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BRIEF DEFINITIVE REPORT

Inferior T cell immunogenicity of a *Plasmodium berghei* model liver stage antigen expressed throughout pre-erythrocytic maturation

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ABSTRACT

Sporozoite antigens are the basis of a number of malaria vaccines being tested, but the contribution of antigens expressed during subsequent liver stage development to pre-erythrocytic stage immunity is poorly understood. We previously showed that, following immunisation with radiation attenuated sporozoites (RAS), a model epitope embedded in a sporozoite surface protein elicited robust $CD8^+$ T cell responses, whilst the same epitope in a liver stage antigen induced inferior responses. Since RAS arrest early in their development in host hepatocytes, we hypothesised that extending parasite maturation in the liver could considerably improve the epitope-specific $CD8^+$ T cell response. Here, we employed a late liver stage arrested parasite model, azithromycin prophylaxis alongside live sporozoites, to increase expression of the model epitope until full liver stage maturation. Strikingly, this alternative immunisation strategy, which has been shown to elicit superior protection, failed to improve the resulting epitope-specific $CD8^+$ T cell responses. Our findings support the notion that liver stage antigens are poorly immunogenic and provide additional caution about prioritising antigens for vaccine development based solely on immunogenicity.

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INTRODUCTION

The malaria pre-erythrocytic stages are comprised of the extracellular sporozoites, which are inoculated by infected mosquitoes to the mammalian host, followed by the intracellular exo-erythrocytic forms (EEF; also known as liver stages), resulting from the development and maturation of sporozoites within a parasitophorous vacuole (PV) in hepatocytes[1]. Whilst there is an abundance of investigations delineating *Plasmodium* sporozoite antigens, the immune responses they induce and their potential for use in malaria pre-erythrocytic vaccines[2-9], little is known about antigens solely expressed in EEFs.

Vaccination-induced protection against pre-erythrocytic stages was first shown to be feasible in animal models and humans using whole sporozoite vaccine approaches, particularly with radiation attenuation sporozoites (RAS)[10-12], considered the benchmark for anti-malaria vaccines. Sterile protection induced by RAS has been shown to be mediated primarily by $CD8^+$ T cells[13,14]. Efficient recall of $CD8^+$ T cell responses following presentation of parasite antigens on hepatocytes is crucial due to the short duration that the parasites are in the liver (~48-52 hours for *P. berghei*[15]). Despite the high level of protection achieved, *P. berghei* RAS do not develop into large, mature hepatic schizonts but are arrested prior to onset of replication and growth approximately 24 hours post immunisation[16,17]. This early hepatic arrest raises questions as to the magnitude of contributions of EEF-specific over sporozoite antigens in immunity.

Enhanced protection has been reported following the administration of anti-malarial chemoprophylaxis and live sporozoites in both animal models and in humans[18-21]. Similar results have been reported after immunisations with late arresting genetically arrested sporozoites in preclinical studies in mice[22,23]. Given that this alternative whole sporozoite vaccine approach ensures full EEF development, the results suggest that the longer the parasites are allowed to develop and mature before arrest, the greater the protection induced by the immunisation strategy. This outcome has been initially interpreted as having more antigens expressed and an increase in antigen biomass during extended parasite development, eliciting a broader range of EEF-specific immune responses

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needed for protection[22,23]. However, immunogenic proteins expressed in the late EEF, which may increase $CD8^+$ T cell responses, are currently not well defined.

