	547	9	458	83.72943327	Db	0.826	6.57	0.01
467	VSFMYSRKL						**0.00831**	yes	1	3204	9	1982	61.86017478	Kb	0.744	16.04	0.03
468	CSISNPTYI						**0.00811**	no	0	1396	9	1344	96.27507163	Db	0.843	5.47	0.01
469	SLYNYFFNL						**0.008**	no	0	3018	9	1805	59.80781975	Kb	0.77	12.11	0.03
470	TGYARYFAL						**0.008**	no	0	256	9	168	65.625	Kb	0.776	11.35	0.01
471	SNYIKYNQL						**0.008**	yes	0	1354	9	295	21.7872969	Kb	0.792	9.44	0.01
472	YAQTNPLPL						**0.008**	no	0	1038	9	138	13.29479769	Db	0.806	8.16	0.01
473	KSIKNTDNI						**0.008**	yes	0	1109	9	982	88.54824166	Db	0.775	11.35	0.03
474	IIYNFFINNL						**0.00795**	no	0	6521	10	3221	49.39426468	Kb	0.717	21.37	0.05
475	ISYSFQNEL						**0.00791**	no	0	797	9	779	97.74153074	Kb	0.825	6.68	0.01
476	FALCNSNFHII						**0.00783**	no	0	2420	11	135	5.578512397	Db	0.689	28.93	0.07
477	YTMCNYTLM						**0.00774**	no	8	1272	9	950	74.68553459	Db	0.866	4.26	0.01
478	YNYYFSYL						**0.00765**	no	4	2996	8	2649	88.41789052	Kb	0.844	5.41	0.01
479	YSYLYTPL						**0.00763**	no	0	610	8	166	27.21311475	Kb	0.861	4.52	0.01
480	STFFFTLL						**0.00763**	no	2	533	8	270	50.65666041	Kb	0.821	6.97	0.01
481	ITYQRHIPF						**0.00761**	yes	3	1149	9	40	3.481288077	Kb	0.766	12.64	0.03
482	INYIYNGI						**0.0076**	yes	1	1809	8	1249	69.04367054	Kb	0.731	18.37	0.05
483	YALENKSLLPI						**0.00758**	yes	0	479	11	257	53.65344468	Db	0.685	30.05	0.07
484	ITYKYSLL						**0.00752**	yes	0	2773	8	415	14.96574107	Kb	0.852	4.96	0.01
485	LAIQNNMPTM						**0.0075**	yes	1	1791	10	890	49.69290899	Db	0.736	17.49	0.05
486	ASFEFISHL						**0.00749**	no	0	1168	9	502	42.97945205	Kb	0.779	10.93	0.01
487	SIFLFTPL						**0.00746**	no	9	1936	8	866	44.73140496	Kb	0.827	6.5	0.01
488	YAINNPNFNNL						**0.00707**	no	0	1388	11	788	56.77233429	Db	0.7	25.69	0.05
489	YSIVNEDIV						**0.00678**	no	0	415	9	404	97.34939759	Db	0.877	3.76	0.01
490	SSLLNEIEI						**0.006725**	no	0	219	9	91	41.55251142	Db	0.844	5.41	0.01
491	FTITNNHSPL						**0.006665**	no	0	154	10	46	29.87012987	Db	0.705	24.33	0.05
492	VSYALFALL						**0.00649**	yes	1	625	9	609	97.44	Kb	0.831	6.26	0.01
493	VSLLFFSYL						**0.00647**	no	13	585	9	530	90.5982906	Kb	0.823	6.79	0.01
494	KAISNFLPL						**0.00644**	no	0	3796	9	2645	69.67860906	Db	0.89	3.29	0.01
495	ASYERFINIL						**0.00643**	no	0	757	10	601	79.39233818	Kb	0.681	31.38	0.1
496	INYSRLFVSFL						**0.00637**	no	6	4789	11	600	12.52871163	Kb	0.641	48.37	0.17
497	IAYYFSVL						**0.00636**	no	0	659	8	332	50.37936267	Kb	0.841	5.59	0.01
498	YAISNFLSQTI						**0.00635**	no	0	1758	11	1653	94.02730375	Db	0.704	24.6	0.05
