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Development of *Plasmodium*-specific liver resident-memory CD8$^+$ T cells after heat-killed sporozoite immunization in mice

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Abbreviations: RAS, radiation-attenuated sporozoites; HKS, heat-killed sporozoites; T_{RM}, resident-memory CD8$^+$ T cells; T_{EM}, effector-memory CD8$^+$ T cells; T_{CM}, central-memory CD8$^+$ T cells; α-GalCer, α-galactosylceramide.

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Abstract:

Malaria remains a major cause of mortality in the world, and an efficient vaccine is the best chance of reducing the disease burden. Vaccination strategies for the liver stage of disease that utilise injection of living radiation-attenuated sporozoites (RAS) confer sterile immunity, which is mediated by $CD8^+$ memory T cells, with liver-resident memory T cells (T_{RM}) being particularly important. We have previously described a TCR transgenic mouse, termed PbT-I, where all $CD8^+$ T cells recognize a specific peptide from *Plasmodium.* PbT-I form liver T_{RM} cells upon RAS injection and are capable of protecting mice against challenge infection. Here we utilize this transgenic system to examine whether non-living sporozoites, killed by heat treatment (HKS), could trigger the development of *Plasmodium*-specific liver T_{RM} cells. We found that HKS vaccination induced the formation of memory $CD8^+$ T cells in the spleen and liver, and importantly, liver T_{RM} cells were fewer in number than that induced by RAS. Crucially, we showed the number of T_{RM} cells was significantly higher when HKS were combined with the glycolipid α-galactosylceramide as an adjuvant. In the future, this work could lead to development of an anti-malaria vaccination strategy that does not require live sporozoites, providing greater utility.

Introduction:

Malaria is a devastating disease which still kills more than 400,000 people a year [1]. In humans, this parasitic disease is caused by five species of *Plasmodium* (*P.falciparum, P.vivax, P.ovale spp., P.malariae and P.knowlesi*) and is transmitted via infected female Anopheline mosquitoes. Different tools have been used to limit infection, including insecticide impregnated bed nets, residual insecticide spraying, and early diagnosis for treatment. Despite these tools considerably reducing malaria morbidity and mortality in the past decade [1], the infection still threatens the lives of millions of people due to emerging drug resistance. Thus, the development of an effective malaria vaccine is still of paramount importance. While viruses and bacteria, for which we have effective

vaccines, generally have relatively simple lifecycles within mammalian hosts, the complexity of *Plasmodium* parasites, and particularly their lifecycle, makes the development of vaccines a considerable challenge. *Plasmodium* parasites, in the form of sporozoites, are introduced into the skin following the bite of an infected mosquito and rapidly migrate to the liver via the bloodstream where they infect hepatocytes. This is the pre-erythrocytic phase of the infection which lasts for approximately one week in humans, or two days in mice, and does not trigger any symptoms. In the liver, sporozoites undergo asexual replication and maturation. Hepatocytes can then release a massive number of parasites (as merozoites) into the blood and initiate the erythrocytic phase of the infection [2].

Different vaccination strategies against malaria have been developed and tested in pre-clinical models and in clinical trials in humans [3]. Of these, approaches using whole sporozoite vaccines have been the most successful. One of the most powerful aspects of whole parasite approaches is that they contain all sporozoite antigens (Ag) and therefore initiate priming for immunity to a broad range of malaria epitopes. The whole parasite approach that has been tested most extensively is the use of radiation-attenuated sporozoites (RAS), whereupon intravenously injected (iv) can protect mice from challenge infection with rodent *P.berghei* sporozoites [4]. The radiation dose delivered to the sporozoites results in attenuated parasites that can still invade hepatocytes to establish infection but prevents differentiation into merozoites and initiation of blood stage infection. It was subsequently found that humans immunized via the bites of irradiated *P. falciparum* infected mosquitoes were protected against the pre-erythrocytic stage of infection when challenged within 9 weeks of immunization [5, 6]. The first human anti-malaria injectable vaccine was manufactured in 2010 through the aseptic purification and cryopreservation of *P. falciparum* sporozoites from irradiated mosquitoes [7]. Intradermal and subcutaneous inoculation of this vaccine resulted in minimal protection in humans [8] but intravenous injection elicited high level and long-lasting sterile protection [8, 9].

The sterile protection elicited by RAS vaccination is $CD8^+$ T cell dependent. In humans, vaccination correlates with an increase in the number of circulating $CD8^+$ T cells that produce effector cytokines [9, 10]. In mice, depletion of $CD8^+$ T cells or neutralization of interferon-gamma after vaccination, but before challenge infection, results in a loss of sterile immunity [11, 12]. Among all $CD8^+$ T cells, intra-hepatic memory $CD8^+$ T cells are particularly important for providing long-lasting liver-stage immunity after intravenous RAS vaccination [13, 14]. Recently, we confirmed the existence and importance of liver-resident memory $CD8^+$ T (T_{RM}) cells in immunity against the rodent parasite *P.berghei* ANKA [15]. T_{RM} cells developed after intravenous inoculation of *P.berghei* ANKA RAS, remained resident in the liver without recirculating to other tissues, and provided protection against sporozoite challenge. Despite the high efficacy of RAS vaccines, this strategy is not optimal for deployment on a large scale in the field as it suffers from logistical difficulties. Indeed, the cost of irradiation and cryopreservation, two essential steps in the production of RAS vaccines, is significant; which limits its implementation as a vaccine on a broad scale. Moreover, the radiation dose is a determining factor in the success of this strategy [16-18]. Insufficient irradiation of sporozoites could lead to infection instead of immunity [19] a considerable concern in terms of safety. Genetically attenuated parasites, which initiate infection but are unable to differentiate into blood stage parasites offer an additional alternative and have been used in safety trials in humans [20] with modest levels of protection observed against controlled human malaria infection in some vaccinated individuals [21].Inactivated vaccines, which are produced by destroying a pathogen with chemicals, heat or other processes, represent a potential solution for these logistical issues. Inactivated vaccines do not require cryopreservation for transport, and inactivation of the microorganism makes the vaccine more stable. However, investigations into inactivated vaccines in the context of malaria have demonstrated that immunization with heat-killed sporozoites (HKS) conferred limited protection against live sporozoite challenge [22], likely due to insufficient expansion of parasite-specific $CD8^+$ T cells [23]. This suggests that the induction of protective immunity against malaria liver stages is dependent on live parasites but the reasons for this remain unclear. Nevertheless, it is

clear that memory CD8$^+$ T cells specific for *Plasmodium* are induced after HKS immunization [23] suggesting that it may be possible to improve these responses in a vaccination setting.

Whether or not protective liver T_{RM} are generated by HKS immunization is still unknown. This is an important issue as lack of protection induced by HSK may relate to an inability to induce liver T_{RM} cells, or if it does, perhaps insufficiently to be protective. Understanding why the response is inadequate may provide direction on how the suboptimal vaccine can be manipulated to boost the generation of T_{RM} cells. Herein, we examined the memory CD8$^+$ T cell response induced by non-living *P.berghei* ANKA sporozoite vaccination. We took advantages of the T cell receptor (TCR) transgenic mice, termed PbT-I mice, that we previously described [24]. PbT-I mice have been engineered so that all CD8$^+$ T cells recognize a specific *Plasmodium* antigen [25]. We found that after vaccination with HKS, memory PbT-I T cells developed in the spleen and liver, but the number of these cells was significantly reduced relative to RAS immunization. Importantly, we were able to detect liver T_{RM} cells after HKS immunization, albeit at lower numbers than induced by RAS vaccination. By combining HKS vaccination with the adjuvant α-galactosylceramide, the number of liver T_{RM} cells was improved, raising the possibility that with the right adjuvant systems it may be possible to provide some level of protection against malaria via vaccination with non-living *Plasmodium* parasites.

