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Cell Host Microbe. Author manuscript; available in PMC 2012 June 16.

Published in final edited form as:
Cell Host Microbe. 2011 June 16; 9(6): 451–462. doi:10.1016/j.chom.2011.05.008.

Superior anti-malarial immunity after vaccination with late-liver-stage arresting genetically attenuated parasites

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Summary

While subunit vaccines have shown partial efficacy in clinical trials, radiation-attenuated-sporozoites (RAS) remain the "gold-standard" for sterilizing protection against *Plasmodium*-infection in human vaccinees. The variability in immunogenicity and replication introduced by the extensive, random DNA damage necessary to generate RAS could be overcome by genetically attenuated parasites (GAP) designed via gene deletion to arrest at defined points during liver-stage development. Here, we demonstrate the principle that late-liver-stage arresting GAP induce larger and broader CD8 T cell responses that provide superior protection in inbred and outbred mice compared to RAS or early-arresting GAP immunizations. Late-liver-stage arresting GAP also engender high-levels of cross-stage and cross-species protection and complete protection when administered by translationally-relevant intradermal or subcutaneous routes. Collectively, our results underscore the potential utility of late-liver stage arresting GAP as broadly protective next-generation live-attenuated malaria vaccines and support their potential as a powerful model for identifying antigens to generate cross-stage protection.

Introduction

Malaria is a devastating disease caused by *Plasmodium* infection that impacts nearly 40% of the world's population. 300 million new cases and more than 750,000 malaria-related deaths are reported annually (W.H.O. World Malaria Report, 2010). The enormity of this public health crisis underscores the need for novel interventions, such as efficacious vaccines, to combat human infection and break the transmission cycle of malaria.

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Plasmodium infection occurs when sporozoites are transmitted by infected *Anopheles* mosquitoes. Following deposition in the dermis, sporozoites transit to the liver where they replicate and differentiate in hepatocytes over a period of 2–3 days in rodents or 6–10 days in humans when exoerythrocytic merozoites are released, infect red blood cells, and initiate the erythrocytic replication cycle that drives the clinical manifestations of malaria (Kappe et al., 2010; Menard et al., 2008). Halting *Plasmodium* infection during the clinically silent liver-stage represents an attractive goal of anti-malarial vaccination (Hill et al., 2010; Kappe et al., 2010). However, and despite decades of effort, currently there are no licensed anti-malarial vaccines, and the most promising pre-erythrocytic sub-unit vaccine candidate (RTS,S) has proven only partially effective in clinical trials (Aide et al., 2010; Cohen et al., 2010; Greenwood, 2011).

On the other hand, it has been known for many years that vaccination with radiation attenuated sporozoites (RAS) induces sterilizing liver-stage specific immunity in rodents (Nussenzweig et al., 1967), humans (Clyde, 1975; Hoffman et al., 2002), and non-human primates (reviewed in (Hafalla et al., 2006)). RAS parasites, attenuated via random DNA damage, undergo intrahepatocytic developmental arrest at the onset of replication. In rodents and non-human primates, RAS-induced protection is largely mediated by CD8 T cells, with activity directed against *Plasmodium*-infected hepatocytes (Hafalla et al., 2006; Krzych and Schwenk, 2005; Overstreet et al., 2008; Tsuji, 2010). Despite the efficacy of RAS vaccination, this approach has yet to be translated into a licensed vaccine. Logistical hurdles aside, one major concern relevant to translating RAS vaccination to the clinic involves the necessity to attenuate *Plasmodium* sporozoites with a dose of radiation to induce DNA damage that is both sufficient to prevent progression to blood-stage infection and maintain sporozoite infectivity and immunogenicity (Chattopadhyay et al., 2009). Despite these issues, RAS have recently progressed to clinical evaluation (Crompton et al., 2010).

By comparison, genetically attenuated parasites (GAP), generated by targeted gene deletion(s), are an attractive alternative because parasites can be “designed” to arrest at specific points of liver-stage development (Kappe et al., 2010). GAP have been generated by deleting genes such as *SAP1* (encoding Sporozoite Asparagine rich Protein) which lead to arrest prior to extensive parasite replication in hepatocytes (Aly et al., 2008). Importantly, early-arresting GAP can induce CD8 T cell-dependent sterilizing immunity in vaccinated mice (Aly et al., 2008; Labaied et al., 2007; Mueller et al., 2005a; Mueller et al., 2005b; Tarun et al., 2007; van Dijk et al., 2005). More recently, deletion of the *FabB/F* gene, encoding an essential enzyme of the apicoplast-localized type II fatty acid biosynthesis (FAS II) pathway, was shown to arrest *P. yoelii* (*Py*) at a late-liver-(schizont)-stage (Vaughan et al., 2009). However, it is not known whether late-arresting *fabb/f*$^{-}$ parasites, similar to early-arresting *sap1*$^{-}$ sporozoites, induce sterilizing protection against sporozoite challenge in rodent models.

Although GAP were first described in 2005 (Mueller et al., 2005b), no studies have directly determined if RAS, early- or late-arresting GAP induce larger CD8 T cell responses or enhanced protective immunity. Of note, *Plasmodium* transcriptome and proteome analyses predict an increase in shared antigens between late-liver-stage parasites and blood-stage parasites (Tarun et al., 2008). This suggests that immune responses directed at the late-liver-stage expressed, shared antigens might simultaneously induce protective immunity against both the liver- and blood-stages of *Plasmodium* infection. Consistent with this hypothesis, recent studies showed that immunization of mice with blood-stage parasites under chloroquine cover elicited T cell responses capable of reducing liver stage parasite burden after sporozoite challenge (Belnoue et al., 2008). Indeed, cross-stage protection by immunization with a single vaccine would be an optimal outcome to prevent malaria. Importantly, early liver stage-arresting GAP have also recently moved into clinical

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evaluation as vaccine candidates (VanBuskirk et al., 2009; Crompton et al., 2010). Here, we directly test the hypothesis that late arresting GAP elicit enhanced CD8 T cell responses and better liver-stage immunity than early arresting GAP or RAS. This question is of critical importance, as the potential complexity of GAP vaccine candidates will require priority ranking in order to streamline their potential selection for clinical evaluation.

Results

Vaccination with a late-liver stage arresting GAP affords enhanced protection in outbred mice

We initially tested our hypothesis with outbred mice because they represent a stringent vaccine model that mimics the immunogenetic complexity of humans, and are difficult to protect from *P. yoelii* (*Py*) sporozoite challenge, even following multiple RAS-immunizations (Schmidt et al., 2010). Groups of 20 outbred Swiss Webster mice were vaccinated with RAS, *sap1*⁻ (early-liver-stage arresting GAP) (Aly et al., 2008), or *fabb/f*⁻ (late-liver-stage arresting GAP) (Vaughan et al., 2009) *Py* sporozoites. Greater than sixty days post-vaccination, or -homologous boost, when numerically and phenotypically stable memory CD8 T cell populations are established (Kaech et al., 2002; Schmidt et al., 2010), immunized and naïve mice were challenged with virulent *Py* sporozoites. We observed little protection in RAS (5%) and *sap1*⁻ (20%) singly vaccinated mice (Table 1), where protection is defined as the absence of blood stage parasitemia following challenge with a stringent dose of 1000 virulent sporozoites. In contrast, a single *fabb/f*⁻ vaccination provided significantly better protection (40%) compared to RAS-vaccination (Table 1, P=0.019). Although protection against sporozoite challenge increased in all three groups following homologous boost, *fabb/f*⁻ prime-boosted outbred mice were again significantly better protected (90%) compared to RAS (45%) or *sap1*⁻ (42%) immunized mice (Table 1, P=0.006 and P=0.002, respectively). Thus, a late-liver-stage arresting GAP induces superior protective immunity against sporozoite challenge in outbred hosts compared to both early-liver-stage arresting GAP and RAS sporozoite vaccines.