The circumsporozoite protein (CSP) is the major surface coat antigen of sporozoites and upregulated in sporozoites 4 (UIS4) is a protein mainly associated with the parasitophorous vacuole membrane (PVM) surrounding the EEF[24,25]. Upon *P. berghei* sporozoite infections of H-$2K^b$ restricted C57BL/6 mice, no immunodominant epitopes in either CSP or UIS4 were identified[26]. Thus, in the absence of known EEF epitopes allowing for the quantification of specific T cell responses following sporozoite immunisation, we and others have used a surrogate marker, that is the upregulation of CD11a, in combination with the downregulation of CD8α, as a durable and 'accurate' phenotyping method for infection or vaccine induced antigen-experienced T cells[27,28]. To understand the immunogenicity of EEF-specific antigens, as proxies for detecting $CD8^+$ T cell epitopes in sporozoite and EEF antigens, we previously generated *P. berghei* transgenic parasites that express the SIINFEKL epitope from ovalbumin as part of either CSP or UIS4, $CSP^{SIINFEKL}$ and $UIS4^{SIINFEKL}$, respectively[29]. Following RAS immunisation, a striking difference between the larger SIINFEKL-specific $CD8^+$ T cell response elicited by $CSP^{SIINFEKL}$ and the smaller response induced by $UIS4^{SIINFEKL}$ was found. This divergence could be due to the abrupt cessation of or limited UIS4 expression following early arrest of RAS.

In this report, we tested the hypothesis that prolonged PVM protein expression increases $CD8^+$ T cell responses against EEF vacuolar membrane proteins. Using $CSP^{SIINFEKL}$ and $UIS4^{SIINFEKL}$, we compared the resulting $CD8^+$ T cell responses, both in the spleen and the liver, following RAS immunisation or infection with live parasites under azithromycin (AZ/Spz) prophylaxis, which is an apicoplast-targeting, delayed death-inducing anti-malarial drug allowing for the complete maturation of EEFs in the liver[30].

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MATERIALS AND METHODS

Ethics and animal experimentation

Animal experiments performed in the United Kingdom were conducted under licence from the United Kingdom Home Office under the Animals (Scientific Procedures) Act 1986. Animal experiments in Germany were 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. All protocols were approved by the Animal Welfare and Ethics Review Board of the London School of Hygiene and Tropical Medicine and the ethics committee of the Max Planck Institute for Infection Biology. 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.

P. berghei ANKA immunisation

P. berghei WT, CSP^{SIINFEKL} and UIS4^{SIINFEKL} (strain ANKA; clone c15cy1) parasites[29] were maintained by continuous cycling between murine hosts (NMRI or CD-1) and *Anopheles stephensi* mosquitoes, as previously described[29]. Mice were immunised with 10,000 freshly-isolated sporozoites intravenously in the lateral tail vein. Sporozoites were either γ-irradiated at 1.2 x 10^4 cGy or administered under prophylactic AZ cover. Azithromycin (Pfizer) was administered at a dose of 240 mg/kg intraperitoneally on the same day as parasite inoculation and one day after[30].

Infection of Huh7 hepatoma cells with *P. berghei* sporozoites

In vitro infections were performed in Huh7 cells that were grown in 8-well Labtek slides (30,000 cells/well). Freshly dissected sporozoites (10,000) were added to the cells in complete DMEM medium (10% FCS, 1% Pen/Strep) in duplicates. Parasites were added in medium containing AZ (1.5 μM; Pfizer) or were subjected to γ-irradiation (1.2 x 10^4 cGy) prior to cell infection. Untreated sporozoites served as control. Huh7 cells were incubated for 1 hour at room temperature to allow sporozoite sedimentation and at 37°C

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for additional two hours to permit parasite entry. Infected cells were incubated in complete medium at 37°C for 48 hours. Incubation was terminated by fixation in 4% paraformaldehyde. The infected cells were stained with a monoclonal mouse anti-*Pb*HSP70 antibody[31] followed by a goat Alexa Fluor 488–labelled anti-mouse secondary antibody to visualise parasites, and with a rabbit anti-*Pb*UIS4 serum[32] followed by goat Alexa Fluor 546–labelled anti-rabbit secondary antibody to delineate the PV. Hoechst 33342 was used to locate the nuclei. >20 individual images of EEFs per group were acquired using a Zeiss Axio Vision microscope at 63x magnification and 100ms exposure at an excitation wavelength of 590nm and an emission wavelength of 617nm.The UIS4 fluorescence signal intensities, as proxies for protein amounts, were measured with Fiji ImageJ (NIH, Bethesda, USA). UIS4 stained liver stages were surrounded with the free hand tool; "RawIntDen" value for each EEF (representing the sum of intensity units in the selected areas) was determined.

