Hepatocyte determinants of susceptibility to pre-erythrocytic *Plasmodium* infection

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Abstract

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Malaria is caused by eukaryotic parasites of the genus *Plasmodium*. They have coevolved with their mammalian hosts, significantly shaping both parasite and host genomes. It is the most deadly parasitic infection in the world, with approximately 200 million clinical episodes annually and nearly half a million deaths, mostly of small children. Disease is caused by parasite infection of host red blood cells; however, prior to this clinical phase, the parasite undergoes a clinically silent liver phase. While our understanding of this part of the *Plasmodium* life cycle has undergone tremendous development within the past two decades, much of the basic biology remains unknown.

Previous work strongly suggests that the hepatocyte intracellular environment is critical for successful development of liver stage parasites and evasion of host defenses such as apoptosis of infected cells. Moreover, there is evidence to suggest that parasites are able to seek out certain hepatocytes that provide a more permissive environment for development. This work seeks to describe hepatocyte phenotypes that show increased susceptibility to *Plasmodium* liver

stage infection, and to interrogate these hepatocytes to discover host molecular factors driving this differential susceptibility. We describe two phenotypes of differential susceptibility to *Plasmodium* in hepatocytes. First, *Plasmodium* preferentially infects and develops in polyploid hepatocytes. This increased susceptibility of highly polyploid hepatocytes is conserved for multiple *Plasmodium* species including the human malaria parasite *P. falciparum.* Susceptibility cannot be explained by differences in hepatocyte size or DNA replication. However, highly polyploid hepatocytes show increased density of surface proteins known to be important for parasite infection at the point of invasion. Secondly, phenotypic variability in infection exists between two closely related mouse substrains, BALB/cJ and BALB/cByJ. A consistent five-fold increase in liver stage burden is found in BALB/cByJ mice when compared to BALB/cJ. This difference is due to increased hepatocyte susceptibility to initial infection rather than immune system clearance or differential development in the liver and cannot be fully explained by previous known infection factors. A broad, unbiased search for host factors contributing to this susceptibility using RNA-Seq transcriptomics identified gene candidates including several known host membrane proteins for potential future work. Finally, interrogation of these phenotypes by reverse-phase microarray identified intracellular host factors, particularly the phosphorylated version of Ribosomal Protein S6 (RPS6) as being significantly and strongly upregulated in susceptible host hepatocytes. Blocking RPS6 phosphorylation by small molecule kinase inhibitors led to a drop in *Plasmodium* infection of hepatocytes. Additionally, mice with mutant RPS6 lacking phosphorylatable residues show lower average liver stage parasite burden than wild type littermates.

Taken together, this body of work shows that *Plasmodium* parasites do not find hepatocytes to be equal. Hepatocytes with distinct molecular characteristics are clearly preferred

as host cells. By inspecting the molecular differences found in hepatocytes of differential susceptibilities, we might identify new host factors important to malaria parasite liver infection and provide novel mechanistic insights into the processes of host cell selection and susceptibility to infection. Understanding the needs of the parasite during liver stage infection provides information useful for the development of new interventional strategies critical for the prevention and elimination of the early stages of malaria parasite infection.

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CHAPTER ONE:

INTRODUCTION AND LITERATURE REVIEW

OVERVIEW

This chapter provides an in-depth review of current literature regarding the biology *Plasmodium* liver stages. The life cycle of the *Plasmodium* parasite is described briefly, followed by relevant characteristics of the hepatocyte-infecting form of the parasite, the sporozoite. The mechanism of sporozoite targeting to the liver is described, as are various mechanisms proposed for sporozoite entry into hepatocyte. Finally, current knowledge of parasite molecular interactions with the host hepatocyte is reviewed.

In addition, this chapter discusses the aims of this thesis, including the hypothesis driving the work and a short descriptions of the chapters to follow.

LITERATURE REVIEW

Malaria is caused by eukaryotic parasites of the genus *Plasmodium*. It is the most deadly parasitic infection in the world, killing 240,000-630,000 people annually, mainly children in Sub-Saharan Africa [1]. Malaria is an acute illness spread through the bite of a female *Anopheles* mosquito. Uncomplicated malaria is characterized by high fever, chills, headache, and sweating, and is often challenging to differentiate from other febrile diseases. In around 1-2% of cases, the disease develops severe and life-threatening complications including cerebral swelling, anemia, metabolic acidosis, and organ failure [2]. Five species of *Plasmodium* infect humans, with the majority of clinical disease caused by *Plasmodium falciparum*, which is most prevalent in Africa and which causes the most mortality, or *Plasmodium vivax*, which is more prevalent in non-African regions [1]. Because *Plasmodium* parasites have a complex life cycle, including passage through the mosquito and multiple vertebrate host tissues, malaria might be controlled in either the insect vector or in the human host. Malaria eradication has been attempted, most notably in the 1950s and 60s; however, these attempts have ultimately failed due to problematic implementation, rising drug and insecticide resistance, and the lack of effective vaccines [3]–[5].

The liver stage of *Plasmodium* infection is an attractive interventional target since the number of parasites within the host is low and the host remains asymptomatic. Recent efforts at vaccine development have shown that intervention at this stage can be highly effective at preventing the development of clinical malaria and subsequent transmission. However, since so little is known about the interactions between the parasite and hits host at this stage designing effective interventions remains challenging [6]. A greater understanding of the role of host hepatocyte factors in malaria parasite liver infection will provide novel mechanistic insights into

malaria pathogenesis and may provide new pathways for the prevention and treatment of this deadly disease.

The *Plasmodium* lifecycle

Plasmodium parasites have a complex lifecycle requiring adaptation to highly varied environments, including two host species and numerous tissues within each. The lifecycle can be broken down into three main periods of replication and development—the insect stages, during which the parasite develops within an *Anopheles* mosquito; the pre-erythrocytic stages, including the infectious sporozoite and liver stages; and the blood stages, during which the disease of malaria develops. The body of work described in this thesis focuses primarily on the pre-erythrocytic stages. However, many general mechanisms, though not specific proteins, involved in navigating and establishing infection are conserved throughout different life stages. Therefore, a brief overview of the entire parasite lifecycle (Fig. 1.1) is useful to put the work in context.

The insect stage of the life cycle begins with a female mosquito taking a blood meal from an infected vertebrate host, which contains the sexual gametocyte forms of the parasite. Within the mosquito midgut, the gametes leave the infected red blood cells and mate forming a zygote, which transforms into a motile ookinete. This form penetrates the midgut wall and develops into a sessile oocyst which grows and undergoes rapid DNA replication and forms sporozoites [7]. After 10-14 days, the oocyst releases motile sporozoites into the hemocoel. These are transported to the mosquito salivary glands where they attach to and subsequently traverse the basal lamina and secretory cells to reach the secretory cavity [8], [9]. Here they remain until the mosquito once again feeds on a mammalian host, at which point hundreds of infectious sporozoites are injected into the skin [10].

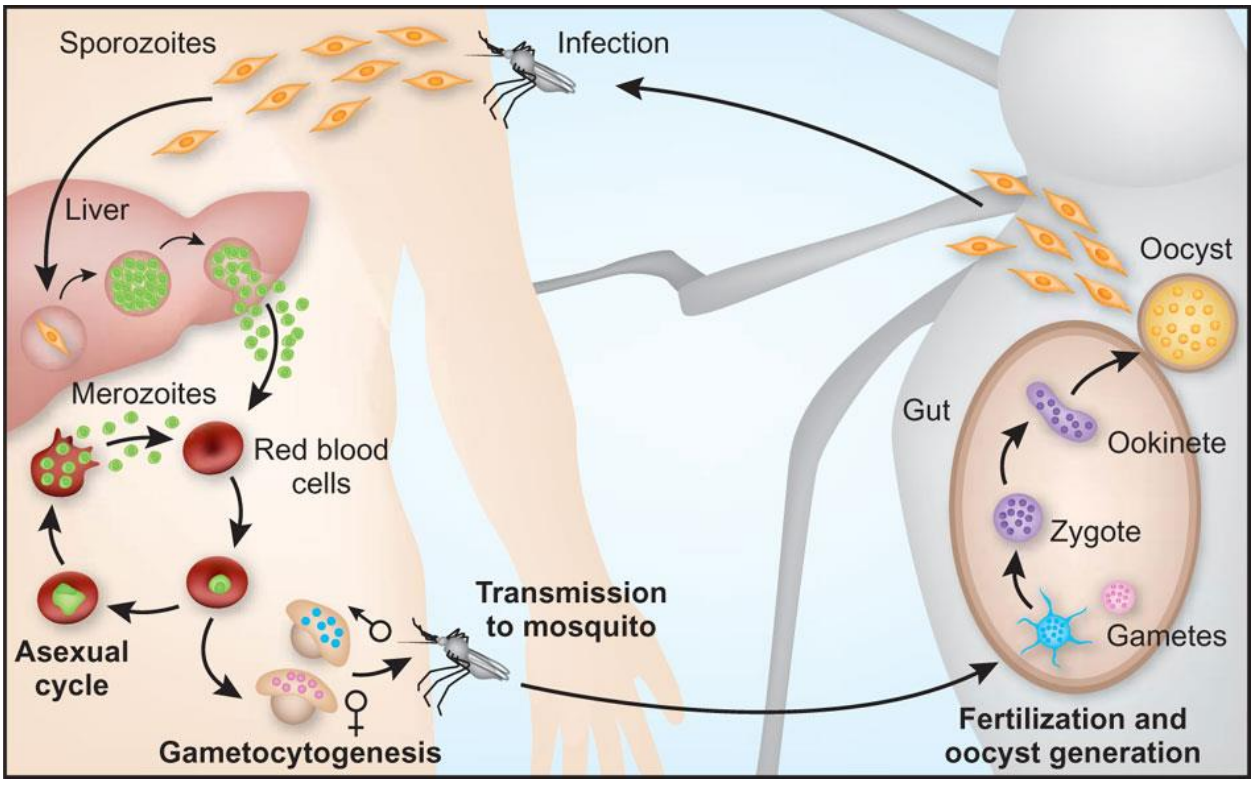

Figure 1.1: Lifecycle of the Plasmodium parasite

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Once injected, sporozoites move through the skin using gliding motility, which uses a parasite actin-myosin motor complex to drive parasites across a surface such as an epithelial cell. Unlike other forms of locomotion, gliding motility relies neither on whip-like structures, such as flagella or cilia, nor on deformation of the cell, such as with amoeba. Upon encountering a capillary, they rapidly enter the bloodstream and travel to the liver of the host [10], [11]. Upon reaching the liver sinusoid, sporozoites exit the blood stream, either by traversing through liver sinusoidal endothelial cells (LSECs) or resident macrophages known as Kupffer cells [12]–[15], and reaches the space of Disse where they can directly interact with its ultimate host cell, the

hepatocyte. Sporozoites travel through numerous hepatocytes in a process known as traversal, during which a parasite enters a cell then exits by wounding the cell plasma membrane [16]–[18]. After traversal, each sporozoite invades a single hepatocyte for residence and development, which constitutes "productive invasion." The host membrane involved in this process is heavily modified by the parasite to form a protective parasitophorous vacuole (PV) [19] within which the sporozoite ceases motility and transforms into sessile liver stage forms [18]. Here, the newly-formed liver stage parasite undergoes a remarkable course of growth and replication called exo-erythrocytic schizogony, during which the size expands by orders of magnitude and a single liver stage can develop into tens of thousands of progeny. After 2-10 days, depending on parasite species, the liver stage forms individual exo-erythrocytic merozoites [20]. In the human parasites *P. vivax* and *P. ovale*, as well as a non-human primate parasite *P. cynomolgi*, a subset of liver stage parasites do not replicate but rather go into a "suspended animation", developing into a form known as the hypnozoite [21], [22]. These hypnozoites can persist for weeks or months until they are triggered by an unknown signal to begin development into a standard liver stage schizont [23] containing merozoites. The merozoites contained within the hepatocyte bud into the blood enveloped in membrane-bound structures called merosomes [24], which travel into the pulmonary vasculature where they disintegrate [25], releasing merozoites into the blood where they invade and develop within red blood cells.

This infection of red blood cells is the beginning of the erythrocytic asexual cycle, or blood stage, during which parasite numbers increase exponentially and the onset of disease occurs. Inside the infected red blood cell, or iRBC, the merozoite develops first into a ring form, then a trophozoite, and finally into a schizont of up to a few dozen new merozoites [26]–[28]. Rupture of the iRBC membrane releases the merozoites into the bloodstream where they each

can invade a new cell and begin the cycle again [29]. In a certain proportion of cells, the merozoites develop into new sexual stage gametocytes [30]. At this point, a blood meal taken by a mosquito will begin the lifecycle over again.

Sporozoite cellular structure and gene expression

Sporozoites exist in the mosquito in two forms. Sporozoites initially develop in the mosquito midgut oocyst, then are transported to the salivary gland. Oocyst sporozoites and salivary gland sporozoites are morphologically similar but show markedly different phenotypes, particularly in terms of liver infectivity. Salivary gland sporozoites show 100 to 10,000 times more infectivity in the mammalian liver than oocyst sporozoites [31]. Comparison of the transcriptomes of the two forms of sporozoite has identified approximately 125 genes with increased transcript abundance in salivary gland sporozoites [32]–[34]. In addition, comparative transcriptomes have been analyzed for the hepatocyte-invading sporozoite and the erythrocyte-invading merozoite [35]. Several of these genes, collectively referred to as upregulated in infectious sporozoite, or UIS, genes, have been proven to be critical in infection of the host liver [36]–[40].