We next examined the immunologic basis for enhanced protection. Both RAS- and GAP-mediated protection is largely CD8 T cell-dependent (Doolan and Hoffman, 2000; Jobe et al., 2007). Consistent with this, we found that CD8 T cells play a clear role in protecting *fabb/f*⁻ vaccinated Swiss Webster mice against sporozoite challenge (Figure S1A). Therefore, we longitudinally analyzed the magnitude of the RAS, *sap1*⁻, and *fabb/f*⁻ vaccine-induced CD8 T cell response using our recently described surrogate marker of activation approach based on changes in cell surface expression of CD8α and CD11a (Rai et al., 2009; Schmidt et al., 2010) in responding pathogen-specific T cells (naïve cells are $CD8\alpha^{hi}CD11a^{lo}$, effector and memory cells are $CD8\alpha^{lo}CD11a^{hi}$). *fabb/f*⁻ sporozoites induced a substantially larger total CD8 T cell response after both initial immunization and homologous boosting. In particular, the total CD8 T cell response remained significantly elevated for more than 30 days after boosting in *fabb/f*⁻ vaccinated mice compared to RAS immunized mice and still trended higher at 60 days after boost (Figure 1A and 1B). Of note, outbred mice immunized with early arresting RAS parasites exhibited at least twice the variability in the peak of both the primary and secondary effector CD8 T cell responses compared to late-arresting *fabb/f*⁻ vaccinated mice (Figure 1C). These data suggest the notion that additional antigens expressed by late-arresting *fabb/f*⁻ parasites recruit a broader repertoire of CD8 T cells in outbred individuals, resulting in uniformly high CD8 T cell responses. Lastly, previous work has shown correlations between *Plasmodium*-specific CD8 T cells exhibiting an effector-memory phenotype ($CD27^{lo}$, $CD43^{glyco+}$, $CD62L^{lo}$, and $CD127^{lo}$) and protection against sporozoite challenge (Berenzon et al., 2003; Schmidt et al., 2010). However, RAS, *sap1*⁻ and *fabb/f*⁻ induced memory CD8 T cells displayed similar effector-memory phenotypes in Swiss Webster mice (Figure S1B–D). Collectively, these

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data show that enhanced protection of outbred Swiss Webster mice following *fabb/f*⁻ vaccination is associated with the induction of a larger and less variable peak total CD8 T cell response.

Vaccination with a late-liver-stage arresting GAP affords enhanced protection against sporozoite challenge of highly susceptible C57BL/6 mice

RAS-immunized inbred C57BL/6 mice are substantially more difficult to protect against *P. yoelii* sporozoite challenge compared to RAS-immunized outbred Swiss Webster and inbred BALB/c mice (Doolan and Hoffman, 2000; Schmidt et al., 2010). To extend our studies to this stringent model, we next compared protection by all three sporozoite vaccines in C57BL/6 mice. Greater than sixty days following single or prime-boost immunization with 20,000 RAS, *sap1*⁻ or *fabb/f*⁻ sporozoites, groups of C57BL/6 mice were challenged with 1000 virulent *Py* sporozoites. We observed little protection in RAS (10%) and *fabb/f*⁻ (10%) singly vaccinated C57BL/6 mice (Figure 2A). Strikingly, however, *fabb/f*⁻ prime-boosted C57BL/6 mice were significantly better protected (100%) compared to RAS (15%) or *sap1*⁻ (0%) prime-boosted mice (Figure 2A, $P<0.0001$ and $P<0.0001$, respectively). Of note, the enhanced protection induced by *fabb/f*⁻ sporozoite vaccination of C57BL/6 mice was wholly CD8 T cell-dependent (Figure S2A). Moreover, we observed significantly larger 1° and 2° effector (d6 and d117, respectively) and 1° and 2° memory (d111 and d169, respectively) CD8 T cell responses in *fabb/f*⁻ vaccinated C57BL/6 mice compared to RAS and *sap1*⁻ vaccinated mice (Figure 2B). In addition to these quantitative differences, parasite-specific memory CD8 T cells in *fabb/f*⁻ vaccinated C57BL/6 mice also displayed qualitative differences, with a larger fraction of cells exhibiting an effector memory-like phenotype ($CD27^{lo}$, $CD62L^{lo}$, $CD127^{lo}$) compared to either RAS or *sap1*⁻ induced cells (Figure 2C and 2D). In contrast, there were only minor changes in the phenotype of 1° memory CD8 T cells compared to 2° memory CD8 T cells (Figure 2C versus 2D) within each immunization group. Lastly, specific functional characteristics of memory CD8 T cells could directly explain the differences in protection observed following RAS, *sap1*⁻ or *fabb/f*⁻ sporozoite vaccination. However, *ex vivo* stimulation of $CD8\alpha^{lo}CD11a^{hi}$ memory T cells from RAS, *sap1*⁻ and *fabb/f*⁻ vaccinated mice revealed no differences in IFN-γ, TNF-α or IL-2 expression (or expression of a combination of these cytokines) following either anti-CD3ε cross-linking (Figure S2B and S2C) or PMA + ionomycin stimulation (data not shown). Additionally, we observed no differences in CD8 T cell degranulation, a marker of cytolytic potential, among RAS, *sap1*⁻ and *fabb/f*⁻ induced $CD8\alpha^{lo}CD11a^{hi}$ T cells (Figure S2D and S2E). Collectively, these data suggest that increased protection in *fabb/f*⁻ prime-boost vaccinated C57BL/6 mice is likely due to the numerically larger secondary memory CD8 T cell responses and not due to specific phenotypic or functional differences among memory CD8 T cells induced by each sporozoite vaccine.

Vaccination with a late arresting GAP engenders durable cross-species protection and sterilizing protection when administered via clinically approved routes of immunization

Inbred BALB/c mice have served as the major model for evaluating sporozoite dose, duration of protection, routes of immunization and cross-species protection in RAS- or GAP-induced immunity to *Plasmodium*. Vaccination of BALB/c mice with low numbers (1000) of *Py* GAP sporozoites revealed that prime-boost-boost (3 total) immunizations with either *sap1*⁻ or *fabb/f*⁻ sporozoites resulted in complete (100%) protection. However, single boost (2 total) immunizations with *fabb/f*⁻ elicited significantly enhanced protection compared to the same vaccination with *sap1*⁻ sporozoites (Table 2, top, $P=0.0007$). Of note, *fabb/f*⁻ vaccinated BALB/c mice exhibited long lasting (300 days), CD8 T cell-dependent sterilizing immunity to high-dose challenge (10,000 spz) following immunization regimens that varied by dose, number of boosts or sporozoite challenge intervals (Table 2, middle). Additionally, we also determined that RAS, *sap1*⁻ and *fabb/f*⁻ vaccination elicited

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equivalent anti-sporozoite antibody titers in immunized mice (data not shown). Strikingly, *fabb/f*$^{-}$ vaccination could also engender complete (100%), sterilizing immunity against virulent *Py* sporozoite challenge of BALB/c mice when administered via two clinically approved routes of administration (intradermally or subcutaneously, Table 2, middle), which is not observed following *P. berghei* (*Pb*-RAS) vaccination of C57BL/6 mice (50,000/20,000/20,000 at weekly intervals; 10,000 spz challenge (Douradinha et al., 2007; Kramer and Vanderberg, 1975)). Finally, in contrast to the handful of studies revealing only partial cross-species protection following multiple, high-dose *Pb*- or *Py*- RAS immunizations of inbred mice (Douradinha et al., 2007; Purcell et al., 2008; Sedegah et al., 2007), we observed that BALB/c mice immunized with *Py fabb/f*$^{-}$ sporozoites exhibited 100% sterilizing, cross-species protection following challenge with 10,000 *Pb* sporozoites (Table 2, bottom). Thus, *fabb/f*$^{-}$ vaccination elicited enhanced, complete and durable protection against both homologous and cross-species sporozoite challenge, and also elicited 100% protection when administered via two clinically approved routes of vaccination.