Quantification of SIINFEKL-specific CD8$^+$ T cell responses

Spleens and livers perfused with 5ml PBS were harvested from immunised and naive mice and filtered by passing the organs through 70 μm cell strainers (Corning). Liver-infiltrating lymphocytes were isolated following published protocols using a Percoll gradient[33]. Following red blood cell lysis and resuspension in complete media, cells were diluted 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. A total number of 2-3x10^6 splenocytes or 0.5-1x10^6 liver-infiltrating cells were plated in flat bottom 96 well plates and incubated with peptides at final concentration 10 μg/ml in the presence of Brefeldin A (eBioScience). SIINFEKL peptide was synthesised and purchased from Peptides and Elephants. Cells were incubated at 37°C, 5% CO_2 for 5-6 hours. Cells were initially stained for cell surface markers, then for intracellular IFN-γ. 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: CD8a (53-6.7), CD11a (M17/4), IFN-γ (XMG1.2).

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Statistics

Data was analysed using FlowJo version 10.0.8 (Tree Star Inc., Oregon, USA), Microsoft Excel and GraphPad Prism v9 (GraphPad Software Inc., CA, USA). Statistics were calculated using one-way ANOVA with Tukey's multiple comparison test. Normality was calculated using the Shapiro-Wilk test.

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RESULTS AND DISCUSSION

We first compared the EEF development *in vitro* of RAS *versus* sporozoites with AZ (Figure 1). Irrespective of the method used, sporozoites retained their invasive capacities. 48 hours after infection, as expected, morphological analysis revealed normal EEF differentiation by both untreated sporozoites and sporozoites cultured with AZ; large, mature hepatic schizonts, which underwent multiple nuclear divisions and surrounded by remodelled PV, as shown by UIS4 staining, were observed[30]. In contrast, RAS gave rise to arrested EEFs, which had reduced growth and showed absence of nuclear divisions[17]. Despite this developmental arrest, these small, round intracellular parasites expressed HSP70 in their cytoplasm and are surrounded by a UIS4 containing remodelled PV, comparable to untreated parasites at earlier stages of intracellular development[29] (Figure 1A). The striking differences in parasite maturation in the liver rather suggest a higher amount of EEF-specific antigens upon AZ treatment. We quantified UIS4 protein levels by measuring the UIS4 fluorescence intensity over the area of individual EEFs. We show that UIS4 protein levels were comparable in AZ treated and untreated control EEFs, but significantly lower in RAS (Figure 1B). For the subsequent experiments, we compared the CD8+ T cell responses induced following RAS immunisation or sporozoites with AZ, utilising the *Pb*CSP^{SIINFEKL} and *Pb*UIS4^{SIINFEKL} parasites we previously generated[29].

We then measured the *in vivo* magnitude of antigen-experienced cells after parasite immunisation. Immunisation with both RAS or sporozoites under AZ cover, produced quantifiable CD11a^{hi} CD8^{+} (CD8α^{lo}) T cells, around 8% in the spleen (Figure 2A,C,E) and 30% in the liver (Figure 2B,D,F), two weeks after immunisation as compared to 5% and 20%, respectively, in naïve mice. These findings are consistent with previous work showing that both RAS and AZ attenuation induce comparable high levels of antigen-experienced CD8^{+} T cells in peripheral blood following immunisation[21].

Next, we compared epitope-specific CD8^{+} T cell responses by *ex vivo* stimulation with SIINFEKL peptide (Figure 3). Consistent with our previous work[29], following RAS immunisation, significantly higher proportions and numbers of SIINFEKL-specific IFN-γ-producing CD8^{+} T cells were induced with *Pb*CSP^{SIINFEKL} as compared to *Pb*UIS4^{SIINFEKL} (Figure 3A-D). Notably, extension of antigen availability and/or increased antigen

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biomass, due to prolonged UIS4 expression permitted by AZ administration, did not improve the proportions and numbers of IFN-γ producing $CD8^+$ T cells, as compared to RAS immunisation. Together, these results clearly demonstrate that extending EEF development, resulting in elevated vacuolar membrane antigen expression, does not amplify IFN-γ producing $CD8^+$ T cell responses.