UIS gene products are largely only required once the sporozoite reaches its mammalian host, yet the genes are transcribed within the salivary gland sporozoites [32], [33], [35]. Premature translation of these transcripts will cause early transformation into liver forms and loss of liver infectivity [41], [42]. *Plasmodium* has developed an ingenious way to prevent this while still allowing rapid production of gene products needed for mammalian host cell traversal, invasion, and development. The sporozoite transcribes the UIS genes in the salivary gland, but then sequesters them with an RNA-binding protein, Puf2, until needed in the liver [41]–[43].

The sporozoite an extremely elongated cell, and has been compared to a human eyelash in shape. Under light microscopy, the two ends look similar, but the sporozoite is in fact polarized. The apical end contains a complex of organelles typical of its phylum, Apicomplexa [44], [45]. Included in this complex are a polar ring which anchors cytoskeletal components, and secretory organelles called micronemes, rhoptries, and dense granules. These organelles contain diverse proteins involved in motility, adhesion, host cell traversal and invasion, as well as parasitophorous vacuole formation and modification. Secreted proteins drive parasite gliding motility by secretion of motile and adhesive proteins by the micronemes at the apical end then translocated toward the basal end [9], [46], driven by an actin-myosin motor contained between the sporozoite outer plasma membrane and a second set of internal membranes [20], [47]. Gliding motility in the sporozoite occurs in a seemingly random corkscrew fashion, and is critical for several steps of infection. Within the mosquito, the sporozoite uses gliding motility to move into the salivary ducts in order to be transmitted by a bite [48]. Once transmitted into the mammalian host, *in vivo* imaging of mouse skin has shown sporozoites continuously gliding through the skin to reach a blood vessel [10], [49]. Finally, once the sporozoite reaches the liver, gliding motility is required for both translocation into the space of Disse and for traversal through hepatocytes [11], [17].

Migration of the sporozoite to the liver

Salivary gland sporozoites are exquisitely selective for infection of hepatocytes [50]; while a small fraction of sporozoites have shown development into exo-erythrocytic forms in skin cells [51] the vast majority, if not all, productive infections occur within hepatocytes [52]. The hepatocyte provides a unique intracellular environment conducive in many ways to pathogenic infection. The liver itself is to an extent an immunoprivileged organ, due to

specialized immune cells which inhibit inflammation and T cell activation [53], [54]. This is necessary to prevent detrimental immune activation from harmless antigens derived from intestinal blood [55]; however, suppression of immune mechanisms including hepatocyte-intrinsic tolerogenic MHC-II signaling [56] allows the parasite to avoid host immune responses to an extent. Further, hepatocytes are key regulators of metabolism and thus represent a nutrient-rich environment which can provide a wealth of carbohydrates, lipids, amino acids, and iron to the developing parasite. Finally, hepatocytes are one of the few tissues in the body with high levels of polyploidy [57], which is hypothesized to be protective against toxic stress [58]–[60], and which also increases both the size and protein content of the cell [61]. Even diploid hepatocytes are quite large—a distinct advantage for a parasite that grows by orders of magnitude during its residence.

Plasmodium is unusual in its highly specific tissue tropism, in direct contrast to even closely related Apicomplexans such as *Toxoplasma* or *Theileria* which can develop within numerous cell types [62]–[64]. However, sporozoites in the bloodstream interact with numerous tissue types throughout the body. How do they target the liver, and more specifically, the hepatocyte rather than one of numerous non-parenchymal cells? The answer seems to be within the most abundant surface sporozoite surface protein, circumsporozoite protein (CSP). CSP seems to serve a dual purpose for the sporozoite. First, CSP is heavily immunodominant; the majority of antibody responses arising from sporozoite inoculation target CSP [65]. These antibodies are to an extent protective, as anti-CSP antibodies can block infection both *in vitro* and *in vivo* [66]–[68]. In fact, the only currently approved malaria vaccine RTS,S is based on CSP subunits [69]. Yet despite robust anti-CSP antibody responses natural infection fails to be fully protective [70], [71], and the RTS,S vaccine only prevents 30-65% of infections [69]. This

may be due to the parasite's ability to shed CSP upon interaction with antibodies, leaving immobilized protein behind and replacing it with newly-synthesized protein [31]. In addition, CSP is able to prevent a respiratory burst within Kupffer cells, preventing sporozoite destruction within the macrophage during traversal [72]. CSP thus seems to be a powerful weapon in *Plasmodium*'s evasion of host immunity.

The second property of CSP is its ability to interact with a set of modified proteins known as heparan sulfate proteoglycans, or HSPGs [73], [74]. HSPGs are found throughout the body, but when expressed on hepatocytes include additional sulfations. These highly sulfated HSPGs (hsHSPGs) are negatively charged, and interact with positively-charged residues on both the N-terminus [75] and the main thrombospondin-like repeat region (TSR) of CSP [76], [77] to target the parasite to the liver. It is not entirely clear whether high sulfation of HSPGs on Kupffer cells is sufficient to begin traversal from the liver sinusoid into the space of Disse, or whether hsHSPGs on hepatocytes or in stellate cell-secreted extracellular matrix extend through fenestrations of the endothelium from the space of Disse to interact with parasites in the bloodstream, or if a combination of the two is necessary [14].

Regardless of the source, CSP interaction with hsHSPGs triggers a calcium-dependent signaling cascade within the parasite [78], leading to cleavage of the N-terminal region of CSP by a parasite cysteine protease and exposure of the TSR [79]–[81]. This cleavage triggers a conformational change in the protein [80], leading to switch between a traversal phenotype and productive invasion of the hepatocyte [79]. Constitutive expression of pre-cleaved CSP increases infection with *P. berghei* in mice [80], suggesting that only a subset of parasites achieves this cleavage in natural infection and thereby fails to invade hepatocytes. In contrast, inhibition of cleavage, either by protease inhibitors [79] or antibodies [81], abrogates infection.

Another sporozoite secreted surface protein, thrombospondin-related anonymous protein (TRAP), also contains a TSR domain as well as an integrin-like A-domain, both of which may interact with hsHSPGs [82]–[84]. Recombinant TRAP has been shown to interact with hsHSPGs on the hepatocyte surface [76], [83], [85]. Both domains of TRAP are dispensible for cellular adhesion but are required for sporozoite invasion of hepatocytes, though it is not known whether the interaction with hsHSPGs is specifically required [83], [86].

Sporozoite entry into the hepatocyte

Two types of sporozoite entry into hepatocytes can be distinguished. The first type is cell traversal, during which the parasite enters, then leaves the hepatocyte in a membrane-wounding manner [16]. This process of traversal has been proposed to be critical for proteolytic processing of CSP [79] and discharge of invasion-related proteins from the secretory organelles [87], thereby activating the sporozoite for productive invasion. In addition, wounding of hepatocytes by traversal induces secretion of hepatocyte growth factor, which enhances infection by *P. berghei* [88], though not other species [89]. However, other work has demonstrated that rhoptry discharge does not occur during cell traversal [90], [91]. Moreover, traversal has been shown to stimulate NF-κB inflammatory signaling, which limits *Plasmodium* liver infection [92]. Thus it is not clear that cell traversal is beneficial for parasite invasion with a PVM or survival within the hepatocyte.

Several hepatocytes are usually traversed and can be visualized microscopically using fluorescently-tagged dextran, which can enter cells with wounded membranes but is excluded from those whose membrane is intact [16]. Cell traversal is common in *in vitro* infections, as well as *in vivo* sporozoite injections by tail vein; however, a lower amount of traversal occurs when parasites are injected by mosquito bite [16], so the extent of cell traversal in natural

infections is unclear. Hepatocyte membrane wounding during cell traversal is due to parasite derived pore-forming proteins, including cell-traversal protein for ookinetes and sporozoites (CelTOS), which is critical for cell traversal in both the mosquito and in the liver [93], and two proteins called sporozoite microneme protein essential for cell traversal (SPECT1/2). SPECT-deficient parasites are able to productively invade a hepatocyte but are entirely deficient in traversal [17], [94]. Due to this lack of traversal, SPECT-deficient parasites fail to efficiently exit the skin at the site of injection, as well as failing to leave the liver sinusoid. However, if the parasite is intravenously injected into mice depleted of Kupffer cells, thus bypassing both barriers, SPECT-deficient parasites form a robust infection in the liver [17], [94].

Until recently, the model of traversal was generally thought to involve membrane wounding at both entry and exit. Recently, it has been demonstrated that instead, traversal involves an invagination of the membrane to produce a transient vacuole. This vacuole merges with lysosomes, lowering the pH. The membrane is then perforated by SPECT2 (also known as PLP1) releasing the sporozoite into the hepatocyte cytoplasm [90]. The parasite then exits the cell by membrane wounding. *Spect2*- knockout parasites fail to exit their vacuole, leading to further lysosomal fusion and degradation of the parasite [90].

The hepatocyte membrane in traversal does not seem to be modified by parasite proteins, nor does traversal trigger release of the rhoptry proteins [90]. In contrast, productive invasion begins with creation of the PVM through remodeling of the host membrane by proteins excreted by the invasion organelles. The specific basis for this switch between traversal and PVM-based productive invasion is not fully elucidated but several key molecular players have been found, both in the parasite as well as the host. First, the cleavage of CSP triggered by hsHSPG interaction has been shown to drive this switch, as noted previously. Treatment of cells with

chlorate, which desulfates HSPGs, decreased productive invasion and increased traversal in a dose-dependent fashion [78]. Antibodies to the N-terminus of CSP, which block this cleavage event, show increased inhibition of parasite infection when compared to antibodies to the central repeat region [81], long considered the gold standard of parasite blocking antibody.

The tetraspanin CD81 was first specific hepatocyte surface protein discovered to be involved in sporozoite productive infection [95]. CD81 resides on the hepatocyte plasma membrane in a network of protein-protein interactions that organize cellular receptors and adhesion molecules into tetraspanin microdomains [96], [97]. CD81 is a known viral entry factor for Hepatitis C through direct interaction with viral protein E2 [98], but does not seem to directly interact with the parasite [99]. Knocking out CD81 abrogates infection with *P. yoelii*, and anti-CD81 antibodies, which disrupt CD81 microdomains, can block infection with both *P. yoelii* and *P. falciparum* both *in vitro* [95], [100] and *in vivo* [95], [101]. CD81 is not required for infection with *P. berghei*; however, the mechanisms of entry in this parasite may be somewhat different, as it is much less specific in host cell tropism than other *Plasmodium* species [102]. CD81 is not required for formation of the traversal vacuole [90] but is necessary for rhoptry discharge and thus formation of the PV [91].

Another known host factor for *Plasmodium* entry is the scavenger receptor B1 (SR-B1). SR-B1 mediates lipid transport, particularly cholesterol, from the bloodstream into the hepatocyte and is also known to be a Hepatitis C viral entry factor. Knockout of SR-B1 lessens, but does not abrogate, *Plasmodium* infection [103]. This is likely due to its role in hepatocyte membrane cholesterol content, as CD81 is dependent on cholesterol to maintain its tetraspanin microdomains [104]. In addition, the hepatocyte growth factor receptor c-Met has been shown to facilitate *P. berghei* infection [88], though this critical role is not conserved in other species

[89]. Like CD81 and SR-B1, c-Met does not appear to be a direct receptor for the parasite. Rather, c-Met-driven signaling affects the intracellular environment in ways beneficial to *P. berghei*, including hepatocyte actin cytoskeletal remodeling [88] as well as anti-apoptotic signaling through the PI-3K/Akt pathway [105].

Discharge of parasite rhoptry and micronemal proteins is critical for the productive invasion with a PVM, though the specific functions of many of these proteins is unclear. However, two micronemal proteins, P36 and P52 (also known as P36p) have been shown to be critical in PVM formation. P36 and P52 are part of a large family of parasite proteins containing a fold that usually contains six cysteine residues, and therefore are called 6-Cys proteins [106]. Sporozoites deficient in both P36 and P52 cannot form a PVM [39], and double knockout of both genes in *P. yoelii* and in *P. falciparum* renders parasites unable to develop as liver stages [38], [107]. However, this total protection may be species-specific [108].

The 6-Cys fold of *Plasmodium* proteins is structurally similar to the metazoan Ephrin-like fold found on many membrane proteins [109]. Ephrin-like domains interact with Eph receptor tyrosine kinases in cell-cell adhesions [110], which suggests that 6-Cys proteins may interact with these host receptors directly. Recently, it was shown that the mammalian receptor EphA2 plays a critical role in PVM formation, likely through directly binding parasite P36 [111]. Mice lacking the EphA2 receptor are refractory to infection [111], indicating that a 6-Cys-Eph receptor interaction is important for establishing the replication-permissive niche during invasion.

Rhoptry and micronemal proteins are needed for the sporozoite to productively invade the hepatocte. The precise mechanism is not fully understood, though it is known to be driven by the same parasite actin-myosin motor that drives gliding motility, rather than internalization by the host cell [11]. It is thought that after secretory organelles discharge, the excreted proteins

attach to ligands on the hepatocyte surface, which leads to the formation of a tight ring around the parasite [112], [113]. The complex is then translocated away from the apical end using an actin-myosin motor similar to that used in sporozoite gliding motility [112], creating a moving junction which excludes host surface proteins, creating the PVM [113]. Host F-actin has been shown to co-localize to the moving junction [114], but does not appear to actively internalize the parasite; rather it may stabilize the complex. Interestingly, host actin and the actin-related protein 2/3 complex (Arp2/3) appears to be recruited to the moving junction, though what signals may promote this recruitment is unknown [114].