499	FSFCNSIPL						**0.00633**	no	0	2957	9	2413	81.60297599	Db	0.878	3.74	0.01
500	YSYNFYSTL						**0.00632**	no	0	1174	9	8	0.681431005	Kb	0.875	3.89	0.01
501	SIYYFFSKL						**0.0063**	yes	16	1292	9	649	50.23219814	Kb	0.849	5.12	0.01

Novel Peptide number	Peptide sequence	NetMHCpan 4.0 data				Expression data											
											Microarray		SSH		Microarray	
		MHC allele restriction	1-log50k	nM	%Rank score	**TARUN 2008 EEF** Mass Spec (LS40-50h)	**LINDNER 2013 Spz** Mass Spec	**LASONDER 2008 spz** Mass Spec	**LASONDER 2008 Spz** (only day 18-22) Mass Spec	**LINDNER 2013 Spz** Mass Spec	**TARUN 2008 spz**	**Tarun 2008 EEF**	**UIS genes Matuschewski 2002** (oocyst spz v sg spz)	**S genes Kaiser 2004** (sg spz v merozoites)	**DSK hi** (highly downregulated in SLARP KO) **Silvie, unpublished**	**DSK lo** (lowly downregulated in SLARP KO) **Silvie, unpublished**
						+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-
						orthologs of *P. yoelii*		orthologs of *P. falciparum*			orthologs of *P. yoelii*				*P. berghei* origin	
459	KSIKNNDEI	Db	0.8246	6.6708	0.0053	-	+	-	-	+	-	-	-	-	-	+
460	SSYLSYYLL	Kb	0.821	6.9344	0.0099	+	+	+	+	+	-	-	-	-	-	-
461	NSLLNVDEI	Db	0.8115	7.6908	0.0057	-	+	-	-	+	-	-	-	-	-	-
462	ISYNFYRIF	Kb	0.8295	6.3247	0.0094	+	+	+	+	+	-	-	-	-	-	-
463	RTFYYFHGL	Kb	0.8569	4.7022	0.0079	+	-	-	-	+	-	-	-	-	-	-
464	YANYNNTYI	Db	0.8222	6.8464	0.0054	-	-	-	-	+	-	-	-	-	-	-
465	INYNKYIHL	Kb	0.8812	3.6178	0.0066	+	-	-	-	-	-	-	-	-	-	-
466	VSIENYHLI	Db	0.7775	11.103	0.0067	-	-	+	+	-	-	-	-	-	-	-
467	VSFMYSRKL	Kb	0.875	3.8661	0.0069	-	-	+	+	-	-	-	-	-	-	-
468	CSISNPTYI	Db	0.8193	7.0642	0.0054	-	-	+	+	+	-	-	-	-	-	-
469	SLYNYFFNL	Kb	0.8577	4.6644	0.0079	-	-	-	-	-	+	-	-	-	-	-
470	TGYARYFAL	Kb	0.8233	6.7637	0.0098	+	+	+	+	+	-	-	-	-	-	-
471	SNYIKYNQL	Kb	0.8195	7.046	0.01	+	+	-	-	+	-	-	-	-	-	-
472	YAQTNPLPL	Db	0.8029	8.4339	0.0059	+	+	+	+	+	+	-	-	-	-	-
473	KSIKNTDNI	Db	0.7668	12.4713	0.007	-	-	-	-	-	-	-	-	-	-	+
474	IIYNFFINNL	Kb	0.8281	6.4212	0.0095	-	+	-	-	+	-	-	-	-	-	-
475	ISYSFQNEL	Kb	0.8587	4.6115	0.0078	-	-	-	-	-	-	+	-	-	-	-
476	FALCNSNFHII	Db	0.7254	19.5102	0.0083	-	+	-	-	+	-	-	-	-	-	-
477	YTMCNYTLM	Db	0.834	6.0288	0.005	-	-	-	-	+	-	-	-	-	-	-
478	YNYYFSYL	Kb	0.9059	2.7692	0.0052	-	+	-	-	+	-	-	-	-	-	-
479	YSYLYTPL	Kb	0.9139	2.5374	0.0048	-	+	-	-	+	-	-	-	-	-	-
480	STFFFTLL	Kb	0.8904	3.2737	0.0061	-	+	-	-	+	-	-	-	-	-	-
481	ITYQRHIPF	Kb	0.7305	18.4742	0.0416	+	-	-	-	-	-	-	-	-	-	-
482	INYIYNGI	Kb	0.8573	4.6813	0.0079	-	-	-	-	+	-	-	-	-	-	+
483	YALENKSLLPI	Db	0.7592	13.5329	0.0073	-	-	+	+	+	-	-	-	-	-	-