Results:

Priming of Plasmodium-specific CD8$^+$ T cells is impaired after HKS immunization.

To examine if non-living *Plasmodium* parasites are able to prime CD8$^+$ T cell responses *in vivo*, we adoptively transferred *Plasmodium*-specific CD8$^+$ T cell receptor (TCR) transgenic T cells (termed PbT-I T cells) [24] into wild-type B6 mice that were then immunized with *Plasmodium berghei* ANKA. We compared the early proliferation of PbT-I T cells after immunization with *P. berghei* ANKA radiation attenuated sporozoites (RAS) or heat-killed sporozoites (HKS). As expected, almost all transferred PbT-I T cells proliferated in the spleen 5 days after immunization with RAS (Figure 1A). In

mice immunized with HKS, proliferation of parasite-specific $CD8^+$ T cells was also observed (Figure 1A). Nevertheless, the number and the proportion of activated PbT-I T cells that accumulated in the spleen were lower in mice immunized with HKS (Figure 1B). A large proportion of the PbT-I T cells that were present in the liver at this time point had also proliferated (Figure 1C), likely reflecting recirculation of cells that were activated and proliferated in the spleen [24]. However, the total number of PbT-I T cells in the livers of the immunized mice was significantly lower after HKS immunization when compared to RAS immunization (Figure 1D).

These data indicated that HKS induced significant priming of *Plasmodium*-specific $CD8^+$ T cells, although their expansion was impaired when compared to the expansion triggered by RAS.

Liver T_{RM} development is relatively poor after HKS immunization

We previously demonstrated that the development of liver tissue-resident memory $CD8^+$ T ($CD8^+$ T_{RM}) cells in response to RAS vaccination is critical for malaria liver-stage immunity [15]. We therefore sought to determine if immunization with HKS can also trigger the development of such T_{RM} cells in the liver. To investigate this, we adoptively transferred PbT-I T cells into B6 mice one day prior to immunization with *P. berghei* ANKA RAS or HKS. 35 days later, we analysed the formation of memory PbT-I T cell subpopulations in the liver (Figure 2.A-D) and the spleen (Figure 2.E). Here we used staining for CD44, CD62L and CD69 to broadly distinguish central memory (T_{CM}) cells ($CD44^+$ $CD62L^+$ and $CD69^-$), effector memory (T_{EM}) cells ($CD44^+$ $CD62L^-$ $CD69^-$) and resident memory (T_{RM}) cells ($CD44^+$ $CD62L^-$ $CD69^-$) (Figure 2.A, B and Figure S1). This revealed that both RAS and HKS induce liver T_{RM} cells. The T_{RM} cell phenotype of cells induced by HSK was further confirmed by their expression of CXCR6 and lack of expression of KLRG-1, contrasting T_{EM} cells (Figure 2.A, B). Few T_{CM} cells were induced in either the spleen or liver by these vaccines (Figure 2, Figure S1). This observation showed that non-living *Plasmodium* parasites induce the development of *Plasmodium-specific* liver T_{RM} cells.

Nevertheless, the absolute number of liver T_{RM} cells in HKS immunized mice was significantly lower than that observed in RAS vaccinated mice (Figure 2C). Interestingly, the proportion of T_{RM} cells present in the liver after HKS immunization was comparable to that detected after immunization with RAS (32% +/-20 of total memory $CD8^+$ T cells after HKS and 32% +/-18 with RAS), suggesting that memory $CD8^+$ T cells are generated in HKS vaccinated mice, although in small numbers (Figure 2.D-E).

Similar findings were evident from examination of the response of endogenous *Plasmodium*-specific memory $CD8^+$ T cells (Figure 3). We used tetramer staining to detect responses in the spleen and liver to two different $CD8^+$ T cell specificities; one specific for an H-$2K^b$-restricted epitope derived from the 60S ribosomal protein L6 of *P. berghei* ANKA (RPL6) that we recently described as the cognate antigen of PbT-I T cells [25] (Figure 3.A-C), and the other specific for a well-characterized H-$2D^b$-restricted epitope within the thrombospondin-related adhesive protein (TRAP) [26] (Figure 3.D-F) of *Plasmodium* parasites. This analysis showed that endogenous memory T cells specific for these two epitopes were expanded after HKS vaccination, yet their absolute numbers were significantly lower than those observed following RAS vaccination. Importantly, they confirmed that liver T_{RM} cells could be generated in HKS immunized mice (Figure 3).

Taken together these data revealed that vaccination with heat-killed *P. berghei* ANKA parasites triggered the formation of *Plasmodium-specific* liver-resident memory $CD8^+$ T cells within the endogenous repertoire.

Liver T_{RM} formation after HKS immunization is improved using α-Galactosylceramide as an adjuvant

Adjuvants are well known to enhance vaccine efficacy by establishing an inflammatory environment that activates dendritic cells to enhance T cell priming [Reviewed in [27]]. Vaccination with sub-unit antigens, including anti-malaria vaccination, confers inadequate protection in the absence of

adjuvants [28]. Therefore, we hypothesised that by combining HKS vaccination with potent adjuvants, we could improve the formation of protective liver T_{RM} cells. We tested three adjuvants known to have potent effects. Firstly, we used a CpG oligonucleotide generated by linking a class-B CpG to a class P CpG [29, 30] that we refer to here as CpG Combo. We previously showed that this CpG Combo enhanced the formation of protective liver T_{RM} cells in a prime-and-trap vaccination strategy [15]. We also used the class B CpG 1668, well known to stimulate TLR-9 in mice and to induce inflammation [31]. CpG 1668 has also shown good efficacy for the induction of protective immune responses with T cell peptide vaccines, particularly in the context of viral infection with lymphocytic choriomeningitis virus [32]. Finally, we employed a synthetic glycolipid, α-Galactosylceramide (α-GalCer), which is a potent stimulator of type I natural killer T (NKT) cells and has been successfully used as an adjuvant with malaria vaccines including RAS [33]. To examine the effect of these adjuvants in the formation of liver T_{RM} cells induced by HKS, we co-administered HKS with each of the adjuvants one day after adoptive transfer of PbT-I T cells and then examined memory T cell numbers in the spleen and liver 35 days later (Figure 4). While neither of the CpG adjuvants significantly altered liver T_{RM} cell formation, α-GalCer significantly increased this population by more than 11-fold. However, this level of T_{RM} cell formation was still suboptimal compared to RAS vaccination (7700 vs 64100, respectively). α-GalCer was also the most effective adjuvant for expanding other memory $CD8^+$ T cells in the liver (Figure 4.B) and spleen (Figure 4.C).