In red blood cell invasion by the merozoite, the moving junction is formed from two parasite proteins—a rhoptry neck protein, RON2, and the micronemal apical membrane antigen 1 (AMA1) [115], [116]. In the sporozoite, a similar rhoptry neck protein, RON4, is known to be required for invasion [117], and AMA1 is expressed in sporozoites [118]. Antibodies against AMA1 inhibit invasion of *P. falciparum* sporozoites *in vitro* [119], as well as *P. yoelii in vivo* [120]. However, in *P. berghei*, AMA1 is not involved in sporozoite invasion [117]. In addition, while RBC tight junction formation appears independent of host proteins, the sporozoite does seem to require the presence of at hepatocyte membrane proteins, thus the process may differ to an extent. Elucidating these interactions could inform vaccines and other efforts to block malaria at the liver stage.

Liver stage development and host cell interactions

After productive invasion, the parasite moves into proximity with the host nucleus and begins a process known as de-differentiation, as the sporozoite transforms from its long "eyelash" shape into a rounded form, and cellular components needed for motility and invasion are disassembled or even ejected from its cell [113], [121]. This process can be phenocopied *in*

vitro by a shift in temperature from 22 C (mosquito body temperature) to 37 C (human body temperature) and exposure serum factors such as albumin or bicarbonate [122], [123], a process which is dependent on parasite calcium signaling [124], followed by protease-based degradation and parasite autophagy [125]. This process takes between 20 to 28 hours in rodent malarias [122] and two to three days in human malarias [107], [126].

During invasion and dedifferentiation, the PVM is modified by parasite proteins which may interact with the host to provide a safe and nurturing environment for the developing parasite within. One primary function provided by the PVM seems to be protective against death of the parasite due to host innate defenses. Apoptosis of the infected hepatocyte, particularly through B-cell lymphoma 2 (Bcl-2) family apoptotic pathways, can eliminate liver stage parasites [127]; however, uncleared hepatocytes show resistance to apoptosis [128]. However, *P36*$^{-}$*/P52*$^{-}$ double knockout parasites, which cannot form a PVM, are rapidly cleared through apoptosis [38], [39]. Likewise, parasites within cells low in the EphA2 receptor, which are nearly universally lacking a PVM, show decreased persistence over 24 hours of infection [111] though it is not clear whether this is due to apoptosis or another mechanism. Host autophagy may also contribute to host clearance of the parasite within the hepatocyte. Early in infection, the autophagy marker LC3 has been observed to insert into the PVM, but is cleared over time [129], [130], presumably through parasite remodeling of the PVM. Cells deficient in autophagy, lacking the Atg5 gene, show decreased clearance of *P. berghei* over 48 hours of infection [130]. However, the role of autophagy specifically in PVM-deficient parasite clearance has not been interrogated.

Plasmodium asexual blood stages substantially remodel their host erythrocyte. In *P. falciparum*, over 10% of all proteins, including the major virulence factor PfEMP1 (P.

falciparum erythrocyte surface protein 1), are exported to the iRBC [131] through an extensive endomembrane system including structures such as Maurer's clefts, J-dots, and a tubovesicular network (TVN) [132], [133]. These proteins change the host cell cytoskeleton and plasma membrane, in part to avoid destruction of the iRBC by the cells of the immune system. For example, P. *falciparum* iRBCs become rigid and covered with adhesion proteins, allowing them to bind to endothelial walls and thus sequester themselves from removal in the spleen [134], [135]. In contrast, P. *vivax*, which infects only newly-formed reticulocytes, actually decrease their host cells' rigidity allowing them to pass through the spleen without clearance [136].

In comparison, the liver stage parasite remodels the hepatocyte only subtly. The TVN has been described in liver stages [137] and at later time points the sheer size of the liver stage schizont physically deforms the hepatocyte as it grows to many times the size of the original cell [138]. However, there is limited evidence that any parasite protein is exported past the PVM [139], [140]. This is likely due to the fact that any parasite proteins within the hepatocyte cytoplasm or membrane risk being exposed to immune cells via Major Histocompatibility Complex (MHC) presentation and subsequent destruction by CD8+ T-cells [141], [142].

Parasite interaction with the host cell, then, may rely entirely on the PV and TVN membranes. Some interaction between membrane proteins and host proteins or structures has been described, and suggest a major role for this interaction to be the scavenging of nutrients. The PVM protein UIS3 interacts directly with liver fatty acid binding protein (L-FABP) [143] which may be importing lipids including cholesterol necessary for parasite growth, although the precise mechanism of the transfer of lipids is unclear [144]. The parasite also incorporates host phosphatidylcholine [145] and phosphatidylinositol phosphate [$PI(3,5)P_2$] [146] into the PV and TVN membranes, with the latter being necessary for fusion with host late endosomes. The

interaction between parasite and host membranous systems is extensive though not fully understood. Both late endosomes and lysosomes are sequestered around the PVM [146], [147], possibly providing nutrients to the parasite through non-specific autophagy of host cellular components [129], [130]. The host nuclear membrane and endoplasmic reticulum are also seen in close association with the PVM [113], though the function of this association is unknown. Interestingly, the PVM also is somewhat porous, allowing passive transfer of molecules up to 855 kilodaltons [113] which might allow for parasite uptake of host metabolites.

Despite the relative dearth of evidence for hepatocyte remodeling, it is becoming increasingly clear that the infected hepatocyte is distinct from uninfected hepatocytes in numerous ways. Interrogation via transcriptomics of infected hepatocytes collected at several time points during infection [148] shows a number of cellular processes that change in both P. *berghei*- and P. *yoelii*-infected cells, particularly those involved in apoptosis, stress responses, intracellular and cell-to-cell signaling, and numerous metabolic and biosynthetic pathways. Recently, it was demonstrated that at least one of the identified stress responses, the unfolded protein response of the host ER, plays a significant role in parasite infection [149], showing the relevance of this global data in current research. Proteomics of infection have been more challenging, but protein lysate microarray interrogation of hepatoma cells was able to detect several cellular signaling disruptions in infected cells, leading to the identification of a previously unknown host factor, the oncogene p53, the suppression of which is critical for parasite survival [149]. Intriguingly, though p53 is known to be pro-apoptotic, the effect of p53 on parasite development seems to be independent of apoptosis [150].

Both transcript and protein-level interrogations identified several proteins in the PI3-Akt-mTOR host pathway [148], [149], which controls a number of pro-survival and anabolic cellular

responses [151], [152], to be dysregulated in infected cells in a manner that would benefit the parasite. In addition, disruption of several pro-apoptotic signaling pathways is also seen in infected cells [128], [148], [149]. The parasite has strong incentive to control hepatocyte autophagy and apoptosis. Both mechanisms of parasite clearance can be induced by hypoxia [153], [154]. However, hypoxia in hepatocytes promotes persistence and development of liver stage parasites [155], possibly due to upregulation of the hypoxia-inducible factor 1-alpha (HIF-1α) [156], which upregulates many of the pathways dysregulated in infected cells including glucose transport and metabolism, cell proliferation, and cellular stress responses [157]. Hypoxia may also be protective against reactive oxygen species, lowering the energy expenditure needed for parasite response to oxidative stress [155].

This raises the intriguing question: does the parasite actively cause these changes via molecular interaction? Or, considering that parasites traverse multiple hepatocytes before productive invasion of one cell, does the parasite seek out hepatocytes that have a predisposition to these parasite-supportive cellular processes? And in either case, what molecular mechanisms are behind the difference in environment between hepatocytes that are conducive to parasite growth, and those that are not? Determining the host molecular players in *Plasmodium* liver infection could have profound implications in the treatment and prevention of malaria. Liver stages of infection are a critical bottleneck in the parasite lifecycle, involving orders of magnitude fewer parasites than blood stages as well as being clinically silent. Stopping infection in the liver prevents transition to blood stage infection, thereby preventing disease in the host and stopping transmission to the mosquito. By increasing our understanding of liver stage *Plasmodium* and its interactions with the host, we can identify key molecular factors in liver stage infection. This, in turn, can drive rational interventions such as genetic attenuation of

parasites for vaccines [37], [38], [158] or antimalarial drugs [159], [160]. In fact, as drug resistance is an increasing concern [161], [162], targeting host factors that promote liver stage development [150] could bypass the rapid evolution of drug resistance seen in malaria.

THESIS AIMS

The liver stage of *Plasmodium* infection has been known for over half a century [50], but only within the last decade have we begun to understand the specific parasite and host factors driving infection and development of the parasite within the hepatocyte. Still, much is yet to be understood. Excellent work has been done to characterize the parasite genome [163], transcriptome [32], [35], [164], [165] , and proteome [164], [166], [167] at both sporozoite and liver stages that has expanded our knowledge of parasite biology during these pre-erythrocytic stages. Less is known about the interaction of parasites and their host hepatocytes, but evidence continues to accumulate that the host cell environment is a critical line of inquiry as well.

Hepatic susceptibility to *Plasmodium* can vary between mice of different genetic backgrounds [168], [169] or between individual people [126]. Susceptibility may be influenced by hepatocyte complement of surface [100], [103], [111] or cytoplasmic proteins [129], [149] which can vary between cells even within a single liver. This variability makes the liver a diverse and nonhomogeneous environment for malaria parasites. *Plasmodium* sporozoites have the ability to traverse through several cells before productively invading. This ability has been shown to be dispensable for hepatocyte infection [11] and both energetically costly and detrimental to immune evasion [92], yet has been evolutionarily conserved [12]. This suggests that *Plasmodium* may use this movement to "select" hepatocytes which provide beneficial environments for parasite survival; changes seen in signaling pathways of infected hepatocytes suggests the parasite may then mold its host to persist and thrive throughout development.

In this work, I hypothesize that specific molecular host factors are critical for determining host susceptibility to infection. This work is primarily focused on the investigation of these unknown molecular host factors involved in malaria parasite infection of hepatocytes, and seeks to identify such host factors that allow for a robust and successful intracellular infection of the host hepatocyte. Three areas of research are described:

1. Susceptibility of hepatocytes with altered ploidy to *Plasmodium* sporozoite infection, showing increased DNA content correlates to increased susceptibility, possibly due in part to changes in receptor complement on these cells.
2. Description of changes in susceptibility of hepatocytes of two closely related mouse strains, BALB/cJ and BALB/cByJ, despite few genomic differences.
3. Identification of a host ribosomal protein, RPS6, as a host factor of parasite development, through post-translational comparison of highly-susceptible to less-susceptible hepatocytes.

This work provides insight into the complex contributions the hepatocyte environment provides to infection and persistence of the *Plasmodium* liver stage parasite. It identifies two phenotypes of hepatocyte susceptibility, and interrogates differential cellular signaling between hepatocytes of greater and lesser susceptibility using small-scale proteomics. The intersection of the differences in the two phenotypes are used to identify a specific hepatocyte factor of infection. Finally, this thesis proposes using further intersections of data derived from both global "-omics" and candidate-driven experiments and analyzed with machine learning tools to identify key players in this parasite life stage. The insights available from this work and future approaches of this type can inform the development of novel, host-oriented interventions for the

treatment and prevention of malaria, thus helping efforts toward eradication of this deadly disease.

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CHAPTER TWO:

SUSCEPTIBILITY TO *PLASMODIUM* LIVER STAGE INFECTION IS ALTERED BY HEPATOCYTE POLYPLOIDY

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Laura S. Austin, Alexis Kaushansky, and Stefan H.I. Kappe

OVERVIEW

This chapter describes the increased susceptibility of polyploid hepatocytes, or those cells with more than two copies of chromosomes. Hepatocytes, unlike most cell types, have a high natural degree of polyploidy. Previous work had shown disruption in cellular pathways involved in cell replication, which are also linked to polyploidy.

To test the functional consequence of polyploidy in liver infection, we infected hepatocytes with the rodent malaria parasite *Plasmodium yoelii* both *in vitro* and *in vivo* and examined the ploidy of infected and uninfected hepatocytes by flow cytometry. In both hepatoma cell lines and in the mouse liver, the fraction of polyploid cells was higher in the infected cell population than in the uninfected cell population. When the data were reanalyzed by comparing the extent of *Plasmodium* infection within each ploidy subset, we found that infection rates were elevated in more highly polyploid cells and lower in diploid cells. Furthermore, we found that the parasite's preference for host cells with high ploidy is conserved among rodent malaria species and the human malaria parasite *Plasmodium falciparum.* This parasite preference for host cells of high ploidy cannot be explained by differences in hepatocyte size or DNA replication. We conclude that *Plasmodium* preferentially infects and develops in polyploid hepatocytes.

This work was originally published in *Cellular Microbiology,* Volume 16(5):784-95, May 2014. Some changes have been made for this thesis; specifically, supplemental figures were either cut or added to the body of the paper, and one new figure has been added to show new data (Figure 2.8 and corresponding text). In addition, the Discussion section has been slightly modified to include the new data.

INTRODUCTION

Parasites of the genus *Plasmodium* are the causative agents of malaria, which remains one of the deadliest infectious diseases worldwide [1]. Infection is transmitted to the mammalian host by the bite of a female *Anopheles* mosquito, which injects the infectious form of the parasite, the sporozoite, into the dermis. Sporozoites then traverse through the skin, wounding cell membranes, until they reach a blood vessel that facilitates their transport to the liver. Here, the sporozoite invades a hepatocyte where it develops for 2-10 days [2]. Once inside the hepatocyte the parasite divides rapidly, eventually differentiating into tens of thousands of merozoites which leave the liver, invade red blood cells and cause symptomatic malaria. Because the liver stage is clinically silent and involves orders of magnitude fewer parasites than the later blood stages of infection, it is a critical target for intervention.