Enhanced protection by a late-liver-stage arresting GAP is linked to diversification of the antigenic targets of the protective CD8 T cell response

The BALB/c model provides an important and unique opportunity to directly examine the mechanism underlying enhanced protective immunity afforded by the late-liver-stage arresting GAP. BALB/c mice mount CD8 T cell responses against a defined *Py* circumsporozoite (CS) protein epitope ($CS_{280–288}$) (Weiss et al., 1992), a sporozoite and early-liver-stage antigen that is highly expressed and can serve as a target for protective CD8 T cells (Butler et al., 2010; Weiss et al., 1992). Indeed, prolonged CS antigen display, detected by adoptive transfer of $CS_{280–288}$-specific CD8 T cell receptor transgenic (CS280 Tg) cells, has been reported after RAS immunization (Cockburn et al., 2010). Thus, one potential explanation for larger, more protective CD8 T cell responses following *fabb/f*$^{-}$ vaccination is prolonged presentation of CS antigen at late time points. To examine this possibility, we transferred naïve, CFSE-labeled CS280 Tg cells to BALB/c mice that had been vaccinated 14, 28 or 45 days earlier with RAS, *sap1*$^{-}$, or *fabb/f*$^{-}$ sporozoites. Analyses of CFSE dilution and accumulation of transferred CS280 Tg cells revealed that CS antigen persisted for at least 45 days in all vaccinated mice (Figure S3A–C). However, we observed no significant difference in CS antigen persistence whether mice were vaccinated with RAS, *sap1*$^{-}$ or *fabb/f*$^{-}$ sporozoites (Figure S3A–C). Thus, larger total CD8 T cell responses and enhanced protection induced by late-liver-stage arresting GAP vaccination are not explained by differences in CS antigen persistence.

At least two other possibilities could explain the differences in the magnitude of the total CD8 T cell response between *fabb/f*$^{-}$ and RAS vaccinated mice. First, *fabb/f*$^{-}$ vaccination may simply be a stronger stimulus, which induces higher numbers of CD8 T cells whose antigen-specificity overlaps with CD8 T cells induced by RAS. The second possibility is that new late-liver-stage antigens may be expressed following vaccination with late-arresting *fabb/f*$^{-}$ sporozoites, thus priming the expansion of additional protective CD8 T cells with non-overlapping antigenic specificities compared to RAS. The lack of published CD8 T cell antigens in rodent *Plasmodium* models precludes direct assessment of these two potential explanations. Thus, in order to distinguish between these possibilities we undertook two alternative approaches. First, we transferred naïve CS280 Tg cells to naïve BALB/c mice and vaccinated recipients with RAS or *fabb/f*$^{-}$ sporozoites the next day. Consistent with our results in Swiss Webster and C57BL/6 mice, we observed a significantly larger total ($CD8\alpha^{lo}CD11a^{hi}$) CD8 T cell response in *fabb/f*$^{-}$ vaccinated BALB/c mice compared to RAS vaccinated mice (Figure 3A and 3B, $P<0.0093$). Importantly, the CS-specific response, measured by enumeration of responding CS280 Tg cells, was equivalent in *fabb/f*$^{-}$ and RAS vaccinated mice (Figure 3C). Thus, *fabb/f*$^{-}$ vaccination does not simply drive a larger

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response against shared antigens but rather induces a larger CD8 T cell response that is partially non-overlapping (i.e. targeting distinct antigens) compared to the CD8 T cell response induced by RAS-vaccination. In support of this, we also observed consistent alterations in the repertoire of TCR Vβ chains utilized by CD8 T cells primed by RAS versus *fabb/f*⁻ sporozoites (Figure S3D), suggesting that these two sporozoite vaccines recruit populations of CD8 T cell exhibiting partially non-overlapping TCR specificities.

Secondly, to more formally demonstrate that *fabb/f*⁻ and RAS vaccine-induced CD8 T cells recognize antigens that are partially non-overlapping, we examined the ability of memory CD8 T cells from RAS- or *fabb/f*⁻ vaccinated mice to respond to homologous or heterologous immunization. Sort-purified, CFSE-labeled RAS- or *fabb/f*⁻ specific memory CD8 T cells were transferred into allelically disparate naïve mice, which were subsequently immunized with the same number of RAS or *fabb/f*⁻ sporozoites (Figure 3D, top). To control for initial antigen input during sporozoite immunization (RAS or *fabb/f*⁻ sporozoite dose), we transferred allelically disparate naïve CS280 Tg cells into separate naïve mice given the same immunizations (Figure 3D, bottom). All mice that received either RAS- or *fabb/f*⁻ induced memory cells, or naïve CS280 Tg cells, were subsequently immunized with RAS or *fabb/f*⁻ sporozoites. Seven days after this subsequent sporozoite immunization, the fraction of CS280 Tg cells that remained undivided was equal after RAS or *fabb/f*⁻ immunization, demonstrating that sporozoite doses and early antigen-display were equivalent (Figure 3E and 3F, right panels or bars, respectively). Furthermore, equivalent division of RAS- specific memory cells occurred after either RAS- (homologous) or *fabb/f*⁻ (heterologous) immunization (Figure 3E, left panels or bars, respectively), demonstrating that RAS memory cells are specific for an overlapping pool of antigens expressed by both RAS and *fabb/f*⁻ sporozoites. In contrast, we observed significantly more undivided *fabb/f*⁻ specific memory CD8 T cells in RAS immunized mice compared to *fabb/f*⁻ immunized mice (Figure 3E and 3F, middle panels or bars, respectively, P=0.007). Thus, more *fabb/f*⁻ specific memory cells remained unresponsive (undivided) following heterologous RAS boost, compared to homologous *fabb/f*⁻ boost. Collectively, these data demonstrate that *fabb/f*⁻ sporozoite-vaccine-induced memory CD8 T cell responses are comprised of a pool of cells whose antigenic specificity is only partially overlapping with RAS-specific memory CD8 T cells.

Vaccination with late arresting GAP sporozoites affords protection against blood stage challenge

Previously it has been shown that late-liver-stage parasites share similar transcriptomes with blood-stage parasites (Tarun et al., 2008), suggesting that many individual parasite proteins could be expressed during both life cycle stages and thus serve as antigenic targets of cross-stage (liver and blood) protective immune responses. If true, vaccination with late-liver-stage arresting *fabb/f*⁻ sporozoites could provide protection against low-dose blood-stage parasite challenge, which is not observed after *Pb*-RAS (75,000 RAS ×1, 1000 infected red blood cell parasite challenge (Nussenzweig et al., 1967)) or early-liver-stage arresting *Pb* GAP vaccinations (10,000 GAP × 3, 10,000 infected red blood cell parasite challenge (Mueller et al., 2005a)). To directly test this, groups of BALB/c mice were vaccinated with *fabb/f*⁻ sporozoites and challenged intravenously with 100 *Py* parasite-infected red blood cells (pRBC) one month later. To assess resistance to blood-stage parasite challenge, peripheral blood was assayed daily for percent parasitemia. Strikingly, *fabb/f*⁻ vaccinated mice were able to control and clear both a non-lethal (*Py* 17XNL, Figure 4A) and lethal (*Py* YM, Figure 4B) pRBC challenge. Of note, sera from BALB/c mice multiply vaccinated with *fabb/f*⁻ sporozoites did not exhibit reactivity against purified recombinant $MSP\text{-}1_{19}$ (Figure S4) or parasitized red blood cells (data not shown). Thus, vaccination of mice with *fabb/f*⁻ late-liver-stage arresting sporozoites can also engender cross-stage protection against

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erythrocytic parasite challenge, likely via the induction of more potent and broadly reactive T cell responses.