484	ITYKYSLL	Kb	0.9156	2.4912	0.0047	-	+	-	-	-	-	-	-	-	-	-
485	LAIQNNMPTM	Db	0.6268	56.7162	0.0148	+	-	-	-	+	-	-	-	-	-	-
486	ASFEFISHL	Kb	0.8672	4.2069	0.0074	-	+	-	-	+	-	-	-	-	-	-
487	SIFLFTPL	Kb	0.8895	3.3053	0.0061	-	-	-	-	+	-	-	-	-	-	-
488	YAINNPNFNNL	Db	0.6978	26.3118	0.0091	+	+	+	+	+	-	-	-	-	-	-
489	YSIVNEDIV	Db	0.8636	4.3749	0.0041	-	+	-	-	+	-	-	-	-	-	-
490	SSLLNEIEI	Db	0.7901	9.6927	0.0063	+	+	-	-	+	-	-	-	-	-	-
491	FTITNNHSPL	Db	0.6764	33.1679	0.0098	+	-	-	-	+	-	+	-	-	-	-
492	VSYALFALL	Kb	0.8875	3.3783	0.0062	-	+	+	+	+	-	-	-	-	-	-
493	VSLLFFSYL	Kb	0.8852	3.4629	0.0064	-	+	+	-	+	-	-	-	-	-	-
494	KAISNFLPL	Db	0.8749	3.8719	0.0038	-	-	-	-	+	-	-	-	-	-	-
495	ASYERFINIL	Kb	0.7217	20.3071	0.0484	-	+	+	-	-	+	-	-	-	-	-
496	INYSRLFVSFL	Kb	0.7239	19.8297	0.0467	-	-	+	+	+	-	-	-	-	-	-
497	IAYYFSVL	Kb	0.9011	2.9143	0.0055	-	-	-	-	-	-	+	-	-	-	-
498	YAISNFLSQTI	Db	0.7878	9.9369	0.0064	-	-	+	+	+	-	-	-	-	-	-
499	FSFCNSIPL	Db	0.8716	4.0107	0.0039	-	+	+	-	+	-	-	-	-	-	+
500	YSYNFYSTL	Kb	0.924	2.2758	0.0042	-	+	-	-	+	-	-	-	-	-	-
501	SIYYFFSKL	Kb	0.9037	2.8355	0.0053	-	+	-	-	+	-	-	-	-	-	-

Novel Peptide number	Peptide sequence	Top NetMHCpan peptides <22nM and <0.05 Rank %	NetMHCpan other peptides 22.381-388.44nM but always %Rank of 0.5 or below	Top NetMHCcons NC and C2	Top NetMHCcons peptides <22nM and <0.05 Rank %	NetMHCcons other peptides 23.05nM-531.38nM but always %Rank of 0.5 or below	Batch purity restimulation % IFN-γ+ CD11a+ / total CD8+ T cells	Signal peptide predicted	No. of transmembrane domains predicted	Protein length	Peptide length	Peptide start position	Percentile of protein	NetMHCcons 1.1 data			
														MHC allele restriction	1-log50k	nM	%Rank score
502	NCYYPYTL						**0.00629**	no	0	420	8	127	30.23809524	Kb	0.248	3398.41	9
503	FSILNENEL						**0.00627**	no	0	2458	9	1121	45.60618389	Db	0.832	6.19	0.01
504	IIYSFYIYL						**0.00625**	no	3	970	9	904	93.19587629	Kb	0.838	5.8	0.01
505	STYYYSMLL						**0.00625**	no	0	426	9	359	84.27230047	Kb	0.814	7.52	0.01
506	TMYYFSISL						**0.00622**	no	0	1013	9	236	23.29713722	Kb	0.801	8.57	0.01
507	IGYYYYPYM						**0.0062**	no	0	870	9	850	97.70114943	Kb	0.84	5.68	0.01
508	VSMVNECFI						**0.00617**	no	0	2057	9	2004	97.42343218	Db	0.859	4.62	0.01
509	VSLSRFFSM						**0.00616**	no	5	245	9	50	20.40816327	Kb	0.757	13.86	0.03
510	FAFKNSCLAPM						**0.00615**	no	0	4800	11	1378	28.70833333	Db	0.72	20.58	0.05
511	MNYSKYLLL						**0.00611**	no	0	414	9	257	62.07729469	Kb	0.824	6.71	0.01
512	YTIQNKDEL						**0.00608**	no	0	1370	9	531	38.75912409	Db	0.8	8.66	0.03