The sporozoites invade host hepatocytes by invagination of the host cell membrane to form a vacuole that ensconces them within the cells. During this process the parasite releases proteins from its apical organelles, remodeling the membrane to facilitate invasion of the host cell. Unlike traversal, which can occur in multiple cell types, productive invasion in the liver is possible only in the hepatocyte. The host factors that contribute to parasite invasion remain largely uncharacterized [3].

Recently, we reported that *P. yoelii* liver stage infection perturbs hepatocyte signaling pathways, including those involved in cell proliferation and replication [4]. Yet, proliferation of hepatocytes is only found at low levels in the normal liver and only increases when the liver has been highly damaged. Thus, these trends raise intriguing questions regarding the perturbation of cellular proliferation pathways in infected cells. Interestingly, cell proliferation pathways are linked to the phenomenon of polyploidy, the presence of more than two homologous sets of

chromosomes in a cell, which is widespread in hepatocytes [5]. Since polyploidy is an unusual but common feature of hepatocytes, and *Plasmodium* liver stage development is restricted to hepatocyte host cells, we asked whether this common feature of hepatocytes might affect the process of liver infection. Here, we show that *Plasmodium* sporozoite infection displays preference for hepatocytes with elevated ploidy.

RESULTS

Plasmodium parasites preferentially infect polyploid cells *in vitro*

While the natural target cell for the malaria sporozoite is the primary hepatocyte, multiple transformed hepatoma cell lines have been developed for *in vitro* experimentation [6]–[8]. We first investigated if levels of ploidy varied between infected and uninfected hepatocytes by infecting HepG2-CD81 cells with *P. yoelii* and using flow cytometry to assess DNA content of infected single cells [9], [10]. An overlay of the DNA histograms of the infected and uninfected cell populations at 2 hours post infection (hpi) showed a noticeable difference in the relative distribution of DNA content between infected and uninfected cells (Fig. 2.1A). The relative distribution of the ploidy differed dramatically between infected and uninfected cells (Fig. 2.1B). We found the percentage of cells with 2n ploidy was significantly lower in the infected cell population than the percentage of 2n cells in the uninfected cell population (Fig. 2.1C, $p=0.002$). In contrast, the percentage of 4n cells was significantly higher in the infected cell population (Fig 2.1D, $p=0.014$). While only a small percentage of cells exist in a >4n state in hepatoma cells,

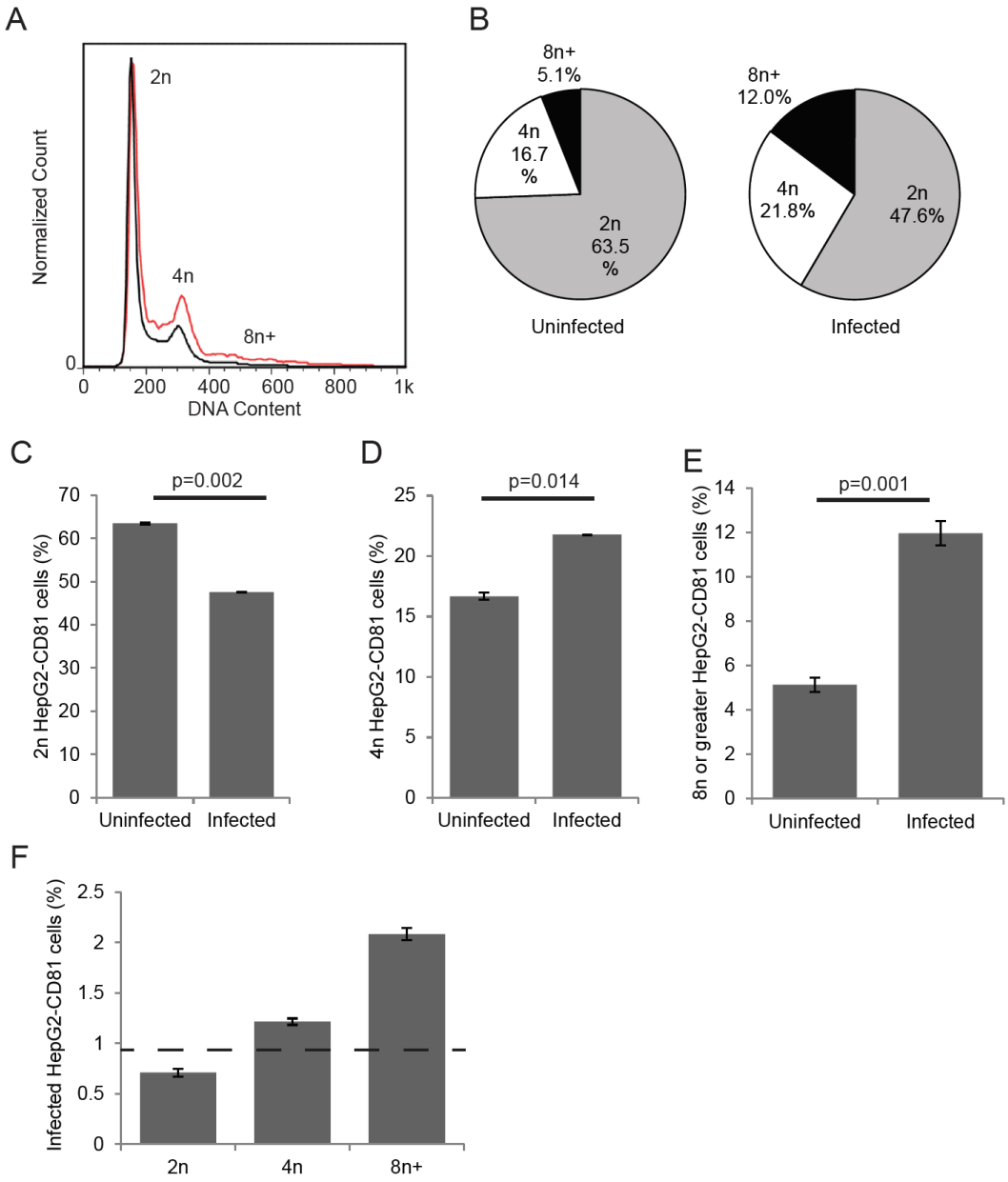

Figure 2.1: Higher ploidy is more prevalent in parasite-infected hepatoma cells *in vitro*

HepG2-CD81 cells were infected with *P. yoelii* sporozoites, harvested at 2 hpi and stained for infection and ploidy. Overlays of histograms of DNA stain for infected (red line) and uninfected (black) show lower peaks for infected 2n cells, and higher peaks for infected 4n and greater cells (A). The relative percentage of cells in each ploidy state greatly differs between uninfected and infected cells (B). A quantitative analysis of the percent of cells in each ploidy state shows significant decrease in cells with 2n chromosomes in infected cell population (C), and a significant increase in cells with 4n (D) or greater (E). Similarly, the rate of infection is lower in 2n cells, and higher in 4n and greater cells (F). Dashed line indicates the overall infection rate. Error bars show S.E.M., biological replicates of n=3.

this population was heavily enriched among infected cells (Fig. 2.1E, p=0.001). In order to clearly delineate between cells of different ploidy, we used a conservative gating strategy that did not count cells with DNA content between the distinct peaks; however, including those cells in a more relaxed gating strategy did not significantly alter the results. When data were reanalyzed by comparing the extent of *Plasmodium* infection within each ploidy subset, we found the rate of infection was lower in 2n cells than the overall infection rate, and was higher in 4n cells and greatly increased in the polyploid cell population (Fig. 2.1F).

We next asked if the observed preference for high ploidy cells was due to parasites that entered their host cell by wounding, or alternatively caused by a preference for cycling cells. To determine if the preference for higher ploidy cells was due to traversing parasites being caught in actively dividing cells, we blocked cell division using the small molecule cell cycle inhibitor nocodazole, which arrested a majority of the cells in G2, as well as the inhibitor L-mimosine, which arrests in G1 (Fig. 2.2A). We found that eliminating cell cycle progression in G2 dramatically increases the rate of infection, while arresting cell cycle before DNA synthesis decreases infection (Fig. 2.2B). Thus, the preference for higher ploidy is not dependent on cell division. Furthermore, when we excluded infected cells that had been entered by cell wounding, we obtained nearly identical results (Fig. 2.2C, D). This suggests that cells that harbor parasites arrested during traversal do not substantially contribute to the observed ploidy distribution in infected cells. Finally, we demonstrated that the shift in ploidy distribution of infected cells is independent of cell proliferation *in vivo*, as actively replicating (Ki-67 positive) cells are no more likely to be infected than quiescent (Ki-67 negative) cells (Fig. 2.2E).

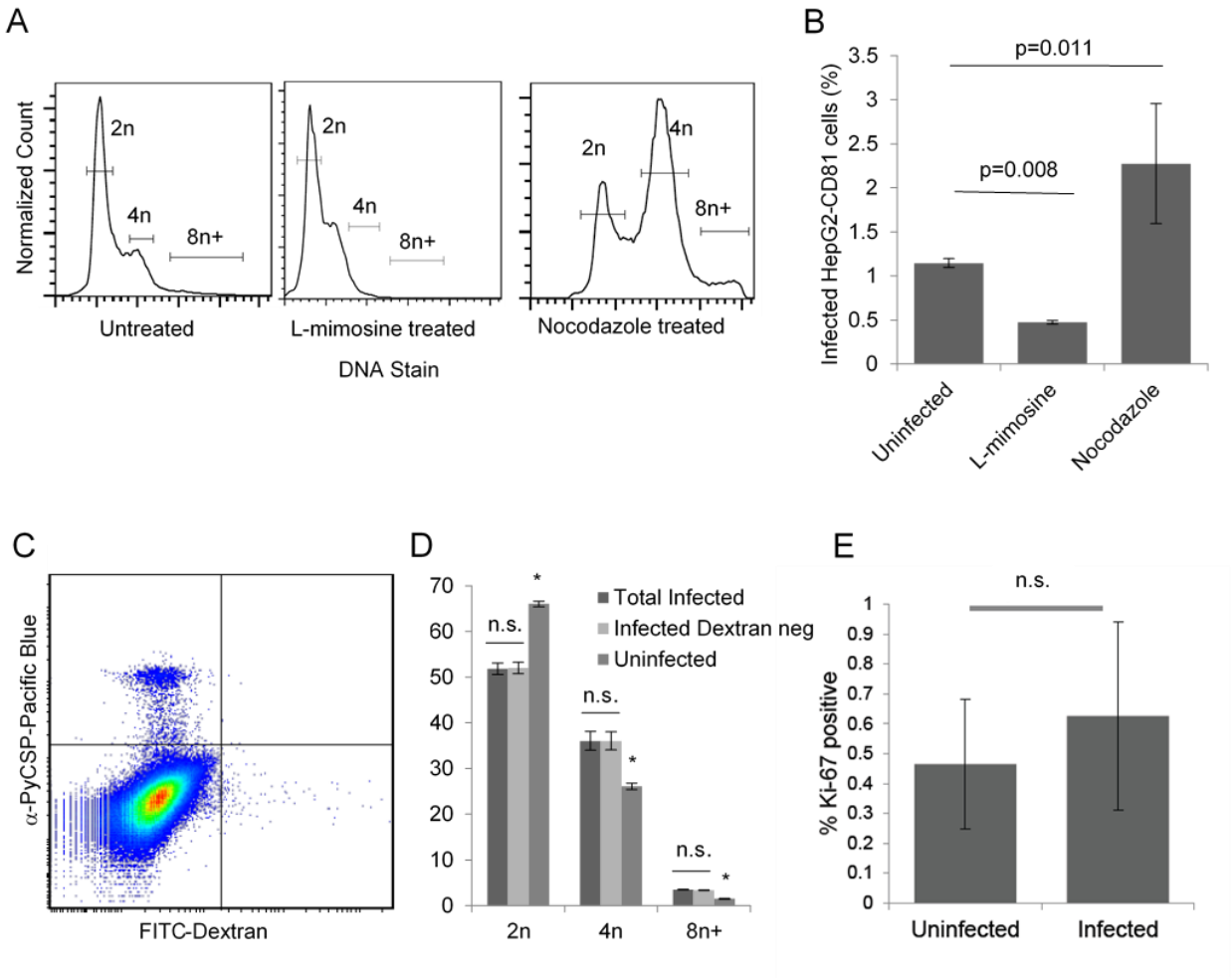

Figure 2.2: Neither traversal nor cell cycling contributes to the sporozoite preference for host cells with high DNA content.

Treatment of HepG2-CD81 cells with the small molecule nocodazole for 24 hours before infection arrested cells in G2 and increased the 4n population (A). Nocodazole treatment of HepG2-CD81 cells increases infection ($p = 0.011$) (B). FITC-dextran was used to distinguish between HepG2-CD81 cells which had been productively invaded (PyCSP high, dextran low) and those in which traversing parasites had arrested (PyCSP high, dextran high) (C). Eliminating traversed cells from the analysis showed that the preference for high ploidy host cells was not affected by sporozoite traversal (D). Cell cycle progression (marked by Ki-67) is on in a similar proportion of infected and uninfected hepatocytes *in vivo* (E).

The differences in the distribution of ploidy were observed only two hours after infection, before liver stage parasites begin to replicate their genomes. Since the amount of parasite-derived DNA at this time is negligible when compared to the DNA of the host cells, this increase in ploidy/DNA content cannot be attributed to parasite DNA (Figure 2.3A-C). Furthermore, the shift in infected cells toward higher ploidy remained constant after 24 hours of parasite development despite substantial parasite replication between 2 and 24 hours, further indicating that the parasite DNA is not falsely presenting as a higher ploidy state of the infected cell (Fig. 2.3A, D). Taken together, these data suggest that hepatoma cells with higher ploidy are more likely to be infected with *P. yoelii* than those with lower ploidy.