Discussion

Here we provide critical insight into the induction of anti-malarial immunity following vaccination with sporozoites that represent distinct attenuation profiles. We show that relative to vaccination with early-arresting RAS and early-arresting *sap1*⁻ GAP sporozoites, vaccination with late-liver-stage arresting *fabb/f*⁻ GAP sporozoites elicits higher magnitude effector and memory CD8 T cell responses and affords superior protection against virulent sporozoite challenge in inbred and outbred mice. Our results also show that late-liver-stage arresting GAP elicit cross-stage immunity against blood stage challenge. Mechanistically, our data link both enhanced liver-stage protection and the induction of larger CD8 T cell responses following late-liver-stage arresting GAP vaccination to diversification of the antigenic targets of responding CD8 T cells.

Unlike *P. yoelii sap1*⁻ GAP and RAS parasites, which arrest very early after infection, *fabb/f*⁻ GAPs undergo extensive intrahepatocytic schizogony with substantial cell mass increase within the vaccinated host, yet fail to differentiate and release infectious exoerythrocytic merozoites (Vaughan et al., 2009) (Model, Figure 5A). Thus, enhanced protection by *fabb/f*⁻ GAP vaccination is associated with the expression of additional parasite gene products that may serve as targets of adaptive immune response (Figure 5B). In support of this, an analysis of early-mid stage (24 hour) versus late (40–50 hour) liver-stage parasites directly isolated from infected mice revealed differential expression of more than 770 transcripts (Tarun et al., 2008). These observations are consistent with our data showing early- (RAS or *sap1*⁻) and late- (*fabb/f*⁻) liver-stage arresting parasites elicit differential protection and CD8 T cell responsiveness. Specifically, our CD8 T cell analyses show that, compared to RAS, *fabb/f*⁻ GAP vaccination induces a population of CD8 T cells with partially non-overlapping antigenic specificity (Figure 5C). These data, together with our *fabb/f*⁻ specific memory CD8 T cell adoptive transfer assays, show that the larger CD8 T cell response induced following *fabb/f*⁻ GAP vaccination is directed against *Plasmodium* antigens not expressed by early-arresting RAS parasites. Interestingly, and consistent with our hypothesis, a recent study showed that CS-specific T cells dominate the protective immune response following vaccination of rodents with either early-arresting RAS or early-arresting *UIS3*⁻ or *uis4*⁻ GAP sporozoites (Kumar et al., 2009). Collectively, these data demonstrate clear differences in the antigenic specificity of CD8 T cells induced by early- versus late-liver-stage arresting whole-sporozoite vaccinations (Figure 5C).

In addition, we also show that vaccination with late-arresting *fabb/f*⁻ sporozoites engenders cross-stage protection against blood-stage challenge. This latter point is of particular interest, as the field of pre-erythrocytic antigen discovery has mainly focused on the identification of sporozoite and liver stage-specific antigens (Speake and Duffy, 2009). Although a few antigens exhibiting expression in both liver- and blood-stage have been investigated for cross-stage protection (Draper et al., 2009; Gruner et al., 2001a; Gruner et al., 2001b; Preiser et al., 2004; Renia et al., 1997; Robson et al., 1988; Silvie et al., 2004; Szarfman et al., 1988), recent proteomic profiling revealed at least 375 *Plasmodium* proteins, which are expressed during both late-liver-stage and blood-stage (Vaughan and Kappe, unpublished observations). Thus, hundreds of additional candidate targets remain to be interrogated to identify the most potent antigens capable of inducing cross-stage anti-malarial immunity. In this regard, our data highlight the clear potential of late-liver-stage-arresting GAP immunizations, in concert with surrogate activation marker-based identification of *Plasmodium*-specific T cells, as a tool for identifying antigenic targets, and suggest that *Plasmodium* genes coordinately expressed during both liver-stage and blood-

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stage may serve as important immunologic targets for anti-malarial vaccination. Lastly, it is important to note that our current study evaluated more than 200 mice immunized with 20,000–50,000 *Py fabb/f*⁻ or *sap1*⁻ GAP and we never observed breakthrough blood stage infection. Thus, exposure of the host to infectious merosomes/merozoites cannot explain the striking cross-stage protection we observe following vaccination with late-liver-stage arresting *fabb/f*⁻ sporozoites.

Recently, both *P. falciparum* RAS and early liver stage-arresting GAP have moved to evaluation in clinical trials (Crompton et al., 2010; Good and Doolan, 2010; VanBuskirk et al., 2009). Although controversy abounds over the utility of rodent models of anti-*Plasmodial* vaccination, it has been known for 35 years that RAS afford sterilizing protection in humans (Clyde, 1975), a result first shown using rodent models (Nussenzweig et al., 1967). Thus, the rodent model predicted the only anti-malarial vaccine ever shown to confer complete, sterile protection in humans. Herein we describe approaches and methods that further illustrate the utility of rodent models of whole-parasite pre-erythrocytic vaccination and should facilitate the rapid identification of the best candidate attenuated sporozoite vaccines using the most stringent experimental systems (*P. yoelii* and highly susceptible C57BL/6 or outbred mice). For example, the surrogate activation marker approach enables tracking of vaccine-induced CD8 T cell responses in the absence of defined parasite antigens or host MHC alleles. This permits direct GAP versus RAS (or GAP versus GAP) comparisons using any parasite species or host strains, including outbred mice, which more closely mimic the immunogenetic complexity of humans. In addition to outbred animals, we show that vaccination with a late-arresting GAP also affords protection to even the most difficult to protect rodents of the C57BL/6 background. In our hands, RAS-immunized C57BL/6 mice cannot readily be protected against *Py* sporozoite challenge at a memory time point (>60 days post-immunization), even following vaccination with three doses of 20,000 RAS (Schmidt et al., 2010). Thus, our data show that in the most stringent of rodent models, the late-arresting GAP vaccination is vastly superior to both RAS and an early-arresting GAP vaccination.

Secondly, although immunity against liver-stage *Plasmodium* infection is mediated primarily by CD8 T cells, the observation that the late-liver-stage arresting *fabb/f*⁻ GAP also engenders protection against blood-stage infection raises the intriguing possibility that substantial CD4 T cell and antibody responses may be elicited following GAP vaccination. Indeed, in models of subunit vaccination with well-defined blood-stage antigens, or convalescent mice, it is widely appreciated that antibodies and CD4 T cells are the primary mechanisms that protect against the erythrocytic parasite cycle (Good, 2001; Taylor-Robinson, 2010). However, our preliminary studies revealed that neither MSP-1_{19}-specific antibody responses nor serum reactivity against *P. yoelii*-infected red blood cells correlated with cross-stage protection. Interestingly, recent data suggest a potentially novel protective role for CD8 T cells with activity against *P. yoelii* blood-stage parasites (Imai et al., 2010). Thus, future studies can now focus on understanding the cellular basis of sporozoite-induced cross-stage protection.