Whole parasite immunisation strategies that allow the parasite to complete EEF development in the liver ensure immunisation against many antigens expressed in the pre-erythrocytic stages. These span from those expressed by the sporozoite to those expressed very late in the EEF prior to merozoite release into the blood, as well as those antigens that are expressed throughout the pre-erythrocytic stages. Given that $CD8^+$ T cells are crucial for protection against pre-erythrocytic stages, these strategies were originally thought to augment the pool of immunisation-induced $CD8^+$ T cells that are specifically targeted against EEF antigens[18]. However, we have demonstrated poor $CD8^+$ T cell responses to an epitope contained within UIS4, a PVM protein expressed constitutively in the liver following sporozoite invasion of a hepatocyte[29]. We show that AZ prophylaxis would have allowed UIS4 to increase in size for the full 48-52 hours of EEF development, in contrast to ~24 hours when RAS were used. Despite these striking differences, adaptive immune responses to sporozoite immunisation remained intact as evidenced by the unaltered numbers of antigen-experienced $CD8^+$ T cells, based on the quantification of CD11a proxy marker co-expression. These results are consistent with our previous findings that responses to UIS4 could not be enhanced by increasing the dose of RAS used for immunisation either[29].

We hypothesised that extending parasite maturation in the liver might improve the epitope-specific $CD8^+$ T cell response. It is noteworthy that in order to obtain direct evidence for prolonged antigen exposure in sporozoites attenuated by AZ cover *versus* RAS immunisations, elution of MHC-bound peptides from infected hepatocytes over time could be performed. Thus far, elution of MHC-bound epitopes from dendritic cells that had been co-cultured with asexual blood stages has been achieved[34]. Establishing this approach for pre-erythrocytic antigens and in a time-course experiment will be considerably more challenging. Our data showing that AZ prophylaxis failed to improve

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the resulting epitope-specific CD8$^+$ T cell responses, lending further support to the notion that liver stage antigens are poorly immunogenic.

AZ has been shown to specifically impede the biogenesis and inheritance of the apicoplast in malaria liver stages; EEFs continue to develop, but blood stage infection is not established[30]. *In vitro* work has indicated that AZ treatment of *P. berghei*-infected hepatoma cells allows for the detachment of merozoite-containing infected liver cells, and these merosomes fail to initiate blood stage patency[30]. The immunogenicity of these merosomes is of interest, particularly because mice immunised with sporozoites under AZ cover are susceptible to blood-stage challenge, demonstrating that protective immunity offered by this form of parasite attenuation is primarily against the pre-erythrocytic stages and that CD8$^+$ T cells are the prime effector mechanisms[21].

In contrast, immunisation with sporozoites under chloroquine (CQ) cover also leads to full EEF development and successful initial blood infection. Accordingly, recent studies revealed the generation of cross-stage protection involving both pre-erythrocytic and blood stages[35,36]. Understanding the extent to which blood stage antigens are exposed to the immune system following immunisation with whole sporozoites is important to the identification of vaccination strategies that can combine T cell responses against the pre-erythrocytic stages and antibodies against sporozoites and blood stages.

This study provides a confirmation that EEF antigens are poorly immunogenic. Yet, CD8$^+$ T cells must recognise peptides directly processed and presented by parasitised hepatocytes to employ their protective functions. Taken together with our recent findings that EEF antigens are nonetheless excellent targets of vaccine-induced CD8$^+$ T cells[29], our results challenge the use of immunogenicity in prioritising antigens for the design and evaluation of next-generation pre-erythrocytic vaccines. Standard immunological assays, endeavoured at discovering highly immunogenic antigens, may fail to identify those candidates with the ability to evoke superior levels of protective immunity. An in-depth characterisation of the complex biology of pre-erythrocytic stages, the immune responses they generate or not, coupled with a strategic identification of vaccine targets, should drive progress towards a highly efficacious malaria vaccine.