T_{RM} cell formation in response to HKS was increased by addition of α-GalCer, but whether this led to protective immunity was yet to be tested. To address this point, we vaccinated a new set of mice with HKS either alone or in the presence of 1nmol of α-GalCer or 10-fold less (0.1nmol, as a similar dose to that used in Figure 4); waited 35 days, and then challenged these mice with live sporozoites (Figure 5A). Examination of protection showed that mice immunized with HKS alone or combined with the lower dose of α-GalCer were all susceptible to infection, whereas 1/8 mice given HKS with the higher dose of α-GalCer was protected. This protection was, however, not significantly different from the control group given HKS alone. Interestingly, 2/5 mice vaccinated with RAS were protected

and this was increased to 4/5 by the addition of α-GalCer, as previously reported [33]. To gain further insight from the protection experiment, challenged mice were also examined for memory T cell populations by analysis of spleen and liver populations. These populations were assessed either immediately after testing positive for parasitemia or on day 12 post-challenge for protected mice (Figure 5B-D). This analysis confirmed a trend to increased T_{RM} cell numbers when α-GalCer is combined with HKS, but that further increasing the dose of this adjuvant was not particularly beneficial. Altogether, these observations showed that the administration of the synthetic glycolipid, α-GalCer, as an adjuvant, significantly improved the formation of protective liver resident memory $CD8^+$ T cells after vaccination with HKS.

$CD8^+$ T cell responses to HKS are not improved by providing innate signals linked to RAS vaccination

We next sought to determine why vaccination with HKS was less efficient at inducing liver T_{RM} cells, and memory $CD8^+$ T cells in general, than vaccination with RAS. While some contribution to RAS superiority is likely to be made by infection of hepatocytes, which is not mediated by HKS, our previous findings indicate that the bulk of this response to RAS is initiated in the spleen [24]. It seemed likely therefore that signals localized to the spleen may also contribute. This view is supported by our day 5 analysis of the PbT-I T cell response to RAS versus HKS in spleen, where RAS vaccination was clearly superior (Figure 1B). We hypothesised that by combining vaccination with RAS and HKS, and therefore using the innate signals triggered by RAS as an adjuvant, we might improve the response to HKS. To investigate this hypothesis, we took advantage of the genetically modified *P. berghei* CS^{5M}-mCherry (CS^{5M}) parasite, which expresses the SIINFEKL epitope from the model antigen ovalbumin (OVA) in place of the endogenous SYIPSAEKI epitope of the circumsporozoite (CS) protein [34]. This parasite is efficiently recognized and eliminated by transgenic ovalbumin-specific OT-I T cells, which recognize the SIINFEKL epitope (Figure S2). Using this parasite allowed us to separate the antigenic signal (SIINFEKL) provided by heat killed CS^{5M}

sporozoites (CS^{5M}-HKS) from the innate signals provided by wild-type RAS (WT-RAS), which lack the SIINFEKL epitope. To determine whether the weak response to OVA triggered by CS^{5M}-HKS could be enhanced by inflammatory signals generated by wild-type RAS, we adoptively transferred mice with OT-I T cells, one day prior to vaccination with 50,000 CS^{5M}-HKS alone or in combination with 50,000 WT-RAS. Five days later, OT-I T cell responses were examined in the spleen (Figure 6.A) and liver (Figure 6.B). Despite a slight increase in the mean number of proliferated OT-I T cells stimulated by CS^{5M}-HKS when co-administered with WT-RAS, this response was not significantly greater than observed for mice given CS^{5M}-HKS alone. These findings indicate that innate signals associated with live sporozoites could not fully recover the response to HKS. Similar conclusions were drawn from experiments using lower doses of CS^{5M}-HKS and WT-RAS that examined both OT-I T cell proliferation on day 5 and long-term memory formation (Figure S3). Together, these findings suggested that innate signals induced by RAS were not sufficient to boost responses to HKS.

Discussion:

In this study, we revealed that live *Plasmodium* parasites are not essential for the generation of *Plasmodium*-specific memory $CD8^+$ T cells. We established that vaccination with non-living, whole sporozoites triggers the generation of *Plasmodium*-specific resident memory $CD8^+$ T cells in the liver, albeit in lower numbers than observed in response to RAS vaccination. Additionally, we demonstrated that as an adjuvant, α-galactosylceramide was able to increase the number of $CD8^+$ T_{RM} cells in the liver in response to HKS vaccination. Finally, we have demonstrated that the presence of RAS is not sufficient to boost the response to HKS expressed antigens.

It is a feature of vaccination that inactivated vaccines produce weaker immune responses when compared to attenuated vaccines, but the reasons for this are not clearly defined. A study comparing two processes of pathogen inactivation showed that lethally irradiated bacteria induced protective T cell immunity, but heat-killed bacteria could not, suggesting that heat destroyed important antigens and thus prevented induction of protective immunity [35]. A similar comparison has been made with

P. berghei, but neither viable over-irradiated sporozoites, whose development stops at an earlier stage than RAS, nor HKS succeeded in conferring protective immunity [18]. The poor protection by over-irradiated sporozoites suggests that heat is not the only factor that reduces *Plasmodium* immunogenicity. The precise requirements for the generation of optimal $CD8^+$ T cell-mediated protective immunity to liver stage *Plasmodium* parasites are not well defined, but there is evidence that genetically attenuated parasite (GAP) vaccination induces higher numbers of memory $CD8^+$ T cells than RAS vaccination [36], suggesting that metabolism and the ability of the parasites to invade and persist within hepatocytes for longer periods of time may be important. This is also relevant in the context of HKS as the heat killed parasites are not metabolically active and cannot migrate to and invade the liver. HKS derived antigens do not persist; as two weeks after immunization, *Plasmodium*-specific $CD8^+$ T cells failed to proliferate [37], a potential contributor to the weak $CD8^+$ T cell memory responses that we observed here.

We have previously shown that the best correlate of protection against infection with *P. berghei* sporozoites in vaccinated mice is the number of T_{RM} cells present in the liver [15]. Here, we showed that only a relatively small number of these cells develop after HKS vaccination, when compared to the number induced by RAS vaccination. Crucially, by combining HKS vaccination with the adjuvant α-GalCer, we improved the development of Plasmodium-specific liver T_{RM} cells and, at the highest dose of α-GalCer, this prevented progression to blood stage in 1/8 mice challenged with 200 liver sporozoites. While this was not significantly better than the 0/8 mice given HKS alone, it supported the view that it may be possible to improve protection induced by HKS by addition of the appropriate adjuvant. α-GalCer is a synthetic glycolipid which binds to the nonclassical MHC-I molecule, CD1d, on antigen presenting cells and activates Type 1 Natural Killer T (NKT) cells. By activating NKT cells, the glycolipid α-GalCer induces maturation of dendritic cells via CD40L presentation and cytokines such as interleukin 4, facilitating subsequent responses by conventional $CD4^+$ and $CD8^+$ T cells [38-40]. We chose α-GalCer as an adjuvant in the context of HKS vaccination because of its proven role in mediating protection against murine malaria [41] by enhancing malaria-

specific CD8$^+$ T cell responses [33]. Furthermore, a synthetic C-glycoside analogue of α-GalCer, α-C-galactosylceramide was reported to exhibit a 1000-fold more potent anti-malaria activity than α-GalCer. This adjuvant was proposed to work by inducing prolonged maturation of APC and an enhanced interleukin-12 release, inducing more potent activation of Natural Killer cells and *Plasmodium*-specific CD8$^+$ T cells *in vivo* [42, 43]. In an analogous approach, the immune response and associated protection against infection after immunisation with genetically attenuated parasites (GAP) was enhanced when an agonistic antibody to OX40 was co-administered, suggesting that the correct adjuvants, given in the right context, have the potential to improve protection against infection [44]. α-galactosylceramide, shown here to improve T_{RM} cell generation to HKS in mice, also has the potential to improve efficacy in humans. Injection of free α-GalCer was well-tolerated in humans in a phase I study at all doses tested [45]. α-GalCer has also been administered with flu vaccines to swine of mixed genetic background (with similar NKT cell frequencies to humans), with improved vaccine efficacy [46]. Furthermore, several studies in the cancer vaccine area show some level of utility by administering α-GalCer to harness NKT cell-dependent immunity (reviewed in [47]).