Finally, our data show that late-arresting GAP are capable of eliciting protection against sporozoite challenge when administered via two clinically approved routes of vaccination (i.e. intradermally or subcutaneously). Importantly, previous work has established that RAS vaccination only elicits complete (100%) sterilizing immunity when administered intravenously (Douradinha et al., 2007; Kramer and Vanderberg, 1975), although partial protection was recently achieved in mice given *Pb* RAS in the presence of a strong TLR7 agonist (Imiquimod) when challenge occurred 15 days after the last immunization (Voza et al., 2010). Whether the partial (or failed) efficacy in the prior studies was due to lack of CD8 T cell induction following inoculation of RAS into the skin, muscle or dermis remains a

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critically important question. By comparison, our results showing that a non-adjuvanted, late-arresting GAP vaccine protects 100% of recipients at memory time points when administered via two translatable routes of immunization suggests that RAS and GAP may fundamentally differ with respect to their potential utility as widely deployable vaccines. It is possible that such biological differences determine whether RAS versus GAP are able to successfully migrate (or move via antigen presenting cell trafficking) from distal sites of injection to areas of T cell priming, such as the spleen and lymph nodes (Chakravarty et al., 2007). Thus, in the future it will be important to examine parasite distribution, CD8 T cell priming and the magnitude of the CD8 T cell response following intradermal, intramuscular or subcutaneous injection of RAS or GAP sporozoites.

In summary, our data strongly suggest that the enhanced protection observed after immunization with late liver stage-arresting GAP results from recognition of a more diverse set of antigens by CD8 T cells, and potentially additional arms of the immune system reacting with multi-stage expressed antigens. Although more work is required to determine if targeting fatty acid biosynthesis pathways (Pei et al., 2010; Vaughan et al., 2009), or other late liver-stage-expressed genes, will provide the most effective GAP for *P. falciparum* vaccines, our results clearly show the principal that late-arresting GAP are superior in inducing protective immunity. Moreover, as additional candidate genes are identified, the murine model and surrogate activation marker approaches can be used to rapidly interrogate and prioritize the new classes of GAP candidates for subsequent evaluation in human clinical trials. Collectively, our data indicate that, in concert with improved identification of vaccine-induced T cells, late-liver-stage-arresting GAP constitute a powerful model for identifying late-liver-stage antigens that might provide cross-stage protection and underscore the potential utility of late arresting GAP as broadly protective second-generation live-attenuated malaria vaccine candidates.

Experimental Procedures

Mice and immunizations

Mice were purchased from the National Cancer Institute (Frederick, MD) and housed at the University of Iowa animal care unit under the appropriate biosafety level. BALB/c mice used in experiments summarized in Table 2 were purchased from the Jackson Laboratory (Bar Harbor, ME) and housed at the Seattle Biomedical Research Institute animal care unit under the appropriate biosafety level. The Institution Animal Care and Use Committee at both locations approved animal experiments. Mice were vaccinated as indicated in the figure legends and tables.

Parasites and sporozoite challenges

A. stephensi mosquitoes infected with WT *Py* (17XNL) were obtained from New York University. *A. stephensi* infected with *Py sap1*$^{-}$ (Aly et al., 2008), *Py fabb/f*$^{-}$ (Vaughan et al., 2009) or *P. berghei* (*Pb*) were generated at Seattle Biomedical Research Institute. WT *P. berghei* and *Py* or gene knockout *Py* (*sap1*$^{-}$ and *fabb/f*$^{-}$) sporozoites were isolated from the salivary glands of infected *A. stephensi* mosquitoes. WT sporozoites were radiation attenuated by exposure to 200 Gy (20,000 rads). Naïve and immunized mice were challenged with the indicated number of WT *Pb* or *Py* sporozoites i.v. Parasitized red blood cells were identified by Giemsa stain 10 days post challenge. Protection is defined as the absence of blood-stage parasites. At least 10 fields of >200 red blood cells were examined for each mouse to be designated as protected.

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Blood-stage challenge

Naïve and immunized mice were infected i.v. with ~100 *Py* YM (lethal) or *Py* XNL (non-lethal) blood-stage parasites. Parasitemia was checked daily until it was undetectable. Mice were euthanized if parasitemia was >60%.

Identification of parasite-specific CD8 T cells

RAS, *sap1*$^{-}$, and *fabb/f*$^{-}$ vaccine induced CD8 T cell populations were identified by staining peripheral blood with anti-CD8α (53–6.7) (eBioscience, San Diego, CA) and anti-CD11a (M17/4) (eBioscience) antibodies. RAS, *sap1*$^{-}$ and *fabb/f*$^{-}$ specific CD8α^{lo}CD11a^{hi} T cells were characterized using anti-CD43^{glyco} (1B11) (Biolegend), anti-CD127 (SB/199) (Biologend),) anti-CD27 (LG.7F9) (eBioscience) and anti-CD62L (MEL-14) (BD Pharmingen, San Diego, CA) antibodies. Cells were analyzed using a BD FACSCanto. Data was analyzed using FLOWJO Software (Tree Star, Inc, Ashland, OR). Animals were pre-bled prior to vaccination to establish individual background circulating CD8α^{lo}CD11a^{hi} T cell frequencies.

RAS versus *fabb/f*$^{-}$ sporozoite induced CS280 TCR Tg CD8 T cell responses

1000 naïve TCR-Tg (Thy1.1/1.2+) CD8 T cells specific for the CS$_{280–288}$ *Py* epitope (Cockburn et al., 2010) were transferred to BALB/c mice (Thy1.2+). One day later mice were vaccinated with 2×10^{4} RAS or *fabb/f*$^{-}$ sporozoites. Six days later the frequency and total number of splenic RAS or *fabb/f*$^{-}$ specific CD8α^{lo}CD11a^{hi} T cells, and CS280 TCR Tg CD8 T cells, was determined.

Identification of a more diverse CD8 T cell response in *fabb/f*$^{-}$ vaccinated mice

BALB/c (Thy1.2) mice were vaccinated and boosted >60 days later (2×10^{4} *fabb/f*$^{-}$ sporozoites for each immunization). Purified CD8α^{lo}CD11a^{hi} T cells obtained >30 days after boosting were labeled with CFSE (1 μM) and transferred (1×10^{5}) into Thy1.1 BALB/c mice. Naïve CFSE-labeled Thy1.1/Thy1.2+ CS280 TCR Tg CD8 T cells (5×10^{5}) were also transferred into naïve Thy1.1+ BALB/c mice. One day later, recipient mice were vaccinated with 2×10^{4} RAS or *fabb/f*$^{-}$ sporozoites. Seven days after vaccination, splenocytes from recipient/vaccinated mice were labeled with anti-Thy1.2-PE (OX-7) (BD Pharmingen) followed by anti-PE microbeads (Miltenyi Biotec) and purified using an AutoMACS (Miltenyi Biotec). Thy1.2-enriched cell suspensions were then stained with anti-CD8, anti-CD4 and anti-CD19. Thy1.2+ CD8α^{lo}CD11a^{hi} T cells were identified by gating on CD8+/Thy1.2+ cells that were CD4/CD19 negative. CS280 TCR Tg CD8 T cells were identified similarly and both subsets were subsequently analyzed for dilution of CFSE.

Statistical Analysis

Data were analyzed using Prism4 software. Specific tests of statistical significance are detailed in figure legends and table footnotes.

Highlights

Genetically attenuated, late liver-stage arresting *Plasmodium* parasites —

- Afford long-lasting sterile protection with small dose vaccination of mice
- Afford enhanced cross-stage and cross-species anti-malarial protection
- Protect when administered via translatable routes of immunization
- Induce larger CD8 T cell responses targeting a diversified pool of antigens

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Supplementary Material

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Acknowledgments

We thank Stanley Perlman for critical review, Lecia Epping, Lisa Hancox and Jemmie Hoang for technical assistance, Fidel Zavala for TCR transgenic mice, Sandra Gonzalez (NYU insectary) and the radiation core personnel (University of Iowa). This work was supported by T32-AI0726024 (N.S.B.), F32-AI084329 (N.W.S.), RO1 AI085515 (J.T.H) and the Foundation for the National Institutes of Health through the Grand Challenges in Global Health initiative (S.H.I.K.).