Plasmodium prefers infection of high ploidy cells in mice

Hepatoma cells rapidly replicate, and most 4n cells are in the G2 stage of the cell cycle. In G2, a number of cellular processes have different activity levels than in the non-replicating hepatocytes. This makes it difficult to uncouple ploidy with changes associated with cell cycle progression. In the healthy adult liver, primary hepatocytes are generally quiescent, with only a fraction of hepatocytes actively going through the cell cycle [11]. To determine the role of polyploidy in primary hepatocytes, we investigated the ploidy distribution of infected and uninfected hepatocytes in mice. We injected seven-week-old female BALB/c mice with one million *P. yoelii* sporozoites intravenously, and analyzed hepatocytes prepared by collagen-mediated perfusion at three or 24 hpi, assessing the infection rates and ploidy using flow cytometry. As *in vitro*, we found that parasite-infected cells had an altered distribution of ploidy (Fig. 2.4A). At three hours post infection, diploid hepatocytes, which comprised 11% of uninfected hepatocytes, were only 6% of infected hepatocytes (Fig. 2.4B). Tetraploid

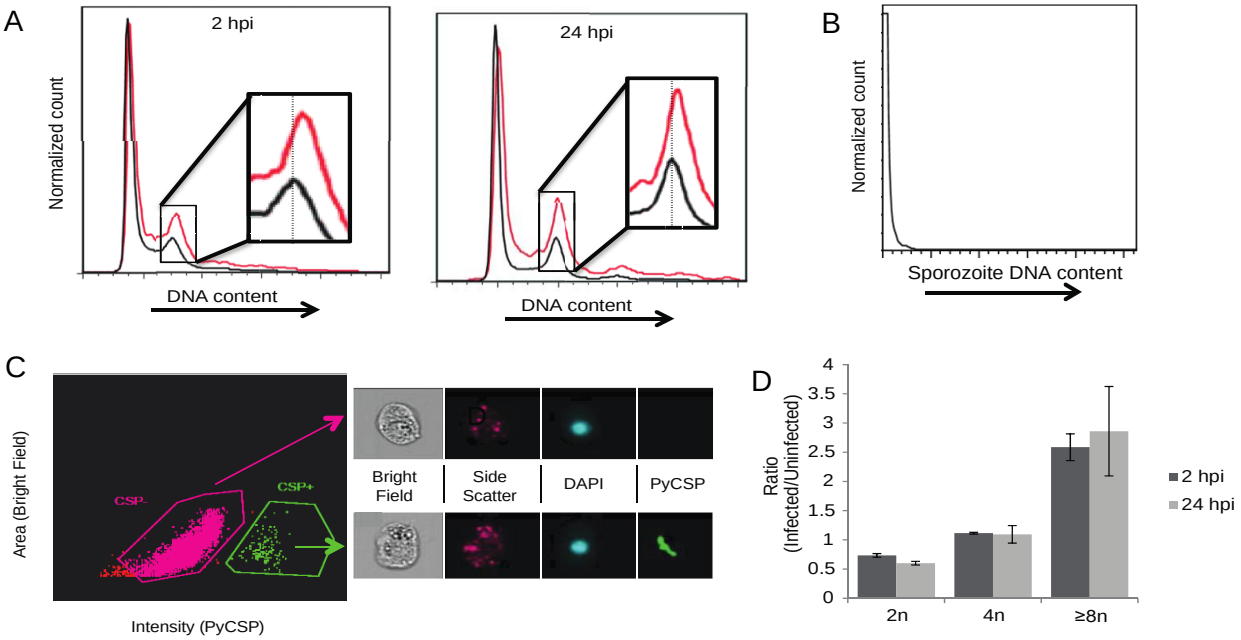

Figure 2.3: Parasite DNA does not contribute to ploidy count

Overlay of DNA histograms of infected (red) and uninfected (black) cells show DNA peaks at approximately the same fluorescence intensity. If parasite DNA contributed substantially, infected peaks would be shifted strongly to the right (A). Sporozoites have equal DNA content to 2 hpi and 3 hpi liver stage parasites. Using the same detecting voltage as used to detect HepG2-CD81 DNA, no parasite DNA can be seen in analysis (B). Imagestream microscopy shows that at 3 hpi, parasite DNA is not visible at an exposure at which the hepatocyte nucleus fluoresces strongly (C). The percentage of HepG2-CD81 cells in each ploidy state is shown as a ratio between infected and uninfected cells. The fold change in ploidy distribution in infected cells remains constant between 2 hpi and 24 hpi. Error bars show propagated errors of the ratios (D).

hepatocytes significantly diminished in the infected population to 49% from 62% of uninfected hepatocytes (Fig. 2.4C). Conversely, the 8n population increased from 23% in uninfected hepatocytes to 36% of infected hepatocytes (Fig. 2.4D), and the population with 16n or greater jumped from 2.5% of the uninfected hepatocyte population to 6.7% of the infected population (Fig. 2.4E). Parasite infection rates correlated positively with ploidy level; in fact the infection rate within hepatocytes 16n or greater was nearly 400% the overall infection rate (Fig. 2.4F). The results were similar at 24 hpi when the genome of the parasite had begun to replicate (see

Fig. 2.3C). Thus, similar to the *in vitro* data, higher ploidy hepatocytes are more susceptible to infection *in vivo*. Since this increased susceptibility is again seen very soon after infection, it is clear that the parasite preference for hepatocytes with elevated ploidy levels is established at the point of infection and is not modified throughout parasite liver stage development.

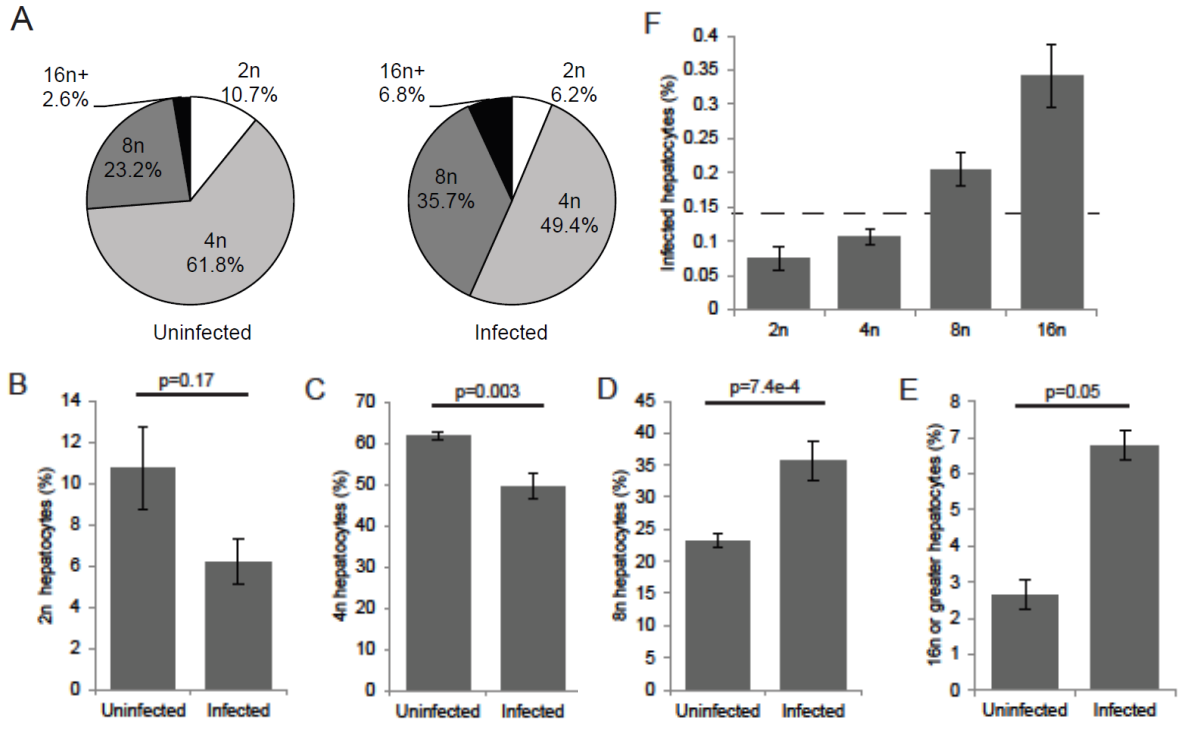

Figure 2.4: Higher ploidy is more prevalent in parasite-infected hepatocytes *in vivo*

Balb/cAnN mice were infected intravenously with 10^6 *P. yoelii* sporozoites. Hepatocytes were isolated at 3 hpi and stained for parasite protein and DNA, then analyzed by flow cytometry. Infected and uninfected cells show greatly different ploidy distributions (A). The percentage of cells with 2n (B) and 4n ploidy (C) is lower in infected populations, while 8n (D) and 16n or higher ploidy cells (E) are significantly higher in infected populations. The rate of *Plasmodium* infection positively correlates with ploidy levels (F). Dashed line indicates the overall infection rate. Error bars show S.E.M., biological replicates of n=3.

Preference for polyploid host cells is conserved across *Plasmodium* species

Plasmodium species are genetically and phenotypically diverse and this diversity extends to their dependence on particular host factors for infection. It has been previously demonstrated that sporozoites of different *Plasmodium* species differ in their preference for host cell invasion factors. For example, *P. yoelii* and *P. falciparum* sporozoites both require hepatocyte CD81 expression for infection but *P. berghei* does not [12]. To address whether sporozoite preference for hepatocytes with higher ploidy is restricted to *P. yoelii*, we infected HC04 hepatoma cells [8] with *P. berghei* sporozoites, and analyzed ploidy levels at 2 hours post-infection. Like HepG2-CD81 cells, HC04 cells are primarily diploid, but are actively cycling, creating a robust 4n population. When we analyzed the percentage of cells with 2n and 4n ploidy in infected versus uninfected cell populations, we found that like *P. yoelii*, *P. berghei* infected cells had a decreased percentage of 2n and an increased percentage of 4n cells compared to the uninfected cells (Figs. 2.5A, B). To further explore the breadth of our findings in the context of human malaria, we infected HC04 cells with the human parasite *P. falciparum* and found a similar ploidy distribution (Figs. 2.5C, D). Thus, the difference in infection between 2n versus 4n cells remained consistent between *Plasmodium* species (Fig. 2.5E), indicating that the mechanism of preferential infection of host cells with higher ploidy is well conserved across parasite species. This is particularly important given that most known host factors involved in parasite infection are not conserved across species [12]–[14] and that the susceptibility of different cell types to infection varies between *Plasmodium* species [15].

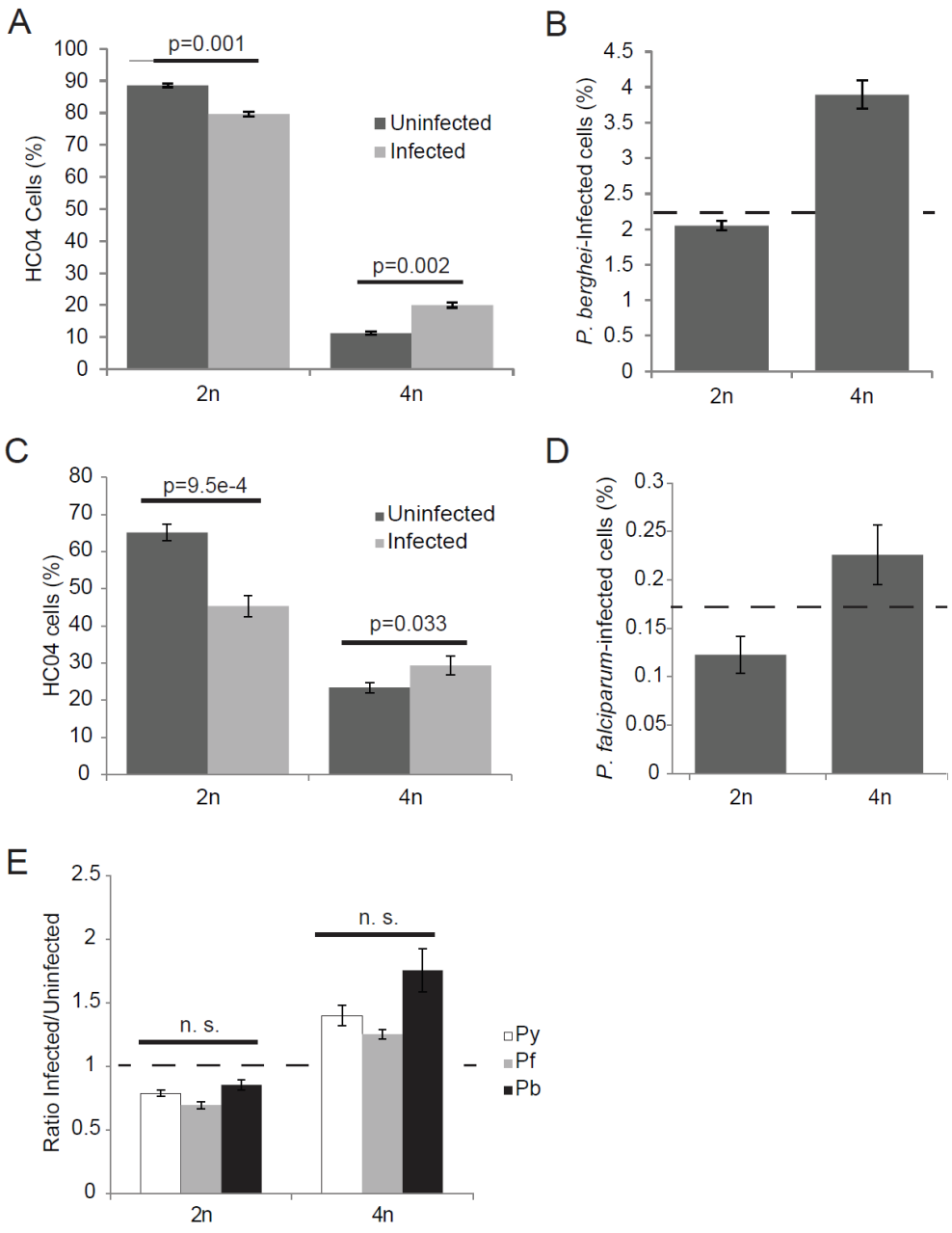

Figure 2.5: Host cell ploidy preference is conserved among *Plasmodium* species

HC04 cells infected with the rodent parasite *P. berghei* have a decreased percentage 2n cells and an increased percentage 4n cells at 2 hpi (A). The infection rate with *P. berghei* increases correlating to ploidy (B). A similar pattern of decrease in 2n cells in infected population holds true for the human parasite *P. falciparum* (C). Similarly, 4n cells had an increased rate of infection (D). The amount of preference away from 2n cells holds very steady in all three parasites. No significant difference in the ratio of infected to uninfected cells is found between species (E). Dashed lines in (B) and (D) indicate overall infection rate.