Surprisingly, our findings showed that concurrent immunization with RAS and HKS did not boost the CD8$^+$ T cell response to antigens present only within heat-killed parasites. This suggests that soluble, inflammatory signals induced by RAS vaccination are not sufficient to improve the response to HKS immunization. This may imply that different antigen-presenting cells (APC) are responsible for the priming of naïve CD8$^+$ T cells after HKS and RAS immunization and that inflammatory signals produced in response to RAS are not sufficient to induce APC activation in trans. This phenomenon was previously investigated in the context of immunization with heat-killed *Listeria monocytogenes* bacteria which induced effector CD8$^+$ T cells that did not have effective cytolytic function [48]. In this study, concurrent immunization of mice with live and heat-killed *Listeria monocytogenes* bacteria did not promote the differentiation of CD8$^+$ T cells against antigens from heat-killed bacteria into effector cells capable of killing via cytolytic function [48]. The authors were not able to trace the

antigen in this study to determine if the same APC subsets were presenting the heat-killed and live bacterial antigens. Similarly, here we cannot determine if the same APC are presenting RAS and HKS antigens, but we would hypothesise that this would improve the HKS response if that were to occur. Lack of augmentation by RAS also supports the view that a major contribution to priming may be provided by antigen presentation associated with infection of the liver, which cannot be achieved under any circumstances by HKS, even with concomitant RAS administration.

In conclusion, here we provide a deeper understanding of why heat-killed sporozoites cannot currently be used as a vaccine against malaria. In parallel, we raise the idea that boosting the expansion of liver $CD8^+$ T_{RM} cells after HKS immunization to a threshold that reaches RAS vaccination levels, could lead to the development of a vaccination strategy that does not require live sporozoites.

Material and Methods:

Mice, mosquitos, parasites and infections

All animal procedures were performed in strict accordance with the recommendations of the Australian code of practice for the care and use of animals for scientific purposes. The protocols were approved by the Melbourne Health Research Animal Ethics Committee, University of Melbourne (ethic project IDs: 1714302, 1814522).

Female C57BL/6 (B6), OT-I-GFP and PbT-I-GFP [24] were used between 6-12 weeks of age and were bred and maintained at the Department of Microbiology and Immunology, The University of Melbourne.

Animals used for the generation of the sporozoites were 4-5-week-old male Swiss Webster mice purchased from the Monash Animal Services (Melbourne, Victoria, Australia) and maintained at the School of Botany, The University of Melbourne, Australia. *Anopheles stephensi* mosquitoes (strain STE2/MRA-128 from The Malaria Research and Reference Reagent Resource Center) were reared

and infected with *Plasmodium berghei* ANKA (*P. berghei*) as described [49]. This included wild-type (WT) *P. berghei* ANKA (*P. berghei*) or *P. berghei* CS^{5M}-mCherry (CS^{5M}) [34]. *P. berghei* CS^{5M}-mCherry (CS^{5M}) are genetically modified to express the SIINFEKL epitope from the model antigen ovalbumin in place of the endogenous SYIPSAEKI epitope in the circumsporozoite (CS) protein [34]. Sporozoites were dissected from mosquito salivary glands and resuspended in cold PBS.

Attenuation of sporozoites (RAS) was accomplished by exposing freshly dissected sporozoites to 20.000 rads using a gamma ^{60}Co source. Heat-killed sporozoites (HKS) were prepared by incubating the parasites at 72°C for 15 min followed by 95°C for another 15 min. *P. berghei* RAS or HKS were injected intravenously (iv) in 200µl of PBS alone, combined with adjuvants or combined with each other by mixing and injecting the combinations simultaneously. Non-immunized mice received no injection. Adjuvants used were either 75 µg of a Combo CpG oligonucleotide, termed Combo (CpG generated by linking (5' to 3') CpG-2006 to CpG-21798 [29, 30]), 0.116 µg of α-galactosylceramide (α-GalCer) for data shown in Figure 4, or 1nmol (0.85µg) or 0.1 nmol (0.085µg) for data shown in Figure 5, or 75 µg CpG1668 dissolved in PBS. Unless otherwise indicated in figure legends, mice were immunized once with 50,000 *P. berghei* sporozoites or left uninjected (Naive).

For challenge experiments, 200 freshly dissected *P. berghei* ANKA sporozoites were injected iv as indicated. Mice were assessed for parasitemia generally on day 6, 7, 8, 10 and 12 using flow cytometry. Briefly, a drop of blood was collected from the mice and stained with Hoechst 33258 dye (ThermoFisher, Scoresby, Victoria, Australia) for 1 hr at 37°C. Samples were analyzed on a LSR Fortessa (BD Biosciences, San Jose, USA) using a violet laser (405 nm) to excite the dye. After gating on Red Blood Cells the percentage of Hoechst positive cells were measured. Values were compared to uninfected controls and typically values of >0.1% were considered positive for parasites. Mice positive for parasites on two consecutive days were euthanized. Mice were considered sterilely protected if they remained parasitemia-negative on day 12 after challenge.

Adoptive transfer of CD8$^+$ T cells

Naïve PbT-I or OT-I CD8$^+$ T cells were negatively enriched from the spleens and lymph nodes of PbT-I-GFP or OT-I-GFP mice, respectively. Briefly, tissues were disrupted by passing through 70 μm cell strainers and red cells lysed. Single cell suspensions were labelled with a cocktail of rat monoclonal antibodies specific for mouse CD4, MHC Class II, macrophages and neutrophils prior to incubating with BioMag goat anti-rat IgG beads (Qiagen) and separated using a magnet. Enriched naïve CD8$^+$ T cells were counted, and their purity analysed by staining with anti-CD8α and anti-V$\alpha_{8.3}$ TCR antibodies for PbT-I T cells or anti-CD8α and anti-Vα_2 TCR antibodies for OT-I T cells. Cell counts were adjusted to the right concentration in PBS and mice were injected with 200 μL iv one day prior to vaccination. To measure cell proliferation, enriched PbT-I T or OT-I T cells were coated with CellTrace^{TM} Violet (CTV, Thermofisher) following manufacturer's instructions, prior to adoptive transfer.

Organ processing for T cell analysis

Tissues were harvested from mice at different time points after immunization and homogenised through 70 μm mesh to make single cell suspensions. For spleen cell preparations, red blood cells were lysed, and remaining cells were filtered through a 70 μm mesh. Liver cell suspensions were passed through a 70 μm mesh and resuspended in 35% isotonic Percoll (Sigma). Cells were then centrifuged at 500g for 20 min at room temperature (RT), the pellet harvested, and the red cells lysed before further analysis.