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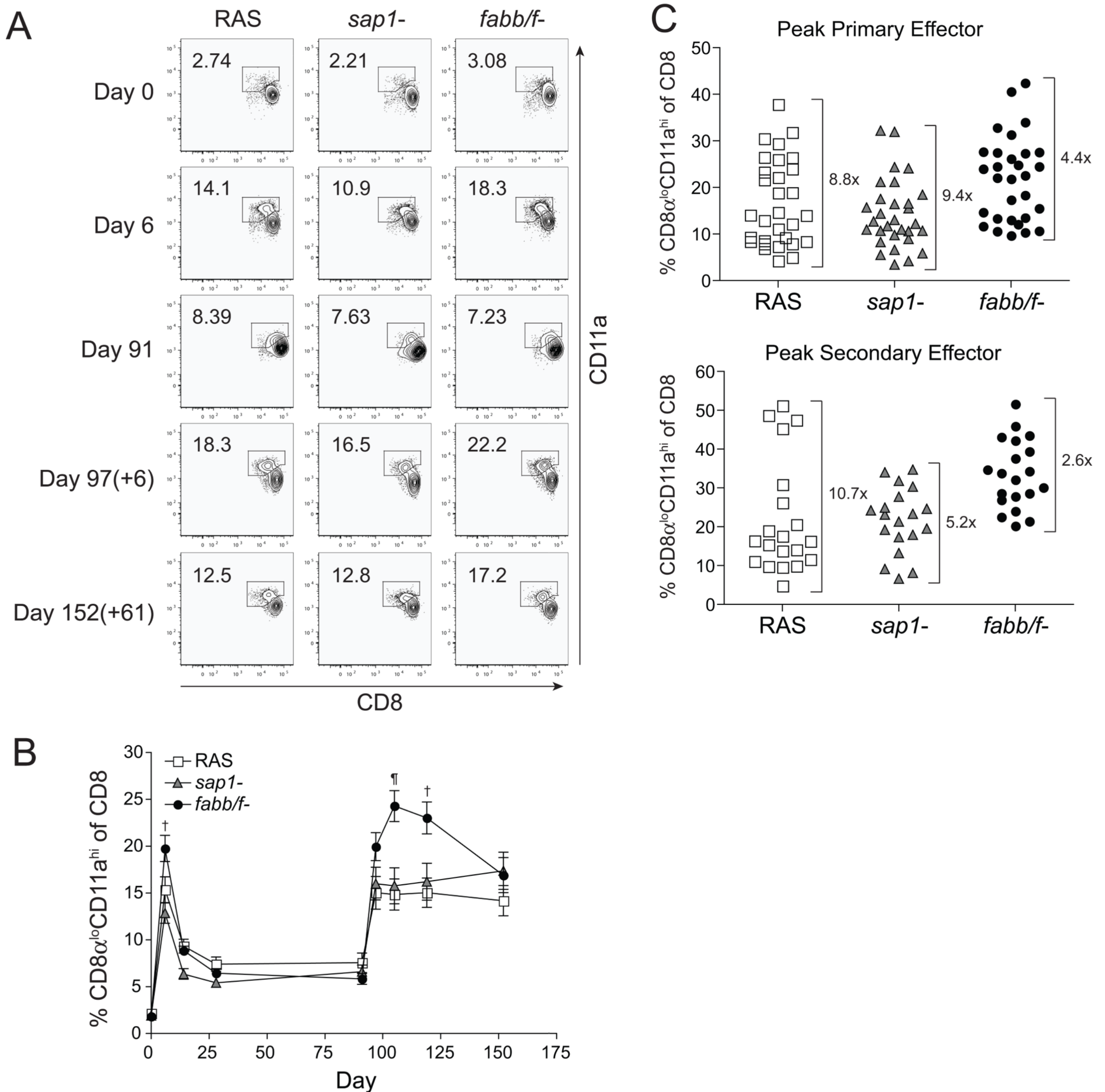

Figure 1. *P. yoelii fabb/f⁻* vaccination of outbred Swiss Webster mice generates a larger and less variable CD8 T cell response than *P. yoelii* RAS or *P. yoelii sap1⁻* vaccination

(**A**) Representative plots showing the percent of circulating CD8 T cells that exhibit the antigen-experienced $CD8\alpha^{lo}CD11a^{hi}$ phenotype before and after immunization with 2×10^4 RAS, *sap1⁻*, or *fabb/f⁻* sporozoites. Mice were given a homologous boost of 2×10^4 sporozoites on day 91. (**B**) Cumulative data showing the percent of circulating CD8 T cells that are $CD8\alpha^{lo}CD11a^{hi}$. Data (mean±S.E.M.) are from 40 to 52 mice/group from two independent experiments. Data were analyzed by One-Way ANOVA († = P<0.01; ¶ = *P*<0.001). (**C**) The fraction of circulating CD8 T cells that exhibit the $CD8\alpha^{lo}CD11a^{hi}$ phenotype from individual mice at the peak of the primary or secondary response. Symbols

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represent each individual mouse examined daily from day 5 to 9 following primary or booster immunization. The absolute peak CD8 T cell response for each individual mouse (which may have occurred on a different day due to genetic variability in outbred mice) was plotted. Numbers to the right indicate the fold difference between the highest and lowest responses within each group. See also Figure S1.

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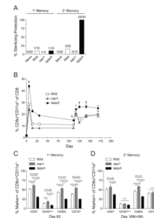

Figure 2. Late-liver-stage arresting *P. yoelii* GAP (*fabb/f*$^-$) vaccinated C57BL/6 mice have a larger CD8 T cell response that exhibits a more effector memory-like phenotype compared to RAS or early-liver-stage arresting GAP (*sap1*$^-$) vaccination

C57BL/6 mice were vaccinated with 2×10^4 RAS, *sap1*$^-$, or *fabb/f*$^-$ sporozoites and given a homologous boost (2×10^4 sporozoites) on day 111. (**A**) Fraction of mice that exhibited complete, sterilizing immunity following single (1° memory) or prime-boost (2° memory) vaccination. Mice were challenged with 1000 virulent *Py* sporozoites and protection was evaluated as described in Experimental Procedures. Numbers refer to no. mice protected/no. mice challenged in each group. Data are cumulative results from two challenges (RAS and *fabb/f*$^-$) or a single challenge (*sap1*$^-$). Results were analyzed by Fisher's Exact Test. $P<0.0001$ for 2° memory RAS versus *fabb/f*$^-$. $P<0.0001$ for 2° memory *sap1*$^-$ versus *fabb/f*$^-$ (n.d. = not determined) (**B**) Cumulative data showing the percent of circulating CD8 T cells that are $CD8\alpha^{lo}CD11a^{hi}$. Data (mean±S.E.M.) are from 10–40 mice per group from three independent experiments analyzed by One-Way ANOVA († = $P<0.01$; ¶ = $P<0.001$). (**C**) Frequency of $CD8\alpha^{lo}CD11a^{hi}$ T cells expressing CD27, $CD43^{glyco}$, CD62L or CD127. Data (mean±S.E.M.) are from 3–9 pooled samples from two independent experiments analyzed by One-Way ANOVA followed by Tukey's Multiple Comparison Test. (n.s. = not significant.) (**D**) Frequency of $CD8\alpha^{lo}CD11a^{hi}$ secondary memory T cells positive for the indicated marker. Data (mean±S.E.M.) are from 3–6 pooled samples from two independent experiments analyzed by One-Way ANOVA followed by Tukey's Multiple Comparison Test. See also Figure S2.