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FIGURE LEGENDS

Figure 1. *Plasmodium berghei* liver stage development 48 hours after co-administration of live sporozoites and azithromycin or administration of irradiation treated sporozoites. (A) Composite (top) and single channel fluorescence micrographs of WT *Plasmodium berghei* liver stages in cultured hepatoma cells. Shown are representative images of liver stages 48 h after infection with untreated sporozoites (control), irradiated sporozoites (RAS), and sporozoites with azithromycin cover (AZ/Spz) visualised by fluorescent staining of the cytoplasm (green; anti-*Pb*HSP70 antibody), the parasitophorous vacuolar membrane (red; anti-*Pb*UIS4 anti-serum), and nuclei (blue; Hoechst 33342). Scale bars: 10 µm. (B) Dot plot illustrating the arbitrary UIS4 fluorescence signal intensity of single liver stages as a proxy for UIS4 protein amounts (n=>20 each) at 48 h after infection with untreated sporozoites (control), RAS, or AZ/Spz . The UIS4 signal was measured as the sum of the fluorescence signal intensity per pixel over the complete liver stage area in ImageJ. Bars and lines represent mean values ± SD. ***, p<0.001; ns, non-significant (One-way ANOVA with Tukey's multiple comparison test).

Figure 2. Induction of antigen-experienced CD8$^+$ T cells to sporozoite and EEF vacuolar antigens following different methods of sporozoite attenuation

(A-B) The flow cytometry gating strategy used to assess the proportion of antigen-experienced CD8$^+$ T cells (CD11a^{hi} CD8α^{lo}) and IFN-γ producing antigen-specific CD8$^+$ T cells (IFN-γ^+ CD11a$^+$) in the (A) spleen and (B) liver. (C-F) Mice were immunised with CSP^{SIINFEKL} (n=3-4) or UIS4^{SIINFEKL} (n=5-9) RAS or AZ/Spz. Spleens and livers from immunised and naïve mice were harvested 14 days later. Graphs show the (C,D) percentage and (E,F) absolute cell counts of CD11a^{hi} CD8α^{lo} cells from the CD8$^+$ T cell compartment in the (C,E) spleen and (D,F) liver. Bar charts show mean values (± SEM). One-way ANOVA with Tukey's multiple comparison test was employed with no statistical significance between groups determined. Values shown are from one representative experiment from at least two independently conducted experiments.

Figure 3. Generation of antigen-specific CD8$^+$ T cells to sporozoite and EEF vacuolar antigens following different methods of sporozoite attenuation

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Mice were immunised with $CSP^{SIINFEKL}$ (n=3-4) or $UIS4^{SIINFEKL}$ (n=5-9) RAS or AZ/Spz. 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,B) percentage and (C,D) absolute cell counts of $CD11a^{hi}$ $CD8\alpha^{lo}$ cells co-expressing IFN-γ in the (A,C) spleen and (B,D) liver. Bar charts show mean values (± SEM). *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; one-way ANOVA with Tukey's multiple comparison test). Values shown are from one representative experiment of at least two independently conducted experiments.