513	YANLNMIHL						**0.00607**	no	0	739	9	361	48.84979702	Db	0.843	5.47	0.01
514	RSFIFYSAM						**0.00605**	no	0	368	9	37	10.05434783	Kb	0.809	7.9	0.01
515	VNYNKFLEL						**0.00602**	yes	8	592	9	257	43.41216216	Kb	0.813	7.56	0.01
516	LSLSNYLFL						**0.006**	yes	0	1354	9	597	44.0915805	Db	0.802	8.47	0.03
517	YALENNDSVL						**0.00585**	no	0	343	10	60	17.49271137	Db	0.806	8.16	0.01
518	YAYINLESL						**0.00581**	no	0	5434	9	2250	41.40596246	Db	0.86	4.55	0.01
519	VSLTNIDSI						**0.00542**	no	0	5317	9	5124	96.37013353	Db	0.847	5.24	0.01
520	YSYKYLAL						**0.00536**	no	0	383	8	365	95.3002611	Kb	0.825	6.68	0.01
521	SSLENMYEM						**0.00526**	no	0	551	9	168	30.49001815	Db	0.887	3.4	0.01
522	SIYLYYYL						**0.00523**	no	0	1471	8	662	45.00339905	Kb	0.824	6.75	0.01
523	YNFSSYFPLL						**0.005**	no	0	3183	10	407	12.78667923	Kb	0.677	32.94	0.1
524	FSYKRIGYL						**0.00491**	no	0	1067	9	76	7.122774133	Kb	0.712	22.68	0.05
525	FIYNFYQGL						**0.00484**	no	0	9556	9	4002	41.87944747	Kb	0.827	6.54	0.01
526	FSHRNLDHI						**0.00467**	no	3	4291	9	1167	27.1964577	Db	0.855	4.83	0.01
527	FSYSYYSNL						**0.00466**	no	0	677	9	41	6.056129985	Kb	0.855	4.83	0.01
528	YNYFYKPL						**0.00463**	yes	0	1272	8	1135	89.22955975	Kb	0.79	9.7	0.01
529	MSIMNFSYI						**0.00462**	no	4	2996	9	2193	73.1975968	Db	0.901	2.9	0.01
530	YSLINYYNL						**0.00445**	no	2	7126	9	2355	33.04799326	Db	0.877	3.76	0.01
531	VSYAKFPPI						**0.00443**	yes	0	786	9	455	57.88804071	Kb	0.815	7.4	0.01
532	VSFNPFSLL						**0.0043**	yes	2	246	9	202	82.11382114	Kb	0.803	8.38	0.01
533	SVMSNLCPI						**0.0043**	no	0	640	9	39	6.09375	Db	0.792	9.44	0.03
534	LSITNLSYI						**0.00429**	no	2	7126	9	4328	60.73533539	Db	0.841	5.62	0.01
535	MAYQNVEEI						**0.00424**	no	0	958	9	546	56.99373695	Db	0.812	7.6	0.01
536	ISMTNELPI						**0.00423**	no	4	1513	9	1338	88.43357568	Db	0.844	5.41	0.01
537	ITYQYYSIF						**0.00411**	no	0	309	9	130	42.07119741	Kb	0.772	11.72	0.03
538	HTYNFYSLM						**0.00408**	no	0	1415	9	913	64.5229682	Kb	0.809	7.9	0.01
539	FSILNNIIL						**0.003895**	yes	0	1478	9	1013	68.53856563	Db	0.77	11.98	0.03
540	KSISNGNTI						**0.00329**	no	0	990	9	58	5.858585859	Db	0.806	8.16	0.01
541	ISFYFYNNKL						**0.00324**	no	0	1194	10	838	70.18425461	Kb	0.732	18.27	0.05
542	VSYGKYSPI						**0.00322**	no	0	511	9	447	87.47553816	Kb	0.827	6.5	0.01
543	ISYVFKSYL						**0.00318**	yes	5	2248	9	252	11.20996441	Kb	0.831	6.22	0.01
544	IAYYRMPL						**0.00315**	no	1	1897	8	583	30.7327359	Kb	0.775	11.41	0.01

Novel Peptide number	Peptide sequence	NetMHCpan 4.0 data				Expression data											