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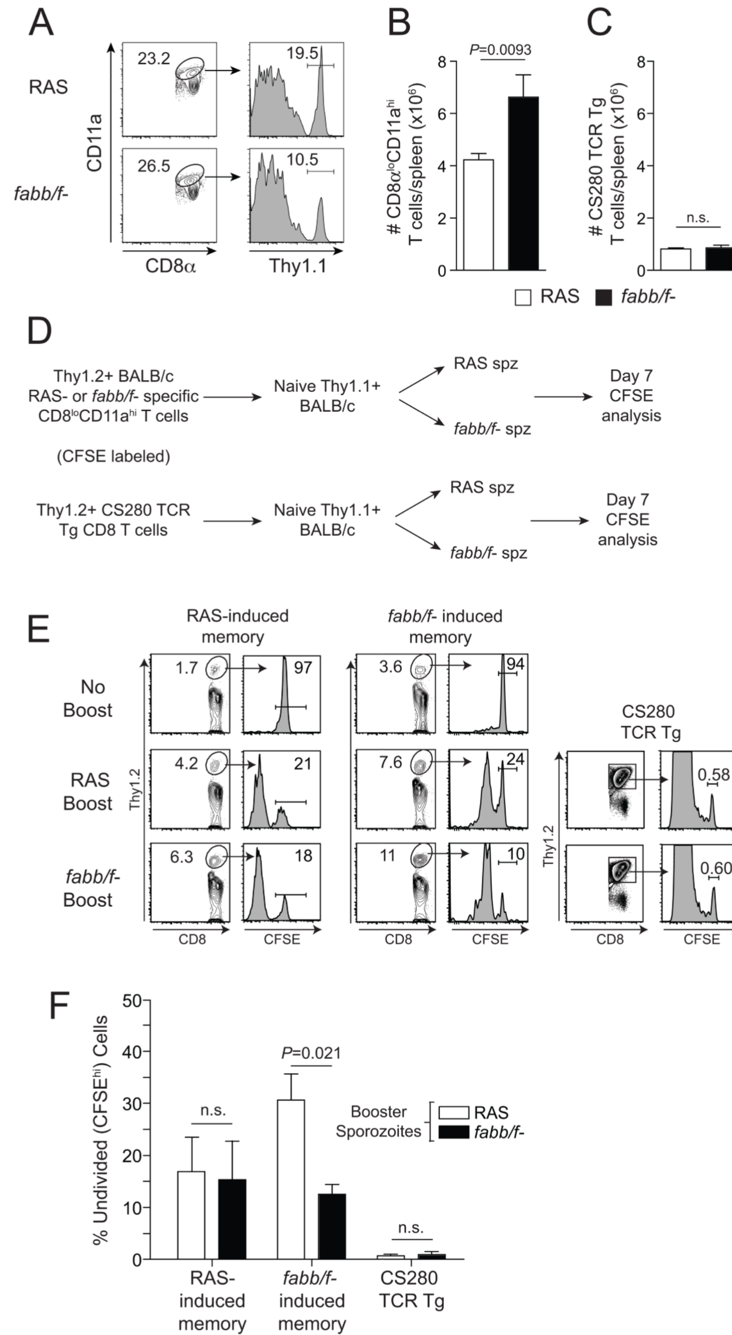

Figure 3. Vaccination with *P. yoelii fabb/f*$^-$ sporozoites diversifies the CD8 T cell response compared to *P. yoelii* RAS vaccination

(**A**) Thy1.1+ CS280 TCR Tg CD8 T cells (1000) were transferred to naïve Thy1.2+ BALB/c mice one day before vaccination with 2×10^4 RAS or *fabb/f*$^-$ sporozoites. Representative plots show the fraction of CD8 T cells that are CD8α^{lo}CD11a^{hi} and the fraction of those that are Thy1.1+ CS280 TCR Tg CD8 T cells on day six post-vaccination. Cumulative data showing the number of CD8α^{lo}CD11a^{hi} T cells (**B**) or Thy1.1+ CS280 TCR Tg CD8 T cells (**C**) per spleen. Data in **B** and **C** (mean±S.D.) are from 3 mice per group analyzed by unpaired student's t-test. Data are representative of two independent experiments. (**D**) RAS or *fabb/f*$^-$ specific memory CD8 T cells (Thy1.2+), or naïve (Thy1.2+) CS280 TCR Tg CD8

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T cells, were CFSE labeled and transferred into separate naïve Thy1.1+ BALB/c mice. Recipient mice were immunized with 2×10^4 RAS or *fabb/f*⁻ sporozoites. Seven days later naïve or memory CD8 T cells were assayed for dilution of CFSE. (**E**) Representative plots show the gating strategy. Numbers in histograms are the percent of RAS, *fabb/f*⁻ or CS280 TCR Tg CD8 T cells that remained undivided ($CFSE^{hi}$) following vaccination with RAS or *fabb/f*⁻ sporozoites. (**F**) Cumulative results showing percent of RAS-specific (left bars) or *fabb/f*⁻ specific memory CD8 T cells (middle bars), or CS280 TCR Tg CD8 T cells (right bars) that remained undivided ($CFSE^{hi}$) following vaccination with RAS or *fabb/f*⁻ sporozoites. Data (mean±S.D.) are from 3 mice per group analyzed by unpaired student's t-test. Data are representative of three independent experiments. See also Figure S3.

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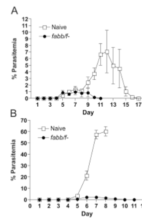

Figure 4. Vaccination of mice with late-liver-stage-arresting *fabb/f*$^{-}$ sporozoites protects against challenge with blood stage parasites

BALB/c mice were immunized with 1×10^5 *fabb/f*$^{-}$ sporozoites on three occasions at two-week intervals. Naïve and immunized mice were challenged one month later with 100 *Py* XNL (non-lethal, **A**) or 100 *Py* YM (lethal, **B**) blood-stage parasites. Parasitemia was measured daily. Mice in **B** were euthanized on day eight when parasitemia reached >60%. Data (mean±S.D.) in **A** and **B** are from 5 mice per group. See also Figure S4.