Flow cytometry

This study adhered to the guidelines on the use of flow cytometry in immunological studies [50]. Lymphocytes were stained with monoclonal antibodies for: CD4 (RM4-5), CD8α (53-6.7), TCRβ (H57-597), from BD, B220 (RA3-6B2), CD44 (IM7), CD62L (MEL-14), CD69 (H1.2F3) from ThermoFisher Scientific (Waltham, MA, USA) , CXCR6 (SA05D1), CX3CR1 (SA011F11) from BioLegend (San Diego,

CA, USA), H2-Kb-PbT-I Ag [25] and H2-Db-PbTRAP130-138 tetramers were made in house. Dead cells were excluded by propidium iodide (PI) staining. For the analysis of memory $CD8^+$ T cell populations in the spleen and liver, cells were gated for tetramer$^+$, PbT-I or OT-I $CD8^+$ CD44hi and cells were subdivided into T_{CM}, T_{EM} or T_{RM} based on CD69 and CD62L expression (T_{CM} $CD62L^+$ $CD69^-$, T_{EM} $CD62L^-$ $CD69^-$ and T_{RM} $CD62L^-$ $CD69^+$). Single-color positive control samples were used to adjust compensation, and cells were analyzed by flow cytometry on a LSR Fortessa (BD Immunocytometry Systems, San Jose, CA, USA) or a Cytek Aurora (CytekTM Biosciences Inc) and by using Flowjo software (Tree Star, Ashland, OR, USA).

Statistical analyses

Figures were generated using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA). Data are shown as mean values ± standard deviation (SD) or standard error of mean (SEM) as indicated in the legends. Statistical analyses were performed using GraphPad Prism 8. The statistical test used is indicated in each figure legend. *P*<0.05 was considered to indicate statistical significance. *,*P*<0.05; **,*P*<0.005; ***,*P*<0.001; ****,*P*<0.0001; n.s.,not significant (*P*>0.05). Unless otherwise indicated, figures show pooled data from all independent experiments performed.

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Conflict of Interest:

The authors declare no commercial or financial conflict of interests.

Data availability statement:

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Figure 1: Priming of *Plasmodium*-specific CD8$^+$ T cells is impaired if sporozoites are heat killed.

C57BL/6 mice were adoptively transferred with 10^6 CTV labelled PbT-I T cells. 1 day later they were immunized iv with 50,000 *P. berghei* ANKA radiation attenuated sporozoites (RAS) or heat-killed sporozoites (HKS). Five days later, spleen (A-B) and liver (C-D) were analyzed for proliferation of PbT-I T cells by flow cytometry. (A) Representative histograms showing percentage proliferation of CTV-labelled PbT-I T cells in the spleen. (B) Numbers (left panel) and percentages (right panel) of proliferating (divided) PbT-I T cells in the spleen. (C) Representative histograms showing percentage proliferation of CTV-labelled PbT-I T cells in the liver. (D) Numbers (left panel) and percentages (right panel) of proliferating PbT-I T cells in the liver. Data (mean ± SD) were pooled from 2 independent experiments with 4 or 5 mice per experiment and compared with a Mann-Whitney U Test. * P<0.05. Naive: Non-immunized.

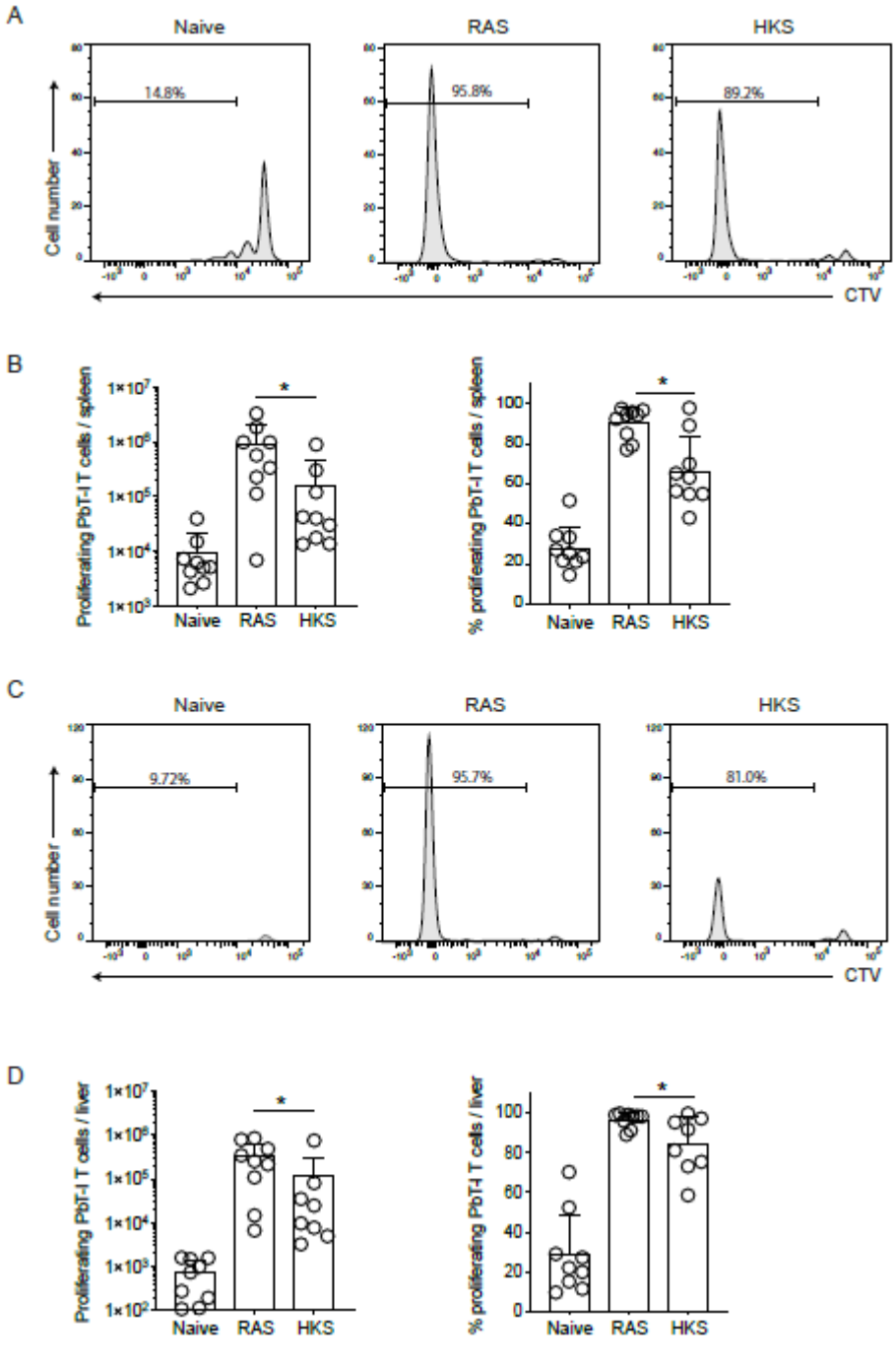


Figure 2: Liver T_{RM} development is drastically decreased after HKS immunization.