Nuclearity and zonation do not influence ploidy preference

Polyploid hepatocytes can be mononuclear (e.g. one tetraploid nucleus which is 4n) or binuclear (e.g. two diploid nuclei, each 2n). Thus, we asked if the number of hepatocyte nuclei dictated the observed differences in infection. It is difficult, however, to simultaneously measure DNA content and nuclearity in low-frequency events such as *Plasmodium* hepatocyte infection. We therefore turned to Imagestream technology [16], which combines flow cytometry with fluorescence and light microscopy. By measuring the aspect ratio of the DNA signal—that is, the ratio between the width of the signal and its length—we were able to distinguish mono- and binuclear cell populations (Fig. 2.6A). We then stratified cells based on ploidy to correct for the increased binuclearity found in cells of higher DNA content, and compared the populations of mononuclear and binuclear cells in infected and uninfected cells in each ploidy group. 2n cells were not analyzed because they are exclusively mononuclear, and 16n cells and above were excluded due to low cell number. When we analyzed the nuclearity of cells of 4n or 8n ploidy, we found that within populations of similar ploidy, no difference in nuclearity existed between infected and uninfected cells (Fig. 2.6B).

In addition to their ploidy, hepatocytes in the liver can be classified based on lobule zonation, depending on whether they are closer to the portal vein (periportal), the central vein (perivenous), or distal to both. Some studies have suggested that polyploidy is differentially distributed within these zones [17], [18] while other reports suggest there are similar distributions of binuclear and tetraploid hepatocytes between zones [19]. To address whether hepatocyte zonation might contribute to the increased *Plasmodium* infection of polyploid hepatocytes, we analyzed tissue sections of infected mouse livers for liver stage parasite distribution in addition to hepatocyte nuclearity. While polyploidy per se is difficult to

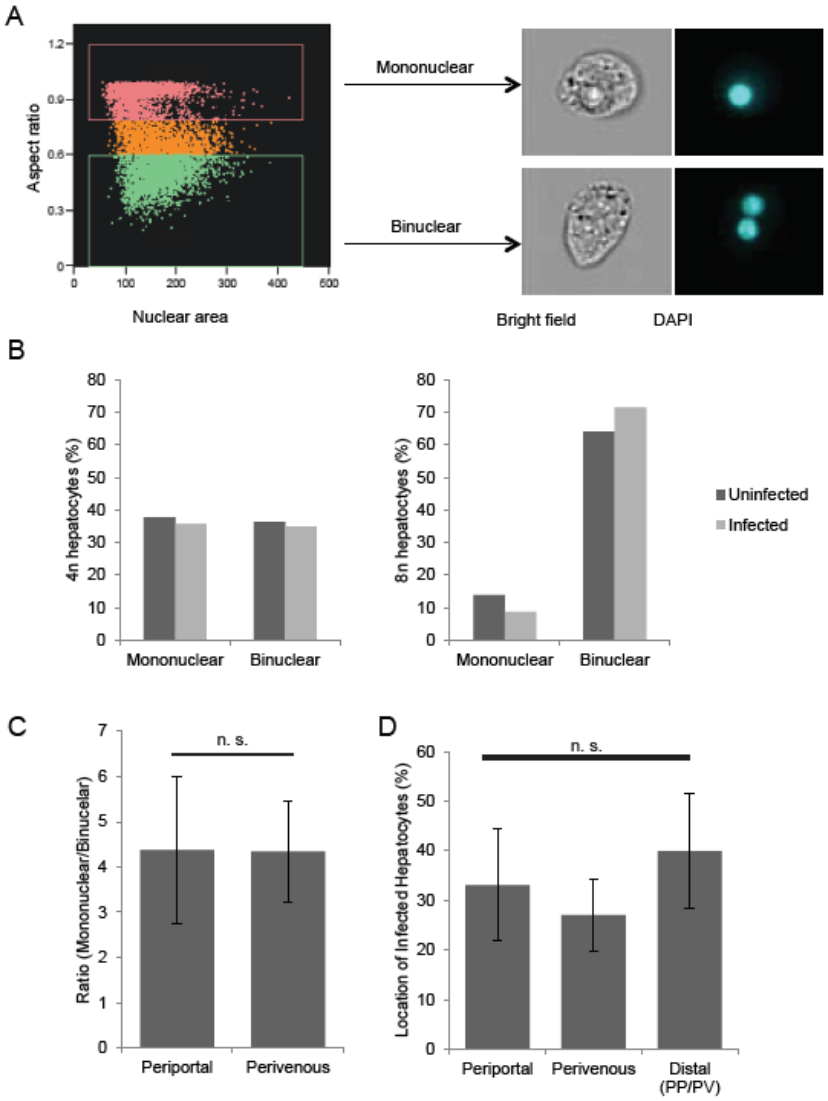

Figure 2.6: Nuclearity and zonation do not influence host cell ploidy preference of parasites

Using Imagestream flow cytometry, infected and uninfected mouse hepatocytes were analyzed by aspect ratio to determine the number of nuclei. Cells were gated into subsets of 2n, 4n, 8n, and 16n or greater. Cells within the 4n and 8n ploidy subsets were analyzed for nuclear number. Nuclearity was confirmed via representative cell images taken from each gate (A). In both 4n and 8n ploidy subsets, the distribution of mononuclear and binuclear cells did not differ between infected and uninfected cells (B).

Nuclearity and infection were also analyzed by liver zonation in H&E stained liver slices. Mouse livers show an equal ratio of mononuclear hepatocytes to binuclear hepatocytes between periportal (PP) and perivenous (PV) regions (C). Infected hepatocytes are likewise evenly distributed between periportal, perivenous, and distal (neither PP nor PV) zones of the liver (D).

determine purely by microscopy, binuclearity is increased with increased ploidy and so was used as a marker for polyploid hepatocytes. We found no significant difference in the ratio of mononuclear to binuclear cells between periportal and perivenous zones of the mouse liver (Fig. 2.6C), in agreement with some previous reports [19]. Moreover, *Plasmodium* parasites showed no preference for infection of hepatocytes in either zone (Fig. 2.6D). Therefore, we conclude that zonation of the liver does not impact the elevated infection rates of polyploid hepatocytes.

Cell size does not account for increased infection of polyploid hepatocytes

Size is also a possible factor in the susceptibility of polyploid cells as increased ploidy positively correlates with cell volume. If cell size fully explained the preference for infection of polyploid cells, it would suggest that the parasite is more likely to interact with large cells due to their increased surface area rather than a specific molecular property of these cells. To assess cell size, we first used the forward scatter measurement of flow cytometry. As expected [20], higher ploidy was associated with larger cell size (Fig. 2.7A). We then stratified the cells by ploidy subset and analyzed the average size of cells within each subset. Cell size was measured by Imagestream microscopy using the average pixel area of each cell image. We reasoned that if increased parasite infection were due primarily to larger host cell size, then within each ploidy level parasites would preferentially invade the larger cells within that subgroup. We found that this was not the case; when stratified by ploidy, infected cells and uninfected cells did not significantly differ in size (Fig. 2.7B). Moreover, when cells were stratified by size using forward scatter, the infected populations still demonstrated altered ploidy distributions with a preference for more polyploid cells (Fig. 2.7C-F). To further assess whether or not the observed

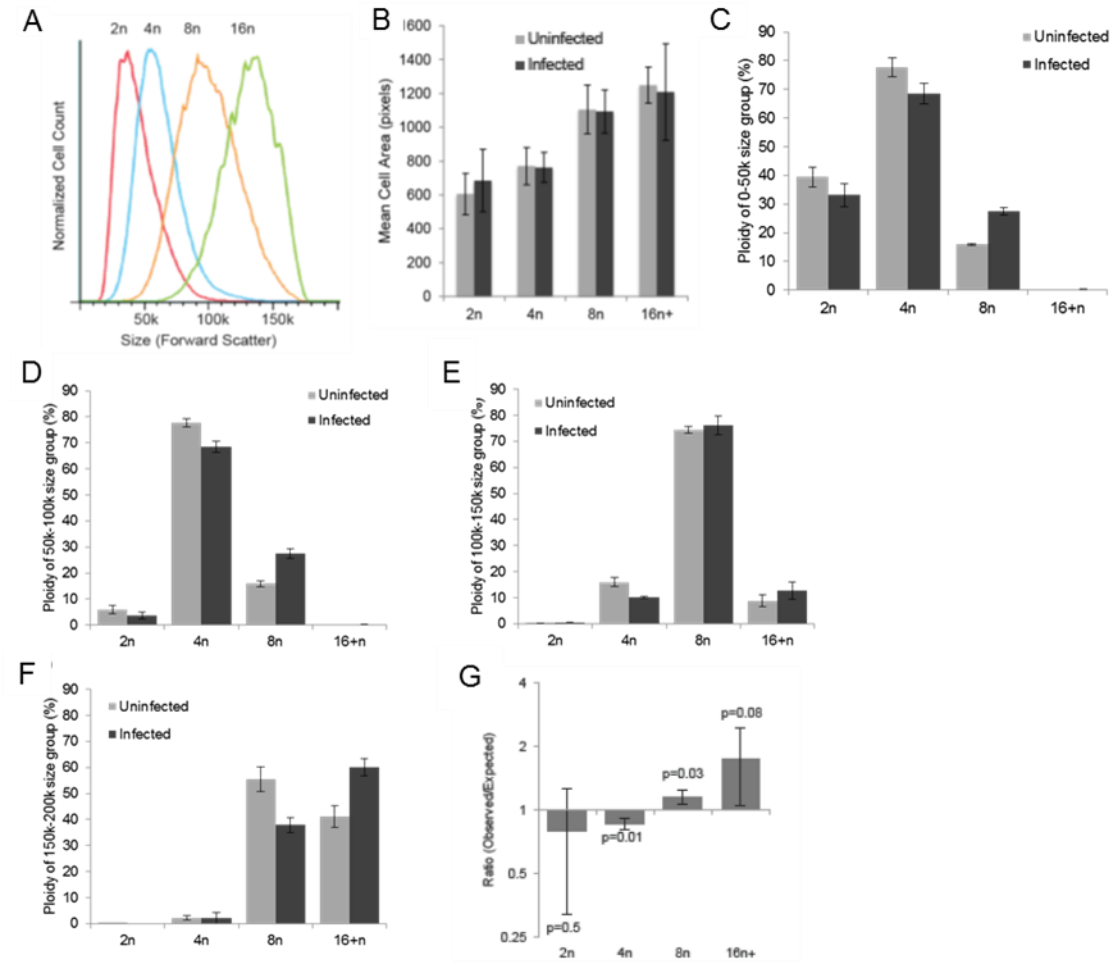

Figure 2.7: Preferential parasite infection of higher ploidy cells is not dependent on their increased size

The size of hepatocytes of different ploidy from infected mice was analyzed using forward scatter during flow cytometry. Cell size increases with increased polyploidy (A). Using Imagestream analysis, cells were gated by ploidy, and the pixel size of infected and uninfected cell images were measured within each ploidy subset. When stratified by ploidy level no difference was found in the average sizes of infected and uninfected cells (B).

Likewise, hepatocytes were stratified by size based on Forward Scatter intensity, then analysed for ploidy distribution of uninfected and infected populations. Cells with a Forward Scatter Intensity of 0–50k (C), 50–100k (D), 100–150k (E), and 150–200k (F) all show the infected population has a smaller percentage of low ploidy hepatocytes, and a larger percentage of high ploidy hepatocytes, than the uninfected population.

Expected rates for hepatocyte infection were calculated based on surface area of each ploidy subset as described in equation (2) and compared to observed infection rates. The observed infection rates were lower than expected rates for 2n and 4n cells, and higher in 8n and 16n and greater cells (G). This shows that preferential infection of polyploid cells is not dependent on the relative surface area of each population.

preference for high ploidy populations is due to increased surface area of polyploidy cells, we calculated the relative percentage of cell surface area in the liver represented by each ploidy subset (see Methods). We then used those percentages to predict invasion rates in each subset if infection was determined stochastically based on exposed surface area. We found that the observed values of infected 2n and 4n cells were less than the expected values, and 8n, 16n and higher polyploid cells were higher than expected (Fig. 2.7G). These data demonstrate that sporozoites preferentially infect hepatocytes with higher ploidy beyond what would be expected based on surface area exposed alone. Thus, the increased susceptibility of polyploid cells cannot be explained simply as a function of cell size.