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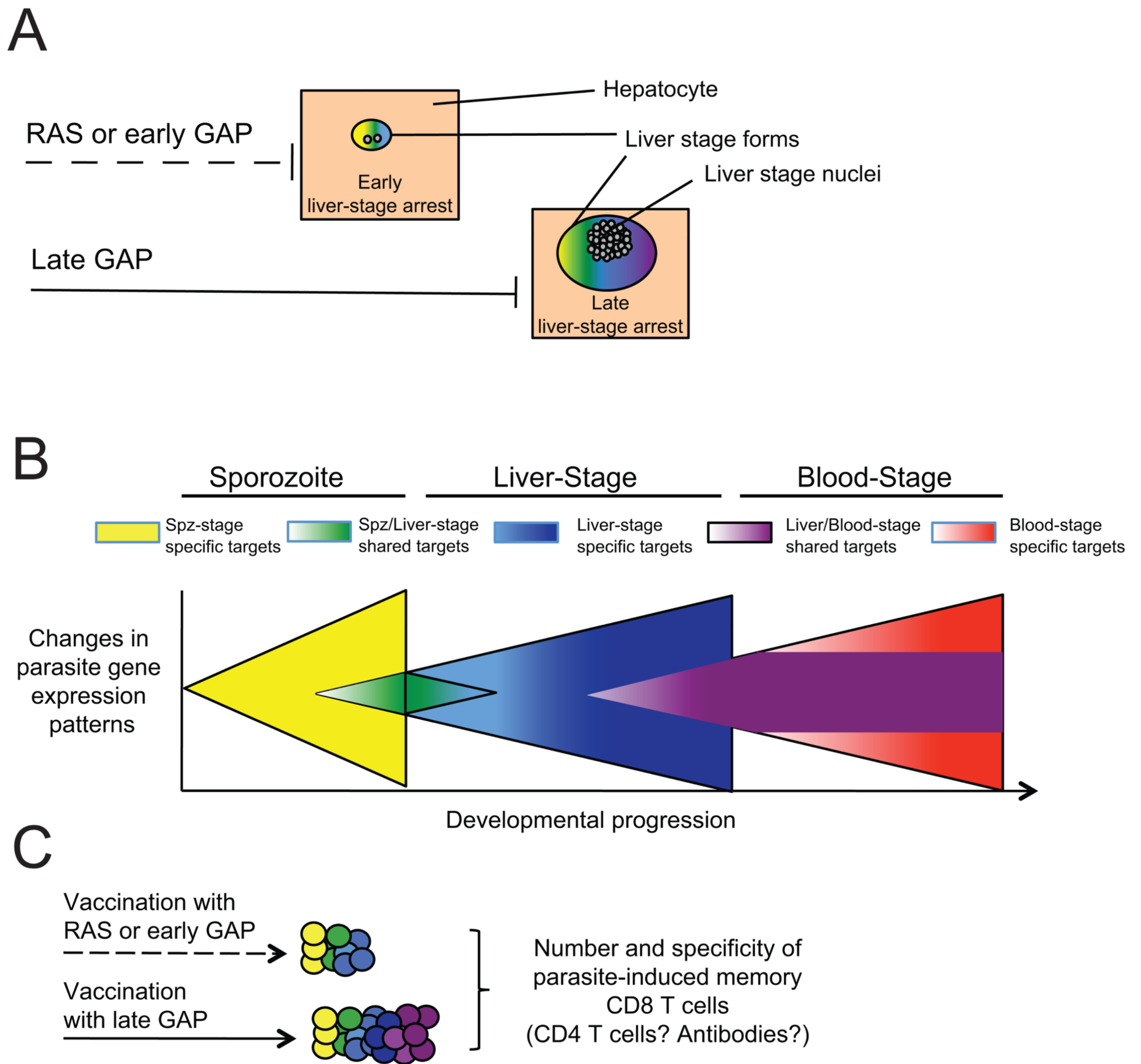

Figure 5. Model for enhanced protective immunity and diversification of antigenic targets by parasite-specific CD8 T cells induced following late-liver-stage arresting GAP vaccination

(**A**) Schematic depiction of liver-stage developmental progression by early (RAS or *sap1*$^{-}$) and late (*fabb/f*$^{-}$) arresting attenuated parasites in rodent malaria models. Early arresting RAS and *sap1*$^{-}$ parasites fail to undergo schizogony, exhibit smaller exoerythrocytic liver stage forms (small parasite biomass) and limited replication as indicated by few parasite nuclei relative to late-liver-stage arresting *fabb/f*$^{-}$ parasites. Colors shown within early and late arresting liver-stage forms depict both overlapping and non-overlapping expression of parasite-derived antigenic targets. (**B**) Schematic depiction of changes in parasite gene expression as a function of liver-stage developmental progression. Yellow, blue and green primary colors indicate expression of developmental stage-specific, parasite-derived antigenic targets, whereas gradients of green and violet secondary colors represent putative

antigens that are coordinately expressed during multiple developmental stages or the transition between stages. (**C**) Relative number and altered antigenic specificity of parasite-specific CD8 T cells induced following vaccination with early- (RAS or *sap1*$^{-}$) versus late- (*fabb/f*$^{-}$) arresting attenuated sporozoites. Colors in **C** correspond to the developmental stages in **B** and represent the relative breadth of antigens targeted by parasite-specific CD8 T cells following vaccination with early- versus late-liver-stage arresting sporozoites. Vaccination with late-arresting *fabb/f*$^{-}$ sporozoites induces a larger population of CD8 T cells whose antigenic specificity is only partially overlapping with CD8 T cells that arise in response to vaccination with early-arresting sporozoites.

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Table 1

Superior induction of protective immunity in outbred Swiss Webster mice by a *P. yoelii* late-liver-stage arresting GAP (*fabb/f*$^{-}$) vaccine

Attenuated sporozoite vaccine	% Protection Primary memory[a,b]	% Protection Secondary memory[a,c]
Naive	0% (0/20)	5% (1/20)
RAS	5% (1/20)	45% (9/20)
sap1^{-}	20% (4/20)	42% (8/19)
fabb/f^{-}	40% (8/20)[d]	90% (18/20)[e,f]

[a]Protection is defined as the absence of blood-stage parasitemia 10 days following challenge with 1000 *P. yoelii* sporozoites. Numbers indicate no. protected/no. challenged × 100. Greater than 90% of age-matched, naïve Swiss Webster mice challenged in parallel developed blood-stage parasitemia.

[b]Mice were challenged >80 days following a single immunization with 20,000 attenuated sporozoites. Data are cumulative results from two separate challenges.

[c]Mice were challenged >60 days following a second homologous immunization with 20,000 attenuated sporozoites. Data are cumulative results from two separate challenges.

[d]P=0.019 (Fisher's Exact Test) for primary memory RAS versus *fabb/f*$^{-}$.

[e]P=0.006 (Fisher's Exact Test) for secondary memory RAS versus *fabb/f*$^{-}$.

[f]P=0.002 (Fisher's Exact Test) for secondary memory *sap1*$^{-}$ versus *fabb/f*$^{-}$.

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Table 2

P. yoelii late-liver-stage arresting GAP (*fabb/f*⁻) vaccination of BALB/c mice affords enhanced protection against homologous and cross-species sporozoite challenge

Gene knockout	Primary immunization[a]	Boosts[a](day after primary immunization)	Challenge dose[b] (days after last immunization)	Protection[c]
sap1−	1,000	1,000 (14)	10,000 (30)	20% (2/10)
	1,000	1,000 (14)/1,000 (28)	10,000 (30)	100% (10/10)
fabb/f⁻	1,000	1,000 (14)	10,000 (30)	100% (10/10)[d]
	1,000	1,000 (14) /1,000 (28)	10,000 (30)	100% (10/10)
fabb/f⁻	10,000	10,000 (14)/10,000 (28)	10,000 (30, 120, 210)[e]	100% (8/8)
	10,000	10,000 (14)/10,000 (28)	10,000 (14, 210)	100% (8/8)
	10,000	10,000 (14)/10,000 (28)	10,000 (100)	100% (5/5)
	10,000	10,000 (14)/10,000 (28)	10,000 (300)	100% (8/8)
	50,000	50,000 (14)/50,000 (28)	10,000 (210)	100% (8/8)
	50,000	50,000 (14)/50,000 (28)	10,000 (30) + rIgG	100% (6/6)
	50,000	50,000 (14)/50,000 (28)	10,000 (30) + anti-CD8	0% (0/8)
	50,000 i.d.	50,000 i.d. (14)/50,000 i.d. (28)	10,000 (30, 210)	100% (5/5)
	50,000 s.c.	50,000 s.c. (14)/50,000 s.c. (28)	10,000 (30, 210)	100% (5/5)
fabb/f⁻	10,000	10,000 (14)/10,000 (28)	10,000 *P. berghei* (150)	100% (5/5)

[a]Mice were immunized by intravenous injection of knockout sporozoites unless otherwise noted. (i.d. = intradermal; s.c. = subcutaneous)

[b]Mice were challenged by intravenous injection of wild-type sporozoites.

[c]100% of age-matched naïve mice developed blood-stage parasitemia following challenge with virulent sporozoites.

[d]*P*=0.0007 (Fisher's Exact Test) for 1000 ×1000(14) × 10,000 (30) challenge *sap1*⁻ versus *fabb/f*⁻.

[e]Mice protected following challenge 30 days after the last immunization were re-challenged at 120 and 210 days and 100% remained protected.