C57BL/6 mice were adoptively transferred with 50,000 PbT-I T cells. 1 day later they were immunized iv with 50.000 *P. berghei* ANKA RAS or HKS. 35 days later, memory PbT-I T cells were analyzed in liver (A-D) and spleen (E) by flow cytometry. (A-B) Representative contour plots of PbT-I memory cell subsets found in the liver after RAS (A) or HKS (B) immunization. The numbers displayed indicate the number of cells in each gate. Histograms show the expression of CXCR6 and KLRG-1 in PbT-I T_{EM} (blue) and T_{RM} (green) cells after RAS (A) or HKS (B) vaccination. The numbers indicate the median fluorescence intensity. (C) Number of PbT-I T_{RM} cells present in the liver. (D) Number of PbT-I T_{CM}, T_{EM} and T_{RM} cells present in the liver. (E) Number of PbT-I T_{CM}, T_{EM} and T_{RM} cells present in the spleen. Data (mean ± SD) were pooled from 4 independent experiments with 5 mice per group for each experiment and data in (C) compared with a Mann-Whitney U Test. **** P<0.0001.

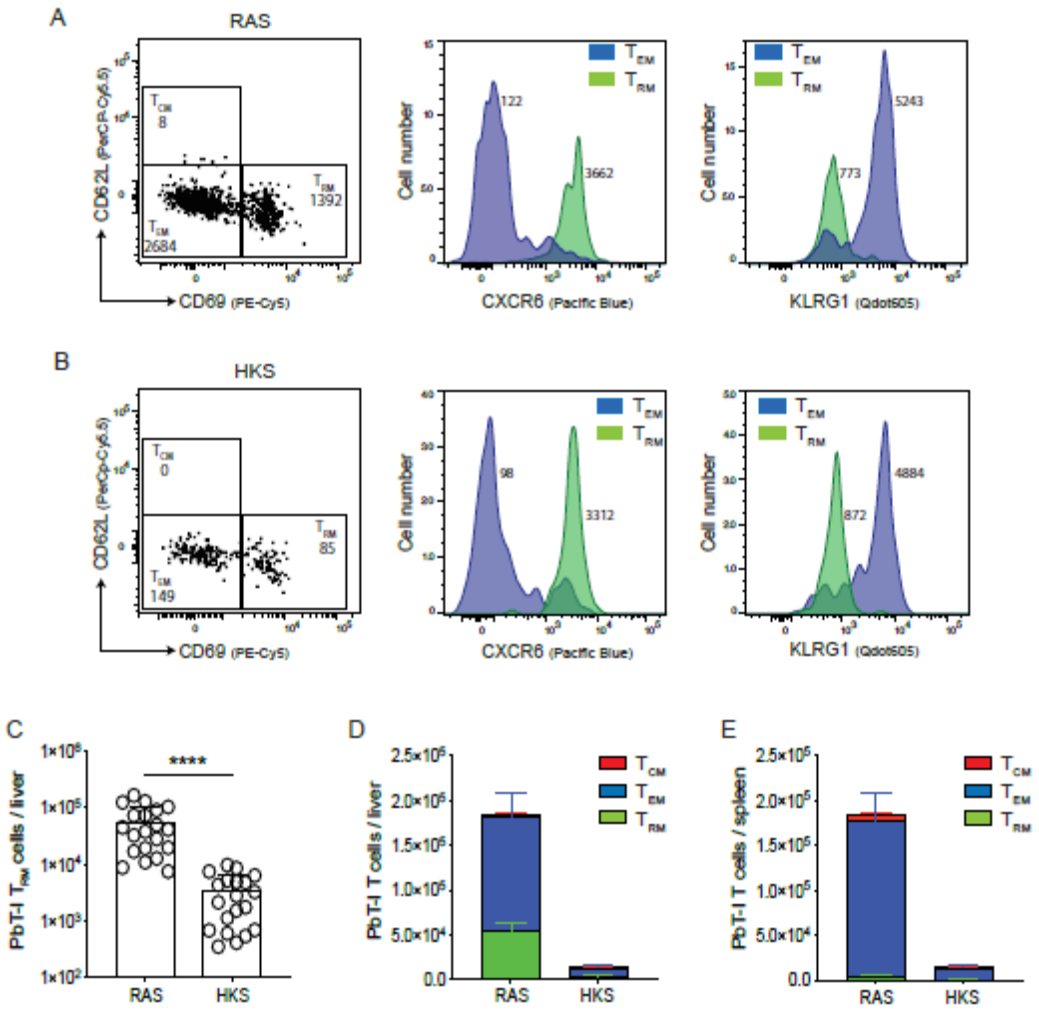


Figure 3: HKS vaccination induces liver T_{RM} formation from the endogenous repertoire.

C57BL/6 mice were immunized iv with 50,000 RAS or HKS. 35 days later, endogenous *P. berghei* ANKA-specific memory $CD8^+$ T cells were analyzed by flow cytometry in liver (A-B and D-E) and spleen (C, F) using two different tetramers. (A) Number of H-$2K^b$-RPL6 $tetramer^+$ T_{RM} cells present in the liver. (B, C) Number of H-$2K^b$-RPL6 $tetramer^+$ T_{CM}, T_{EM} and T_{RM} cells present in the liver (B) or spleen (C). (D) Number of H-$2D^b$-TRAP $tetramer^+$ T_{RM} cells present in the liver. (E, F) Number of H-$2D^b$-TRAP $tetramer^+$ T_{CM}, T_{EM} and T_{RM} cells present in the liver (E) or spleen (F). Data (mean ± SEM) were pooled from 3 independent experiments with 5 mice per group for each experiment and data in (A) and (D) compared with a Mann-Whitney U Test. **** P<0.0001.