											Microarray		SSH		Microarray	
		MHC allele restriction	1-log50k	nM	%Rank score	**TARUN 2008 EEF** Mass Spec (LS40-50h)	**LINDNER 2013 Spz** Mass Spec	**LASONDER 2008 spz** Mass Spec	**LASONDER 2008 Spz** (only day 18-22) Mass Spec	**LINDNER 2013 Spz** Mass Spec	**TARUN 2008 spz**	**Tarun 2008 EEF**	**UIS genes Matuschewski 2002** (oocyst spz v sg spz)	**S genes Kaiser 2004** (sg spz v merozoites)	**DSK hi** (highly downregulated in SLARP KO) **Silvie, unpublished**	**DSK lo** (lowly downregulated in SLARP KO) **Silvie, unpublished**
						+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-
						orthologs of *P. yoelii*		orthologs of *P. falciparum*			orthologs of *P. yoelii*				*P. berghei* origin	
502	NCYYPYTL	Kb	0.3056	1831.71	2.9722	-	+	-	-	-	-	-	-	-	-	-
503	FSILNENEL	Db	0.8312	6.2134	0.0051	-	-	-	-	-	-	-	-	-	-	+
504	IIYSFYIYL	Kb	0.8392	5.6966	0.0089	-	+	-	-	+	-	-	-	-	-	-
505	STYYYSMLL	Kb	0.8369	5.839	0.009	-	+	-	-	+	-	-	-	-	-	-
506	TMYYFSISL	Kb	0.814	7.4804	0.0111	+	+	+	-	+	-	-	-	-	-	-
507	IGYYYYPYM	Kb	0.8992	2.9748	0.0056	-	-	+	+	-	-	-	-	-	-	-
508	VSMVNECFI	Db	0.822	6.8636	0.0054	-	-	-	-	-	-	-	-	-	-	-
509	VSLSRFFSM	Kb	0.7539	14.3393	0.0298	-	+	+	-	+	-	-	-	-	-	-
510	FAFKNSCLAPM	Db	0.7426	16.2014	0.0078	-	+	-	-	+	-	-	-	-	-	-
511	MNYSKYLLL	Kb	0.8453	5.3351	0.0086	-	+	-	-	+	-	-	-	-	-	-
512	YTIQNKDEL	Db	0.7905	9.6491	0.0063	-	+	-	-	+	-	-	-	-	-	-
513	YANLNMIHL	Db	0.7801	10.8029	0.0066	-	+	-	-	-	-	-	-	-	-	-
514	RSFIFYSAM	Kb	0.8703	4.0689	0.0072	-	+	+	+	+	-	-	-	-	-	-
515	VNYNKFLEL	Kb	0.8443	5.3932	0.0086	-	+	-	-	+	-	-	-	-	-	-
516	LSLSNYLFL	Db	0.7257	19.44	0.0083	+	+	-	-	+	-	-	-	-	-	-
517	YALENNDSVL	Db	0.7371	17.1847	0.0079	-	+	-	-	-	-	-	-	-	-	-
518	YAYINLESL	Db	0.8657	4.2756	0.004	-	+	-	-	+	-	-	-	-	-	-
519	VSLTNIDSI	Db	0.8401	5.6389	0.0048	-	+	+	-	-	-	-	-	-	-	-
520	YSYKYLAL	Kb	0.8777	3.7566	0.0068	-	+	-	-	+	+	-	-	-	-	-
521	SSLENMYEM	Db	0.8305	6.256	0.0051	-	+	-	-	-	-	-	-	-	-	-
522	SIYLYYYL	Kb	0.8517	4.9739	0.0082	-	-	-	-	+	+	-	-	-	-	-
523	YNFSSYFPLL	Kb	0.71	23.0544	0.0561	+	+	-	-	+	-	-	-	-	-	-
524	FSYKRIGYL	Kb	0.7292	18.7241	0.0425	-	+	-	-	+	-	-	-	-	-	-
525	FIYNFYQGL	Kb	0.842	5.5273	0.0087	-	+	-	-	+	-	-	-	-	-	-
526	FSHRNLDHI	Db	0.8076	8.0223	0.0058	-	-	-	-	-	-	-	-	-	+	+
527	FSYSYYSNL	Kb	0.9269	2.2047	0.004	+	+	-	-	+	-	-	-	-	-	-
528	YNYFYKPL	Kb	0.8789	3.7092	0.0067	+	+	-	-	+	-	-	-	-	-	-
529	MSIMNFSYI	Db	0.9005	2.9357	0.003	-	+	-	-	+	-	-	-	-	-	-
530	YSLINYYNL	Db	0.8027	8.4576	0.006	-	-	-	-	+	-	-	-	-	-	+
531	VSYAKFPPI	Kb	0.8421	5.5224	0.0087	+	+	-	-	+	-	-	-	-	-	-
532	VSFNPFSLL	Kb	0.832	6.155	0.0093	-	-	-	-	-	-	+	-	-	-	-