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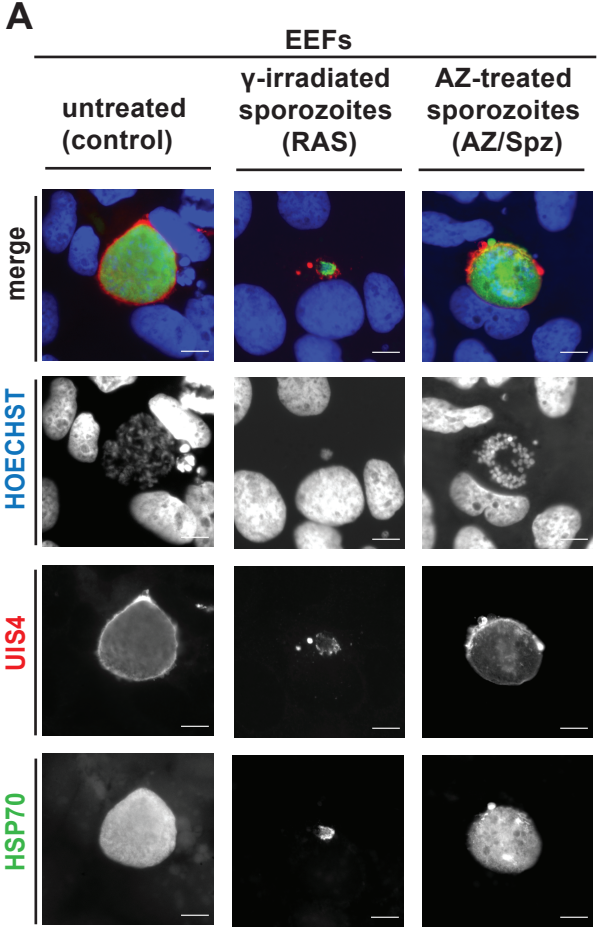
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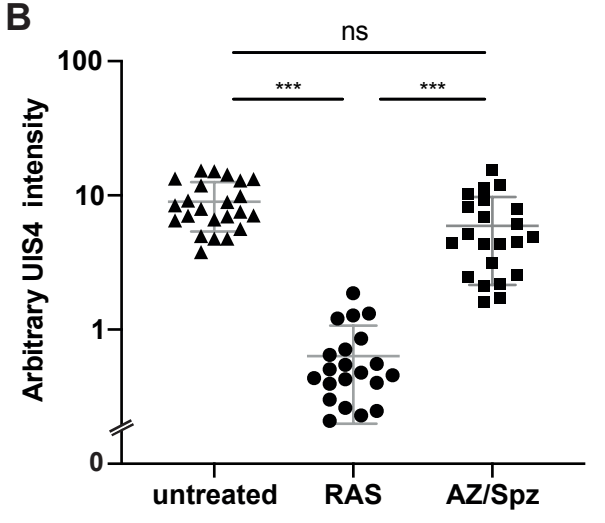