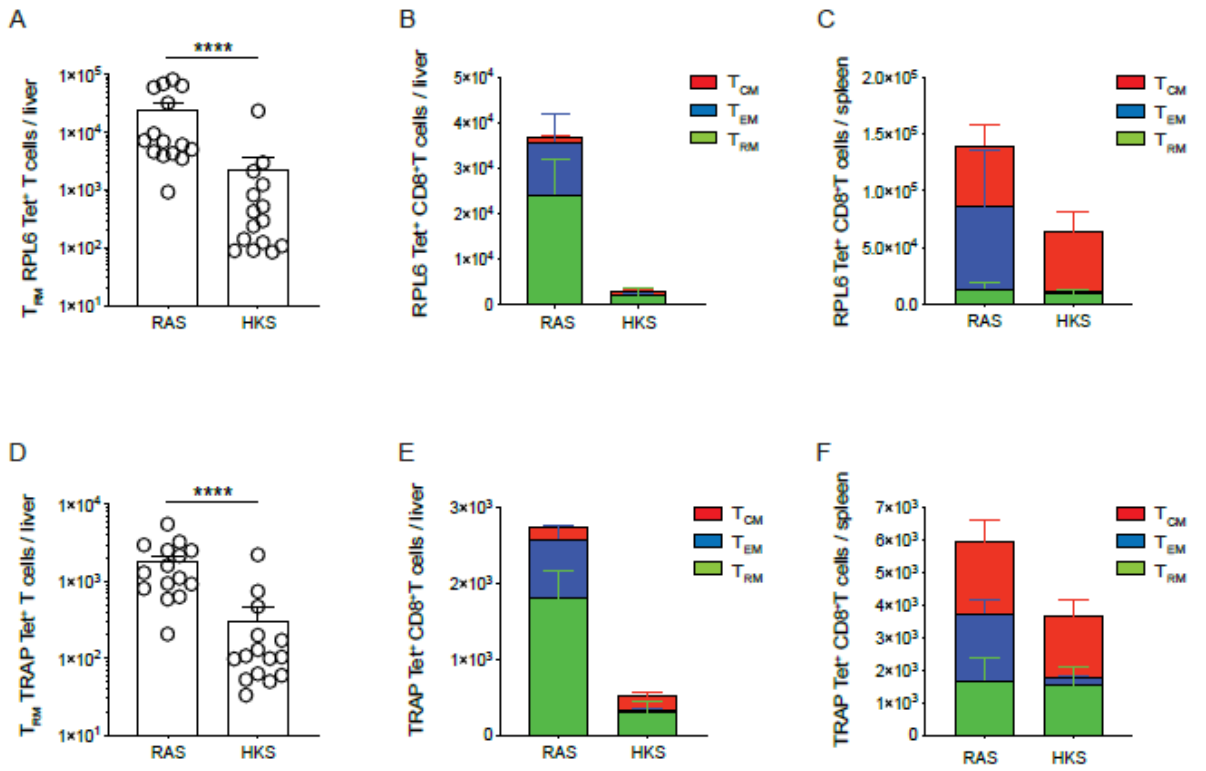


Figure 4: Liver T_{RM} cell formation after HKS immunization is improved by using α-galactosylceramide as an adjuvant.

C57BL/6 mice were adoptively transferred with 50,000 PbT-I T cells 1-day prior to immunization. Mice were then vaccinated with either 50,000 *P. berghei* ANKA RAS or 50,000 *P. berghei* ANKA HKS alone or in combination with adjuvants: CpG Combo, CpG 1668 or α-galactosylceramide (αGalCer). 35 days later, memory PbT-I T cells were analyzed by flow cytometry in liver (A-B) and spleen (C). (A) Absolute number of PbT-I T_{RM} cells present in the liver. (B, C) Absolute number of PbT-I T_{CM}, T_{EM} and T_{RM} cells present in the liver (B) or spleen (C). Data (mean ± SEM) were pooled from 3 independent experiments with 3 to 4 mice per group for each experiment and data in (A) compared with a Kruskal-Wallis test and Dunn's multiple comparisons test. ** *P*<0.005; *** *P*=0.001; **** *P*<0.0001.

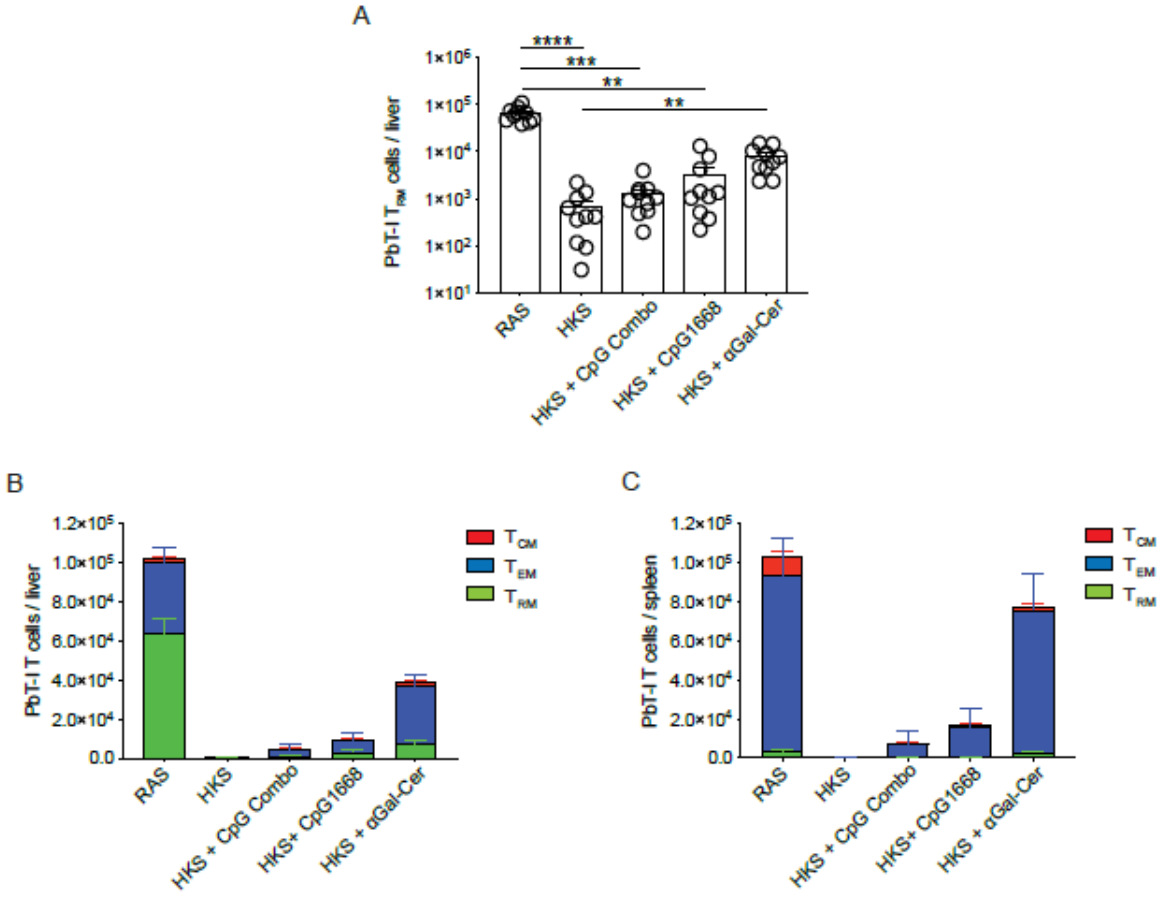


Figure 5: α-galactosylceramide improves protection following RAS immunization but not HKS immunization.

C57BL/6 mice were adoptively transferred with 50,000 PbT-I T cells 1-day prior to immunization. Mice were then vaccinated with either 50,000 *P. berghei* ANKA RAS or 50,000 *P. berghei* ANKA HKS alone or in combination with 0.1nmol or 1nmol of α-galactosylceramide (αGalCer). (A) To measure protection in vaccinated mice, 35 days after immunization, all mice were challenged with 200 live *P. berghei* ANKA sporozoites intravenously. Mice were monitored for parasitemia from day 6 to day 12 by flow cytometry and considered protected if parasitemia was not detected by day 12. On the second consecutive day that parasites were detected, memory PbT-I T cells were analyzed in liver (B-C) and spleen (D) by flow cytometry. (A) Protection against infectious challenge in vaccinated mice. (B) Absolute number of PbT-I T_{RM} cells present in the liver. (C, D) Absolute number of PbT-I T_{CM}, T_{EM} and T_{RM} cells present in the liver (C) or spleen (D). Data (mean ± SEM) were pooled from 2 independent experiments with 3 to 5 mice per experiment. Data in (A) are compared with a Fischer's Exact Test. *P<0.05. Data in (B) compared using a Kruskal-Wallis test and Dunn's multiple comparisons test. * P<0.05; *** P=0.001. Naive: Non-immunized.