533	SVMSNLCPI	Db	0.8021	8.5141	0.006	-	-	-	-	-	-	+	-	-	-	+
534	LSITNLSYI	Db	0.8422	5.5124	0.0048	-	-	-	-	+	-	-	-	-	-	+
535	MAYQNVEEI	Db	0.825	6.6402	0.0053	-	+	-	-	+	-	-	-	-	-	-
536	ISMTNELPI	Db	0.8278	6.4422	0.0052	-	-	-	-	-	-	-	-	-	-	+
537	ITYQYYSIF	Kb	0.8137	7.5051	0.0112	+	+	-	-	+	-	-	-	-	-	-
538	HTYNFYSLM	Kb	0.8177	7.1869	0.0104	-	-	-	-	-	-	-	+	-	-	-
539	FSILNNIIL	Db	0.7509	14.8014	0.0075	-	-	-	-	+	-	-	-	-	-	-
540	KSISNGNTI	Db	0.8052	8.2324	0.0059	-	+	-	-	+	-	-	-	-	-	-
541	ISFYFYNNKL	Kb	0.8651	4.3041	0.0075	-	-	+	-	-	-	-	-	-	-	-
542	VSYGKYSPI	Kb	0.8331	6.084	0.0092	-	+	+	+	-	-	-	-	-	-	-
543	ISYVFKSYL	Kb	0.8994	2.9707	0.0056	-	-	+	+	+	-	-	-	-	-	-
544	IAYYRMPL	Kb	0.8251	6.6326	0.0097	-	-	+	-	+	-	-	-	-	-	-

Novel Peptide number	Peptide sequence	Top NetMHCpan peptides <22nM and <0.05 Rank %	NetMHCpan other peptides 22.381-388.44nM but always %Rank of 0.5 or below	Top NetMHCcons NC and C2	Top NetMHCcons peptides <22nM and <0.05 Rank %	NetMHCcons other peptides 23.05nM-531.38nM but always %Rank of 0.5 or below	Batch purity restimulation % IFN-γ+ CD11a+ / total CD8+ T cells	Signal peptide predicted	No. of transmembrane domains predicted	Protein length	Peptide length	Peptide start position	Percentile of protein	NetMHCcons 1.1 data			
														MHC allele restriction	1-log50k	nM	%Rank score
545	NAMVNNFFTI						**0.00311**	no	0	3439	10	2017	58.65077057	Db	0.753	14.48	0.05
546	YGYTHYLQL						**0.0031**	no	0	5317	9	3369	63.36279857	Kb	0.762	13.06	0.03
547	YNILNSDTI						**0.0031**	no	0	1010	9	355	35.14851485	Db	0.864	4.36	0.01
548	YSYINENEI						**0.00309**	no	0	233	9	131	56.22317597	Db	0.788	9.91	0.03
549	KSMSNLDLL						**0.00304**	no	0	6521	9	5956	91.33568471	Db	0.808	8.03	0.01
550	HSLKNGDTI						**0.00304**	yes	3	583	9	180	30.87478559	Db	0.853	4.88	0.01
551	LSFIFYSLL						**0.00273**	no	10	541	9	497	91.86691312	Kb	0.824	6.75	0.01
552	FNYYHFYKPL						**0.00259**	no	0	529	10	36	6.805293006	Kb	0.711	22.93	0.05
553	VSYNFKSRL						**0.00256**	no	4	3350	9	236	7.044776119	Kb	0.829	6.4	0.01
554	VMFSRASAL						**0.00254**	no	0	264	9	211	79.92424242	Kb	0.792	9.54	0.01
555	INYTKFLSL						**0.00251**	no	0	455	9	241	52.96703297	Kb	0.792	9.44	0.01
556	KSYSKYILL						**0.00247**	no	0	1174	9	162	13.79897785	Kb	0.809	7.9	0.01
557	YSNANMATL						**0.00216**	no	4	2562	9	2082	81.264637	Db	0.879	3.72	0.01
558	NCYKYKNL						**0.00215**	no	2	3724	8	2733	73.38882922	Kb	0.428	487.32	2
559	INYFYLLL						**0.002**	no	8	4154	8	1606	38.66153105	Kb	0.824	6.71	0.01
560	YAFFFYPNL						**0**	no	6	951	9	436	45.84647739	Kb	0.818	7.2	0.01