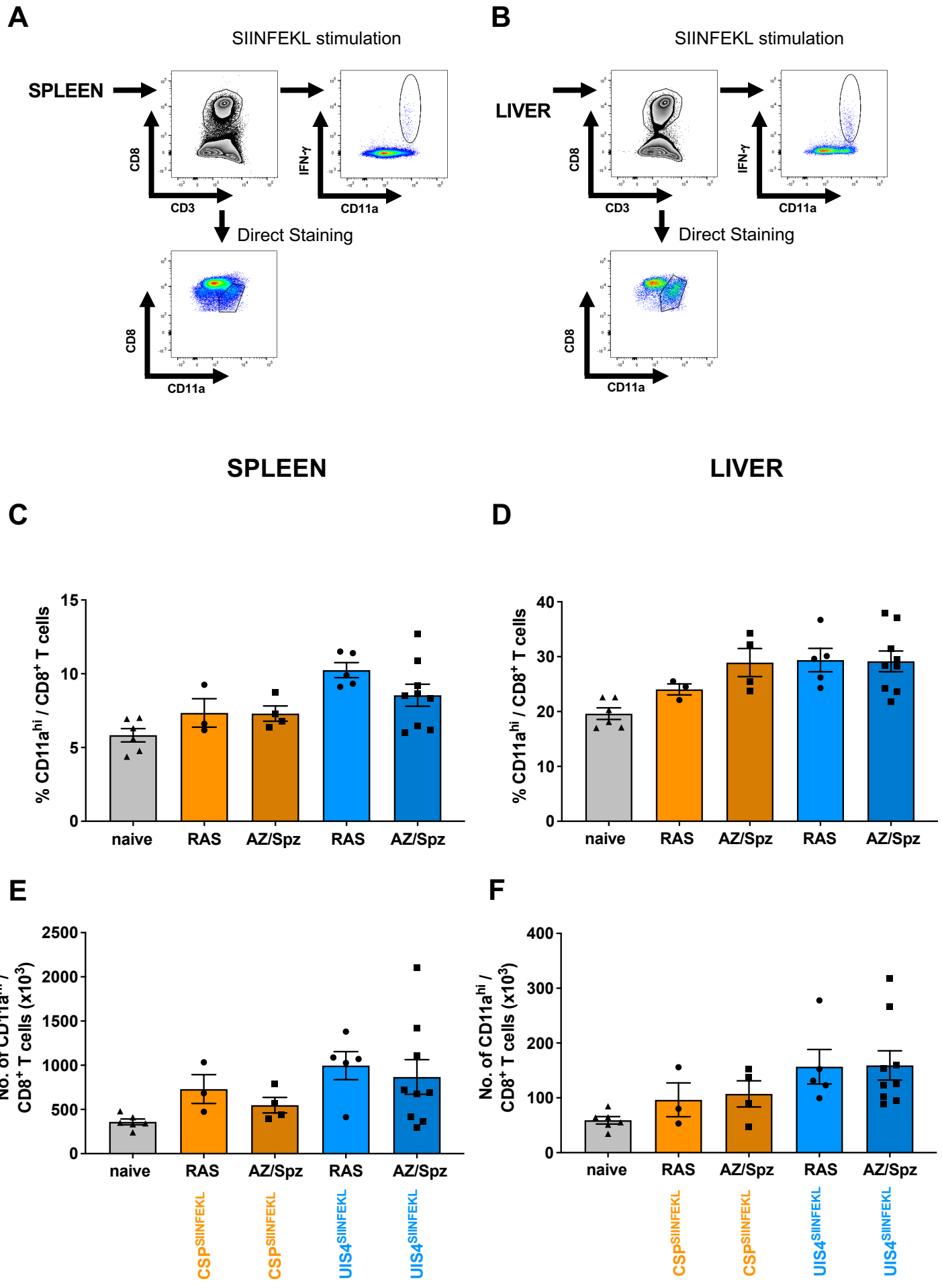
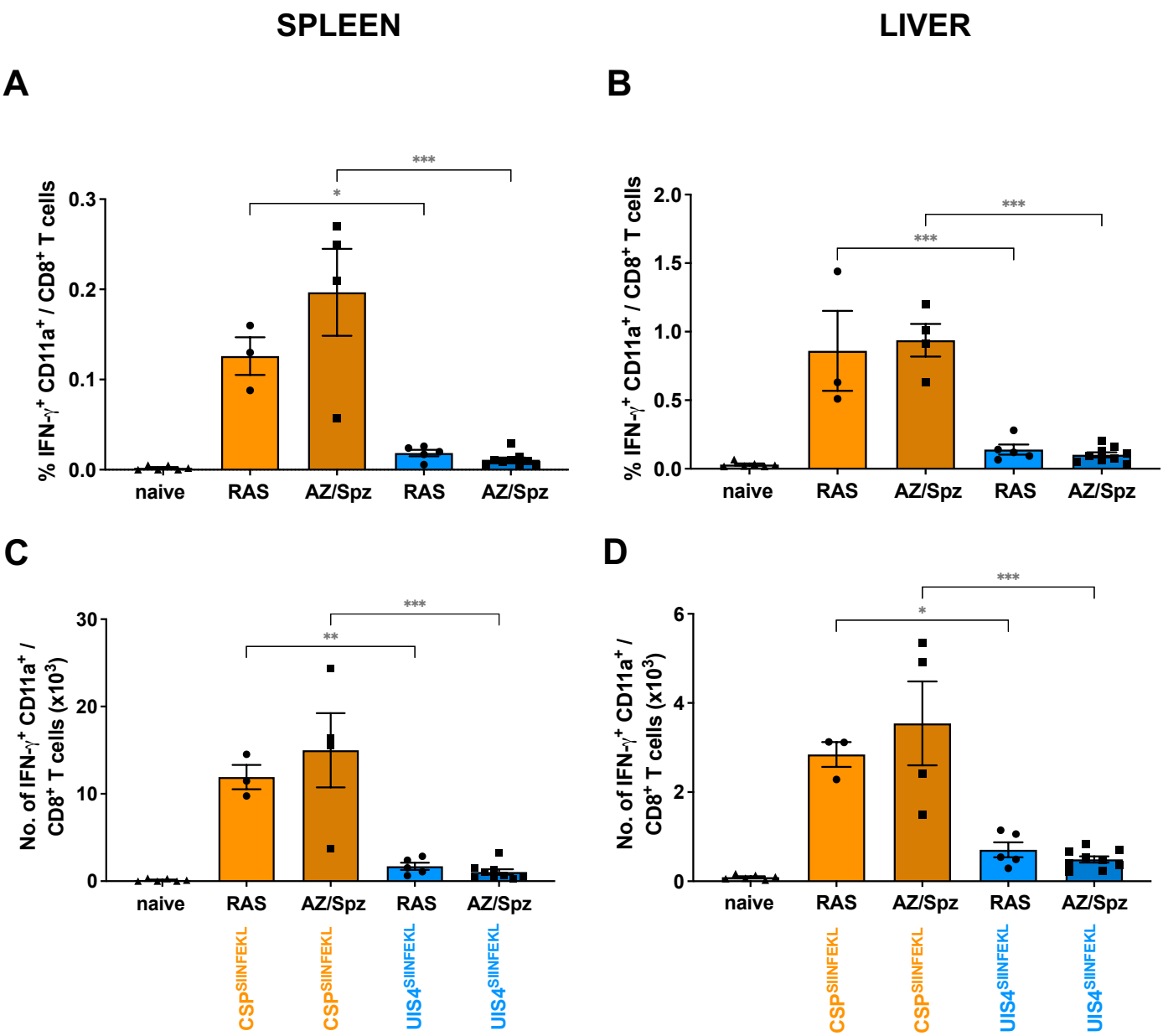
EEFs
untreated (control)
γ-irradiated sporozoites (RAS)
AZ-treated sporozoites (AZ/Spz)
merge
HOECHST
UIS4
HSP70