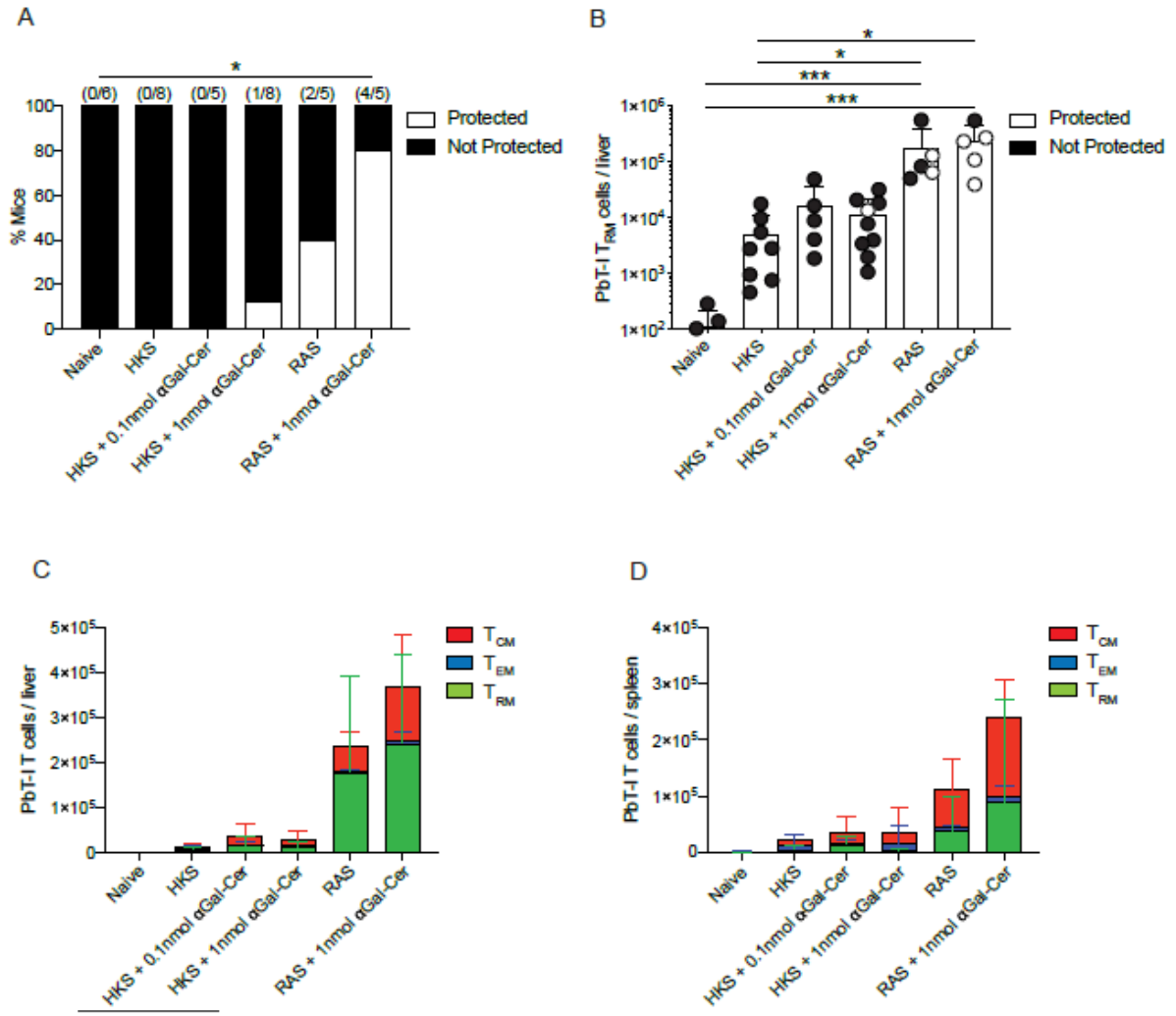
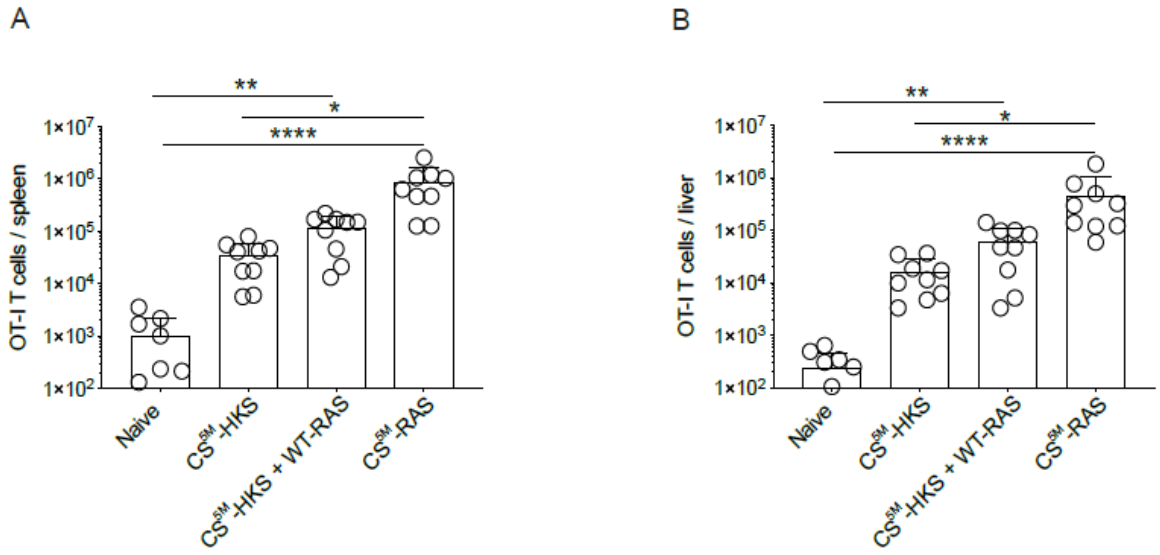


Figure 6: CD8$^+$ T cell responses to HKS are not improved by providing innate signals linked to RAS vaccination.

C57BL/6 mice were adoptively transferred with 10^6 CTV labelled OT-I T cells 1-day prior to immunization with either 50,000 heat-killed *P. berghei* CS^{5M}-mCherry (CS^{5M}-HKS) alone or combined with 50,000 radiation attenuated wild-type *P. berghei* ANKA (WT-RAS). And additional group of mice received 50,000 radiation-attenuated *P. berghei* CS^{5M}-mCherry (CS^{5M}-RAS) or were left uninfected (Naive). Spleen and liver were analyzed 5 days later for proliferation of OT-I T cells by flow cytometry (A, B). Numbers of OT-I T cells in the spleen (A) or liver (B) 5 days after immunization. Data (mean ± SD) were pooled from 2 independent experiments with 4 to 5 mice per experiment and compared using a Kruskal-Wallis test and Dunn's multiple comparisons test. * P<0.05; ** P<0.005; **** P<0.0001. Naive: Non-immunized.



Graphical abstract_text

Vaccination with radiation-attenuated *Plasmodium* sporozoites is known to induce liver resident memory $CD8^+$ T cells (T_{RM}) that confer sterile protection. We show that immunization with heat-killed *Plasmodium* sporozoites also trigger liver T_{RM} formation, but their numbers are suboptimal for sterile protection, even when enhanced by the adjuvant α-galactosylceramide.