561	INYERFNAL						**0**	no	0	439	9	98	22.32346241	Kb	0.842	5.53	0.01
562	YSFSNYYSI						**0**	no	2	3209	9	2638	82.2062948	Db	0.868	4.17	0.01
563	YSMFNLSII						**0**	yes	2	217	9	197	90.78341014	Db	0.853	4.91	0.01
564	YSYLNIDLL						**0**	no	0	1591	9	867	54.49402891	Db	0.871	4.06	0.01
565	YGLINITTI						**0**	yes	1	3204	9	3117	97.28464419	Db	0.904	2.83	0.01
566	YSYQNYSFL						**0**	no	0	252	9	51	20.23809524	Db	0.872	3.99	0.01
567	TALYNTETI						**0**	no	0	881	9	277	31.4415437	Db	0.873	3.93	0.01
568	FALYNVNIM						**0**	yes	0	2150	9	1665	77.44186047	Db	0.826	6.57	0.01
569	SSFNNMHYM						**0**	no	0	812	9	657	80.91133005	Db	0.817	7.24	0.01
570	YSISNDELI						**0**	no	0	1429	9	1356	94.89153254	Db	0.868	4.17	0.01
571	SSIKNVFSL						**0**	no	0	1214	9	343	28.25370675	Db	0.804	8.34	0.01
572	YSPLNYDVL						**0**	no	0	1218	9	724	59.44170772	Db	0.89	3.29	0.01
573	FAIENNMEI						**0**	yes	2	293	9	204	69.62457338	Db	0.855	4.78	0.01
574	YAYNNIFLI						**0**	no	13	2715	9	1866	68.72928177	Db	0.797	8.99	0.03
575	VAPTNITTI						**0**	no	0	655	9	152	23.20610687	Db	0.882	3.57	0.01
576	IALLNCDSI						**0**	no	0	784	9	569	72.57653061	Db	0.843	5.44	0.01
577	TSIANFYLL						**0**	no	0	1083	9	546	50.41551247	Db	0.83	6.29	0.01
578	YMIENLCVI						**0**	no	2	3209	9	1922	59.89404799	Db	0.839	5.68	0.01
579	FAIINVLLL						**0**	no	2	2091	9	1915	91.58297465	Db	0.865	4.31	0.01
580	YAPRNSDNI						**0**	no	0	581	9	233	40.10327022	Db	0.869	4.15	0.01
581	YGAHNYDPI						**0**	no	0	414	9	19	4.589371981	Db	0.821	6.97	0.01
582	RSMHNNIPI						**0**	no	0	1991	9	623	31.29080864	Db	0.81	7.77	0.01
583	YSFNFHVTYL						**0**	no	1	327	10	306	93.57798165	Kb	0.713	22.44	0.05
584	YQLKNVDEL						**0**	no	0	1591	9	1036	65.11627907	Db	0.857	4.72	0.01
585	TAIQNSNNFPI						**0**	no	0	1519	11	685	45.09545754	Db	0.704	24.47	0.05

Novel Peptide number	Peptide sequence	NetMHCpan 4.0 data				Expression data											
											Microarray		SSH		Microarray	
		MHC allele restriction	1-log50k	nM	%Rank score	**TARUN 2008 EEF** Mass Spec (LS40-50h)	**LINDNER 2013 Spz** Mass Spec	**LASONDER 2008 spz** Mass Spec	**LASONDER 2008 Spz** (only day 18-22) Mass Spec	**LINDNER 2013 Spz** Mass Spec	**TARUN 2008 spz**	**Tarun 2008 EEF**	**UIS genes Matuschewski 2002** (oocyst spz v sg spz)	**S genes Kaiser 2004** (sg spz v merozoites)	**DSK hi** (highly downregulated in SLARP KO) **Silvie, unpublished**	**DSK lo** (lowly downregulated in SLARP KO) **Silvie, unpublished**
						+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-