B
ns
***
***
100
10
1
0
Arbitrary UIS4 intensity
untreated
RAS
AZ/Spz


A
SIINFEKL stimulation
SPLEEN
CD8
CD3
IFN-γ
CD11a
Direct Staining
CD8
CD11a
B
SIINFEKL stimulation
LIVER
CD8
CD3
IFN-γ
CD11a
Direct Staining
CD8
CD11a
SPLEEN
LIVER
C
% CD11a^hi / CD8^+ T cells
15
10
5
0
naive
RAS
AZ/Spz
RAS
AZ/Spz
D
% CD11a^hi / CD8^+ T cells
40
30
20
10
0
naive
RAS
AZ/Spz
RAS
AZ/Spz
E
No. of CD11a^hi / CD8^+ T cells (x10^3)
2500
2000
1500
1000
500
0
naive
RAS
AZ/Spz
RAS
AZ/Spz
CSP^SIINFEKL
CSP^SIINFEKL
UIS4^SIINFEKL
UIS4^SIINFEKL
F
No. of CD11a^hi / CD8^+ T cells (x10^3)
400
300
200
100
0
naive
RAS
AZ/Spz
RAS
AZ/Spz
CSP^SIINFEKL
CSP^SIINFEKL
UIS4^SIINFEKL
UIS4^SIINFEKL


SPLEEN
LIVER
A
B
C
D
% IFN-γ+ CD11a+ / CD8+ T cells
No. of IFN-γ+ CD11a+ / CD8+ T cells (x10³)
naive
RAS
AZ/Spz
RAS
AZ/Spz
CSPSIINFEKL
CSPSIINFEKL
UIS4SIINFEKL
UIS4SIINFEKL
0.0
0.1
0.2
0.3
0.5
1.0
1.5
2.0
0
10
20
30
2
4
6
*
**
***