Surface density of *Plasmodium* infection proteins is increased in polyploid hepatocytes

Few host proteins have been shown to play a role in parasite infection of hepatocytes. To date, only three specific host proteins have been demonstrated to be important in *P. yoelii* infection. CD81, a tetraspannin [12], and Scavenger Receptor B1 (SR-B1) [21], a lipid transporter, have been shown to enhance infection, possibly due to an increase in membrane cholesterol. Recently, the receptor tyrosine kinase EphA2 has been identified as an important factor of infection, possibly directly interacting with a parasite protein [22].

To investigate whether these three proteins may contribute to the increased susceptibility of highly polyploid cells, we stained isolated mouse hepatocytes with antibodies against each protein and analyzed the median fluorescence of each ploidy subset, adjusted for relative cell surface area. We found that all three receptors showed significantly higher concentration in highly polyploid cells (Fig. 8A-C). In contrast, an unrelated hepatocyte surface receptor, TrkB, showed no relative increase in polyploid cells after adjusting for size (Fig. 8D). Thus, we reason

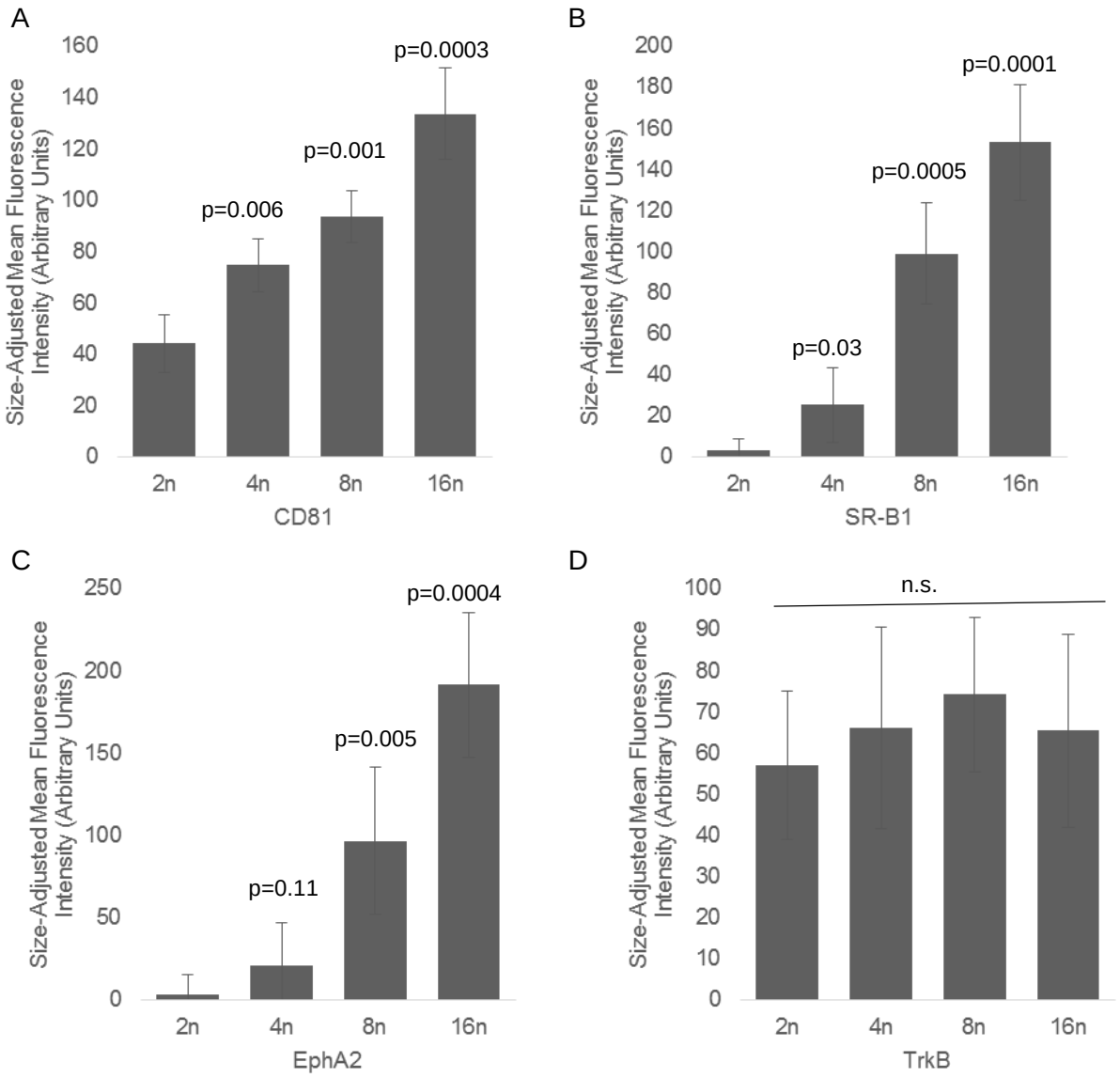

Figure 2.8: Surface density of hepatocyte proteins known to impact *Plasmodium yoelii* infection increases with ploidy

Expression of three surface proteins known to increase infection with *P. yoelii* was analyzed by flow cytometry. CD81 (A), SR-B1 (B), and EphA2 (C) all show increased expression when normalized by size, thus showing an increased protein density on the cellular membrane. An unrelated surface protein, TrkB (D) does not show increased density. P-values are compared to adjusted MFI of 2n in all cases.

that susceptibility of highly polyploid cells is at least partially driven by increased surface protein density.

DISCUSSION

Here, we demonstrate that polyploidy of hepatocytes plays a substantial role in the host cell preference of *Plasmodium* parasites. Since the parasites' selection of hepatocytes with higher ploidy can be seen almost immediately after infection, it is likely that this preference is due to an intrinsic hepatocyte factor that plays a role during the early stages of infection, or the process of invasion itself. We have shown that levels of three known protein factors of infection—CD81, SR-B1, and EphA2—are increased relative to cell surface area in highly polyploid cells. However, it is unlikely that these three proteins are the extent of host receptors used by the parasite to infect hepatocytes. Other cellular surface factors may be playing a role in increased susceptibility to *Plasmodium*. For example, polyploid hepatocytes have been shown to have higher levels of the adhesion molecule ICAM-1 [23], although this is unlikely to a factor in hepatocyte infection as mice with deletions of ICAM-1 are able to support *P. yoelii* liver stage infection [24]. Another possibility is that the preference for high ploidy is due to receptor organization, rather than quantity. CD81 acts as a scaffolding protein for organization of lipid microdomains, which can contain a number of different membrane receptors [25]. The increased density of CD81 on the surface of polyploid hepatocytes may change organization of these microdomains, thus increasing local density of surface receptors that might favor *Plasmodium* infection.

We have previously demonstrated that *Plasmodium* liver stages perturb signaling pathways in their host hepatocytes, including those pathways involved in cell proliferation and replication [4]. However, this analysis was not designed to distinguish between hepatocyte

factors that the parasite selects for during its invasion process and factors that the parasite actively engages and changes throughout infection. Interestingly, several cell proliferation proteins, including p53 [26] and cell cycle transcription factors of the E2F family [27], [28] have been implicated in the generation of polyploidy in hepatocytes. p53 in particular might be playing a role in the relationship between polyploidy and parasite infection. We have previously shown that infected hepatocytes exhibit reduced levels of p53. In turn, elevated p53 levels greatly reduce hepatocyte infection. In contrast, mice with a p53 knockout genotype were significantly more susceptible to liver stage infection [4]. In its role as a cell cycle regulator p53 acts as a transcription factor controlling many factors involved in the cell cycle and polyploidization of hepatocytes. Strikingly, *p53-/-* mice exhibit lower levels of diploid and tetraploid hepatocytes [26]. Thus, it will be of interest to examine the relationship between parasite infection levels, host cell p53 levels and host cell ploidy.

Polyploidy is a ubiquitous feature of mammalian hepatocytes. While the development of hepatocyte polyploidy has been extensively characterized [29]–[31], the functional consequences of polyploidy are still largely unknown. Only a few transcriptional differences have been seen between cells of differing ploidy [32], although this line of inquiry has been sparse. Different species have variable hepatocyte ploidy distributions within a narrow range in healthy adults. While these percentages change over the lifespan of an individual, the distribution of hepatocyte ploidy among healthy animals of the same age are comparatively uniform [5]. It has been described that hepatocytes exist within a "ploidy conveyor", meaning that ploidy is not entirely fixed: diploid cells can become polyploid, or vice versa [33]. Additionally, polyploidy can be altered in response to liver injury via partial hepatectomy [34], oxidative [35] or chemical [36] stress. Interestingly, increased polyploidy due to partial hepatectomy returns to its standard

levels within weeks after the injury [34], suggesting that there is a mechanism of polyploidy homeostasis in the mammalian liver. The conservation of hepatocyte polyploidy both within and among species strongly implies that this phenomenon is of critical importance in liver function.

Hepatocytes are constantly subjected to the stress of toxins and oxidation involved in liver metabolism, and polyploidy might play a role in protection against DNA damage or other environmental stresses [31], [37]. If so, this could be an attractive host cell feature for the intracellular parasite because it depends on survival of its host cell until completion of development and egress. Apoptosis of infected cells prevents parasite development, and *Plasmodium* has developed ways to block host cell apoptosis. Infected hepatocytes are partially resistant to induced apoptosis [38], and several pro-apoptotic pathways are disregulated in infected hepatocytes [4], [39], [40]. However, this protection against apoptosis is incomplete, and a proportion of parasite infected cells still undergo apoptosis [40]. The persistence of parasites within the liver, then, is proposed to depend heavily on the ability of the parasite to suppress host cell stress responses. The preferential infection of polyploid cells inherently more resistant to stress might thus be an important selection mechanism for that increases the likelihood of successful intracellular development.

The skewing of *Plasmodium* infection towards polyploid cells suggests that these cells display an altered molecular state that increases sporozoite infection. The phenomenon is highly consistent throughout experimental cell lines, mouse models, and parasite species. This preference, however, is not complete as parasites are able to infect cells of low ploidy at high numbers. Most of the infected hepatocytes are only 4n cells, which comprise the majority of total mouse hepatocytes. These data suggest that the factor that mediates elevated infection in polyploid hepatocytes is present at some level in hepatocytes of lower ploidy. Thus, further

investigation into quantitative differences between hepatocytes of low and high ploidy might elucidate novel factors that mediate sporozoite invasion or early development.

Both the functional relevance of hepatocyte polyploidy and the specific mechanisms of *Plasmodium* infection are still poorly understood. In a healthy liver, only hepatocytes display significant polyploidy, while other cell types are diploid. Likewise, hepatocytes are the only liver cell type that *Plasmodium* sporozoites will productively infect. While several host factors for infection have been identified, none of them are required for all species of *Plasmodium* [6], [14]. However, the preference for infection of polyploid cells is conserved in three *Plasmodium* species we have tested to date, pointing toward a conserved host factor or factors for parasite infection that are differentially expressed in highly polyploid cells. By inspecting the molecular differences found in different states of host cell ploidy, we might identify new host factors important to malaria parasite liver infection. While modulation of hepatocyte polyploidy by viral hepatitis is known, to our knowledge this investigation is the first description of polyploidy playing an important role in the initiation of infection with a eukaryotic parasite. A greater understanding of the role of hepatocyte ploidy in malaria parasite liver infection might provide novel mechanistic insights into the processes of host cell selection and susceptibility to infection and might also provide insights into the physiological relevance of hepatocyte ploidy in the liver.

MATERIALS AND METHODS

Cell lines, cell culture, and experimental animals

In vitro, HepG2-CD81 cells were used for *P. yoelii* infections, and HC04 cells for *P. falciparum* and *P. berghei*. Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM)

complete media (Cellgro), supplemented with 10% FBS (Sigma-Aldrich), 100IU/ml penicillin (Cellgro), 100mg/ml streptomycin (Cellgro) and 2.5mg/ml Fungizone (HyClone/Thermo Fisher), and split 1–2 times weekly. Female BALB/cAnN mice (6-8 weeks old) were purchased from Harlan Laboratories and maintained in accordance with protocols approved by Center for Infectious Disease Research Institutional Animal Control and Use Committee (IACUC).

Mosquito rearing and sporozoite production

For *Plasmodium* sporozoite production, female 6–8-week-old Swiss Webster mice (Harlan) were injected with blood stage *P. yoelii* (17XNL), *P. berghei* (ANKA), or *P. falciparum* (NF54) parasites to begin the growth cycle. Animal handling was conducted according to the Institutional Animal Care and Use Committee-approved protocols. We used infected mice to feed female *Anopheles stephensi* mosquitoes after gametocyte exflagellation was observed. We isolated salivary gland sporozoites according to the standard procedures at days 14 or 15 post blood meal for *P. yoelii*, day 20 for *P. berghei*, and days 14-19 for *P. falciparum*.