						orthologs of *P. yoelii*		orthologs of *P. falciparum*			orthologs of *P. yoelii*				*P. berghei* origin	
545	NAMVNNFFTI	Db	0.669	35.9413	0.01	-	+	-	-	+	-	-	-	-	-	-
546	YGYTHYLQL	Kb	0.8291	6.3527	0.0095	-	+	+	-	-	-	-	-	-	-	-
547	YNILNSDTI	Db	0.8255	6.6053	0.0053	-	+	-	-	-	-	-	-	-	-	-
548	YSYINENEI	Db	0.8228	6.8042	0.0053	-	+	-	-	+	-	+	-	-	-	-
549	KSMSNLDLL	Db	0.8233	6.762	0.0053	-	+	-	-	+	-	-	-	-	-	-
550	HSLKNGDTI	Db	0.818	7.1685	0.0055	-	+	-	-	-	-	-	-	-	-	-
551	LSFIFYSLL	Kb	0.8918	3.2248	0.006	-	+	-	-	+	-	-	-	-	-	-
552	FNYYHFYKPL	Kb	0.7803	10.7751	0.0205	-	+	-	-	+	-	-	-	-	-	-
553	VSYNFKSRL	Kb	0.8897	3.2983	0.0061	-	+	-	-	+	-	-	-	-	-	-
554	VMFSRASAL	Kb	0.7539	14.3285	0.0298	+	+	+	+	+	-	-	-	-	-	-
555	INYTKFLSL	Kb	0.8691	4.1198	0.0072	-	+	-	-	+	-	+	-	-	-	-
556	KSYSKYILL	Kb	0.7959	9.0994	0.015	-	+	-	-	+	-	-	-	-	-	-
557	YSNANMATL	Db	0.81	7.8129	0.0057	-	-	-	-	+	-	-	-	-	-	+
558	NCYKYKNL	Kb	0.4468	397.7509	0.8498	-	-	-	-	+	-	-	-	-	-	-
559	INYFYLLL	Kb	0.9074	2.7246	0.0051	+	-	-	-	+	-	-	-	-	-	-
560	YAFFFYPNL	Kb	0.8944	3.1362	0.0058	-	+	-	-	+	-	-	-	-	-	-
561	INYERFNAL	Kb	0.8616	4.4681	0.0077	-	+	-	-	+	+	-	-	-	-	-
562	YSFSNYYSI	Db	0.8587	4.6117	0.0043	-	+	-	-	+	-	-	-	-	-	-
563	YSMFNLSII	Db	0.8582	4.6393	0.0043	-	+	-	-	-	-	-	-	-	-	-
564	YSYLNIDLL	Db	0.8447	5.3654	0.0047	-	+	-	-	+	-	-	-	-	-	-
565	YGLINITTI	Db	0.844	5.4057	0.0047	-	-	+	+	-	-	-	-	-	-	-
566	YSYQNYSFL	Db	0.8411	5.5801	0.0048	-	+	-	-	+	-	-	-	-	-	-
567	TALYNTETI	Db	0.8353	5.9435	0.005	-	+	+	-	+	+	-	-	-	-	-
568	FALYNVNIM	Db	0.8267	6.5198	0.0052	-	+	-	-	-	-	-	-	-	-	-
569	SSFNNMHYM	Db	0.8258	6.5824	0.0053	-	+	-	-	-	-	-	-	-	-	-
570	YSISNDELI	Db	0.8238	6.7288	0.0053	-	+	-	-	+	+	-	-	-	-	-
571	SSIKNVFSL	Db	0.8204	6.9838	0.0054	-	+	-	-	+	-	-	-	-	-	-
572	YSPLNYDVL	Db	0.8104	7.7827	0.0057	-	+	-	-	+	-	-	-	-	-	-
573	FAIENNMEI	Db	0.8074	8.0367	0.0058	-	-	+	-	+	-	-	-	-	-	-
574	YAYNNIFLI	Db	0.8042	8.3181	0.0059	-	-	+	-	-	-	-	-	-	-	-
575	VAPTNITTI	Db	0.8028	8.4432	0.0059	-	+	+	-	+	-	-	-	-	-	-
576	IALLNCDSI	Db	0.8027	8.4525	0.006	-	+	-	-	-	-	-	-	-	-	-
577	TSIANFYLL	Db	0.7961	9.0773	0.0061	-	-	+	+	+	-	-	-	-	-	-
578	YMIENLCVI	Db	0.793	9.3859	0.0062	-	+	-	-	+	-	-	-	-	-	-
579	FAIINVLLL	Db	0.7927	9.422	0.0063	-	-	-	-	-	-	-	-	-	-	+
580	YAPRNSDNI	Db	0.7883	9.8811	0.0064	-	+	-	-	-	-	-	-	-	-	-
581	YGAHNYDPI	Db	0.7826	10.5049	0.0066	-	-	+	+	+	-	-	-	-	-	-
582	RSMHNNIPI	Db	0.7681	12.2958	0.007	-	+	-	-	+	-	-	-	-	-	-
583	YSFNFHVTYL	Kb	0.7602	13.3874	0.0275	-	+	+	-	+	+	-	-	-	-	-
584	YQLKNVDEL	Db	0.7593	13.5198	0.0073	-	+	-	-	+	-	-	-	-	-	-
585	TAIQNSNNFPI	Db	0.7231	19.9958	0.0083	-	+	-	-	+	-	-	-	-	-	-