***In vitro* cell ploidy assay**

$2x10^6$ HepG2-CD81 cells were seeded into each well of a 6-well plate and allowed to adhere overnight in DMEM. About 24 hours after plating, cells were infected with $1.5x10^6$ *P. yoelii* sporozoites. One hour post infection (hpi), extracellular parasites were washed off cells and media was replaced. Cells were harvested with 0.25%Trypsin-EDTA at 2 hpi and 24 hpi, and fixed with Cytoperm/Cytofix (BD Biosciences). The cells were blocked with Perm/Wash (BD Biosciences)+2% BSA overnight at 4 C, then stained for one hour at room temperature with antibodies to *P. yoelii* Circumsporozoite protein (PyCSP) conjugated to Alexa Fluor 488 (Life Technologies). The cells were washed in PBS+5 mM EDTA, then resuspended in PBS+5 mM

EDTA+ RNAse A (0.1 mg/mL)+ FxCycle Far Red DNA Dye (Invitrogen). Infection rate and DNA content were measured by flow cytometry on an LSRII (Becton-Dickson) and analyzed by FlowJo (Tree Star).

Traversal analysis of infected cell cultures

$5x10^5$ HepG2-CD81 cells were seeded into each well of a 12-well plate and allowed to adhere overnight in DMEM. 24 hours after plating, cells were infected with $1x10^5$ *P. yoelii* sporozoites in the presence of 1mg/mL FITC-Dextran (Invitrogen). Cells were harvested 2 hpi and analyzed as described above except parasites were stained with PyCSP antibody conjugated to Pacific Blue (Life Technologies).

Cell cycle inhibition of cell cultures

$2.5x10^5$ HepG2-CD81 cells were seeded into each well of a 24-well plate and allowed to adhere overnight in DMEM. About 24 hours after plating, cells were treated with either 400 µM L-mimosine (Sigma) or 100 ng/mL nocodazole (Sigma). 24 hours after treatment, cells were washed for one hour with DMEM, then infected with $5x10^4$ *P. yoelii* sporozoites per well. Cells were harvested 2 hpi and analyzed as described above.

***P. falciparum* and *P. berghei* cell ploidy assay**

$8x10^5$ HC04 cells were seeded into a 12-well plate and infected with $2x10^5$ *P. falciparum* or $4x10^5$ *P. berghei* sporozoites. Cells were harvested 2 hpi and fixed as above, and stained with antibody and FxCycle Far Red DNA dye (Invitrogen). *P. falciparum*-infected cells were stained with anti-PfCSP conjugated with Alexa Fluor 488, and *P. berghei*-infected cells were stained with unconjugated mouse antibodies to PbCSP, and a secondary anti-mouse conjugated to Alexa Fluor 488.

Hepatocyte isolation and analysis

Female BALB/cAnN mice were inoculated with 10^6 *P. yoelii* sporozoites each via tail vein injection. 3 hours and 24 hours post infection, the livers were perfused *in situ* with HBSS supplemented with HEPES and 5 mM EDTA. When the livers blanched, the solution was changed to HBSS with HEPES and 5 mM $CaCl_2$ and the livers perfused until dissociation. The livers were removed, and hepatocytes dissociated by pushing through a 100 µm cell strainer into DMEM. The liver cell suspension was centrifuged at 50x *g*, and the remaining pellet washed in DMEM. This step was repeated twice until the supernatant was clear, to isolate hepatocytes from other parenchymal cells. The hepatocytes were fixed, stained, and analyzed by flow cytometry as above.

Imagestream imaging

One mouse was injected with 10^7 *P. yoelii* sporozoites, and the hepatocytes were isolated at 3 hours post infection and processed as described above. The cells were stained with anti-PyCSP conjugated with Alexa Fluor 488 overnight. The nuclei were then stained with DAPI prior to analysis with an Imagestream Mark II Imaging Flow Cytometer (Amnis-EMD Millipore Corporation). The sample was split into multiple aliquots, and a minimum of 30,000 events per aliquot was collected with 10 mW 488 nm and 10 mW 405 nm laser powers. During data acquisition, cell images of Bright Field, Side Scatter, Alexa Fluor 488, and DAPI were simultaneously collected in different detection channels. During the data analysis with IDEAS software, single cells of best focus were first separated from cell aggregates and debris using several features such Bright Field Area and Aspect Ratio. Then the PyCSP-Alexa Fluor 488 and DAPI intensities were plotted. The DAPI+ CSP+ populations of the data files were identified and merged into one file to maximize the number of CSP+ cells in the file.

Histological analysis of mouse livers

Infected (10^6 *P. yoelii* sporozoites per mouse) BALB/c livers (n=3) were harvested 42 hours post infection, then harvested and fixed in formalin. Livers were embedded in paraffin, sectioned, and mounted on glass slides. Slices were then stained with hematoxylin and eosin. Parasites were visually identified as being periportal, perivenous, or distal to both at 10x power magnification, with a minimum of fifty parasites per mouse quantified. Hepatocyte nuclei were counted at 40x power, with a minimum of 10 fields per zone per mouse counted. Hepatocytes without clearly identifiable nuclei were excluded from the analysis.

Size analysis of infected hepatocytes

The average areas of 2n, 4n, 8n or 16n cells were determined by Imagestream analysis (Amnis Corporation). From this, the average radius in pixels was determined, and the average cell surface area (ASA) calculated using standard geometric formulas. The average surface area of each ploidy was multiplied by the percentage of total cells of that ploidy (e.g. 10.7% of cells were 2n) to get the total surface area (TSA) represented by each ploidy subset (Formula 1). Each TSA was divided by the sum of all ploidy TSAs to get the percentage of TSA represented by each ploidy subset (%SA) (Formula 2). If infection were due to surface area alone, the expected ploidy distribution of infected cells would be identical to the ploidy distribution of %SA. The expected %SA was compared to the observed ploidy distribution as shown in Figure 2. The equation to determine the expected percentage of 2n cells in the infected population was as follows:

(1) $ASA_{2n} * \%cells_{2n} = TSA_{2n}$

(2) $\%SA_{2n} = TSA_{2n} / (TSA_{2n} + TSA_{4n} + TSA_{8n} + TSA_{16n+})$

Cell surface protein analysis

Uninfected BALB/cAnN hepatocytes were isolated and processed as above. Live cells were enriched by 10 minute centrifugation at 50x *g* with no brake through 35% Percoll. Membrane integrity was checked using Trypan Blue exclusion, then cells were fixed on ice in 1% paraformaldehyde. Cells were blocked in PBS+2% BSA, then stained in PBS+2% BSA+primary or conjugated antibodies. Anti-SR-B1 (Abcam) and anti-TrkB (Cell Signal Technologies) staining occurred overnight at 4 C, then cells were washed 3 times and stained with anti-rabbit secondary antibodies conjugated to Alexa Fluor 488 (Invitrogen) at room temperature for 1 hour. Anti-CD81-PE (Beckman Coulter), and anti-EphA2-PE (R&D Systems) staining occurred overnight at 4 C. The cells were washed twice in PBS+5 mM EDTA, then resuspended in PBS+5 mM EDTA+ RNAse A (0.1 mg/mL)+ SYTOX Blue DNA Dye (Invitrogen) and analyzed as above.

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CHAPTER THREE:

SUSCEPTIBILITY TO *PLASMODIUM YOELII* PRE-ERYTHROCYTIC INFECTION IN BALB/C SUBSTRAINS IS DETERMINED AT THE POINT OF HEPATOCYTE INVASION

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OVERVIEW

The previous chapter described differences in susceptibility between hepatocytes within an individual liver. In this chapter, we will discuss differential susceptibility to *Plasmodium* liver infection based on a whole organism. Rodent models of malaria exhibit large differences in the magnitude of liver infection, both between parasite species and between strains of mice. This has been mainly attributed to differences in innate immune responses and parasite infectivity. Here, we report that BALB/cByJ mice are more susceptible to *Plasmodium yoelii* pre-erythrocytic infection than BALB/cJ mice. This difference occurs at the level of early hepatocyte infection, but expression levels of reported host factors that are involved in infection do not correlate with susceptibility. Interestingly, BALB/cByJ hepatocytes are more frequently polyploid; thus, their susceptibility converges on the previously observed preference of sporozoites to infect polyploid hepatocytes. Gene expression analysis demonstrates hepatocyte-specific differences in mRNA abundance for numerous genes between BALB/cByJ and BALB/cJ mice, some of which encode hepatocyte surface molecules. These data suggest that a yet-unknown receptor for sporozoite infection, present at elevated levels on BALB/cByJ hepatocytes and also polyploid hepatocytes, might facilitate *Plasmodium* liver infection.

This chapter was published in *Infection and Immunity*, Volume 83(1): 39-47, January 2015. It is reprinted here without modification except removal of a phrase, "yet neither CD81 nor SR-BI are upregulated on polyploid hepatocytes" which has since been shown to be incorrect, as shown in Figure 2.8.

INTRODUCTION

Malaria is caused by eukaryotic parasites of the genus *Plasmodium*. They have coevolved with their mammalian hosts, significantly shaping both parasite and host genomes. It is the most deadly parasitic infection in the world, with approximately 300 million clinical episodes annually, mainly in the developing world. Initiation of pre-erythrocytic infection commences after parasite transmission by the bite of an infected mosquito, when the sporozoite travels to the liver and invades hepatocytes. During invasion, the parasite surrounds itself with a parasitophorous vacuole membrane (PVM) that protects the intracellular niche and permits liver stage (LS) development. Parasites leave the liver as first-generation red blood cell infectious merozoites and initiate the symptomatic erythrocytic stages of infection by cyclic intraerythrocytic replication and concomitant destruction of erythrocytes [1].

Host factors that mediate malaria parasite erythrocytic infection [2]–[4] have been uncovered in part by examining genetics of susceptible and nonsusceptible populations [5]. For example, African populations are largely protected against *Plasmodium vivax* blood stage infection and also lack Duffy blood group determinants. This has led to the finding that Duffy blood group determinants are critical to allow *P. vivax* merozoite invasion of erythrocytes [6]. However, human populations resistant to pre-erythrocytic *Plasmodium* infection have not been identified. Therefore, mouse strains differing in susceptibility to pre-erythrocytic infection might provide a useful model for the identification of host factors that are important for hepatocyte infection.

Differences in susceptibility to rodent malaria pre-erythrocytic infection have been documented for mice. *Plasmodium berghei* sporozoites are much more infectious to C57BL/6 mice than they are to BALB/cJ mice [7]. Yet BALB/c mice are 2,000 times more susceptible

to *Plasmodium yoelii* sporozoite infection than *P. berghei* infection [8]. While it is difficult to ascertain whether *Plasmodium* species which infect humans exhibit distinct infectivity profiles *in vivo*, hepatocytes from different human donors differ in susceptibility to pre-erythrocytic stage infection *in vitro* [9]. Unfortunately, mechanistic insights into the causes for these differences are lacking.

Candidate-based approaches have revealed several host cell receptors that facilitate sporozoite infection of hepatocytes, such as the tetraspanin CD81 [10], scavenger receptor B1 (SR-BI) [11], [12], and heparin sulfate proteoglycans (HSPGs) [13], [14]. However, none of these receptors are entirely essential for infection *in vivo*, arguing that an unbiased investigation aimed at identifying novel hepatocyte receptors required for sporozoite infection is needed. To begin this process, we took advantage of genetically similar mice with pronounced differences in susceptibility to pre-erythrocytic *P. yoelii* infection.

BALB/c mice are highly susceptible to *P. yoelii* pre-erythrocytic infection [8], but in some cases, hundreds of generations of mice have been bred separately, leading to founder effects within substrains. These breeding arrangements have led to the establishment of BALB/cJ and BALB/cByJ substrains [15]. Here we show that these two substrains exhibit differential susceptibility to pre-erythrocytic *P. yoelii* infection and that this is determined by differences in early hepatocyte infection. None of the described receptors for sporozoite infection are, however, differentially expressed in BALB/cJ and BALB/cByJ hepatocytes. Our data suggest that additional hepatocyte receptors are required for the effective sporozoite infection of hepatocytes.

RESULTS

BALB/cByJ mice display increased susceptibility to *Plasmodium* liver infection

The study of pre-erythrocytic *Plasmodium* infection has been facilitated by the use of suitable mouse models, perhaps the most susceptible of which is the BALB/c mouse for the study of *P. yoelii* [16]. We asked if susceptibilities are similar across BALB/c-derived substrains. BALB/cJ or BALB/cByJ mice were infected with 10^6 *P. yoelii* sporozoites and sacrificed 44 h after infection. LS burden was assessed by histological analysis. We observed that LS density in infected BALB/cByJ mouse livers was approximately five times higher than in BALB/cJ mouse livers (Fig. 3.1A, B). To test if frequencies of infection and/or survival or the LS growth rates were different between BALB/cJ and BALB/cByJ mice, we measured the cell size of LS in sections of infected BALB/cJ and BALB/cByJ mice. We did not observe a significant difference in LS size, indicating that it is frequency of liver infection and/or survival of the parasite in the liver that causes the observed differences in LS burden (Fig. 3.1C).

We further analyzed at what point during pre-erythrocytic infection the difference in susceptibilities arose. We infected BALB/cJ or BALB/cByJ mice with 5 × 104 P. *yoelii* parasites that express a green fluorescent protein (GFP)-luciferase fusion protein under the control of the EF1α promoter [17] and monitored LS burden over the course of liver infection by bioluminescent imaging (Fig. 3.1D). The difference in LS burden remained constant between BALB/cByJ and BALB/cJ mice at 16, 24, and 43 h post infection (Fig. 2A). These data suggest that the increased susceptibility of BALB/cByJ mice is established at a time point between infection and 16 h after infection. Furthermore, the consistent difference in LS burden throughout schizogony (approximately 16 h after infection through the completion of LS

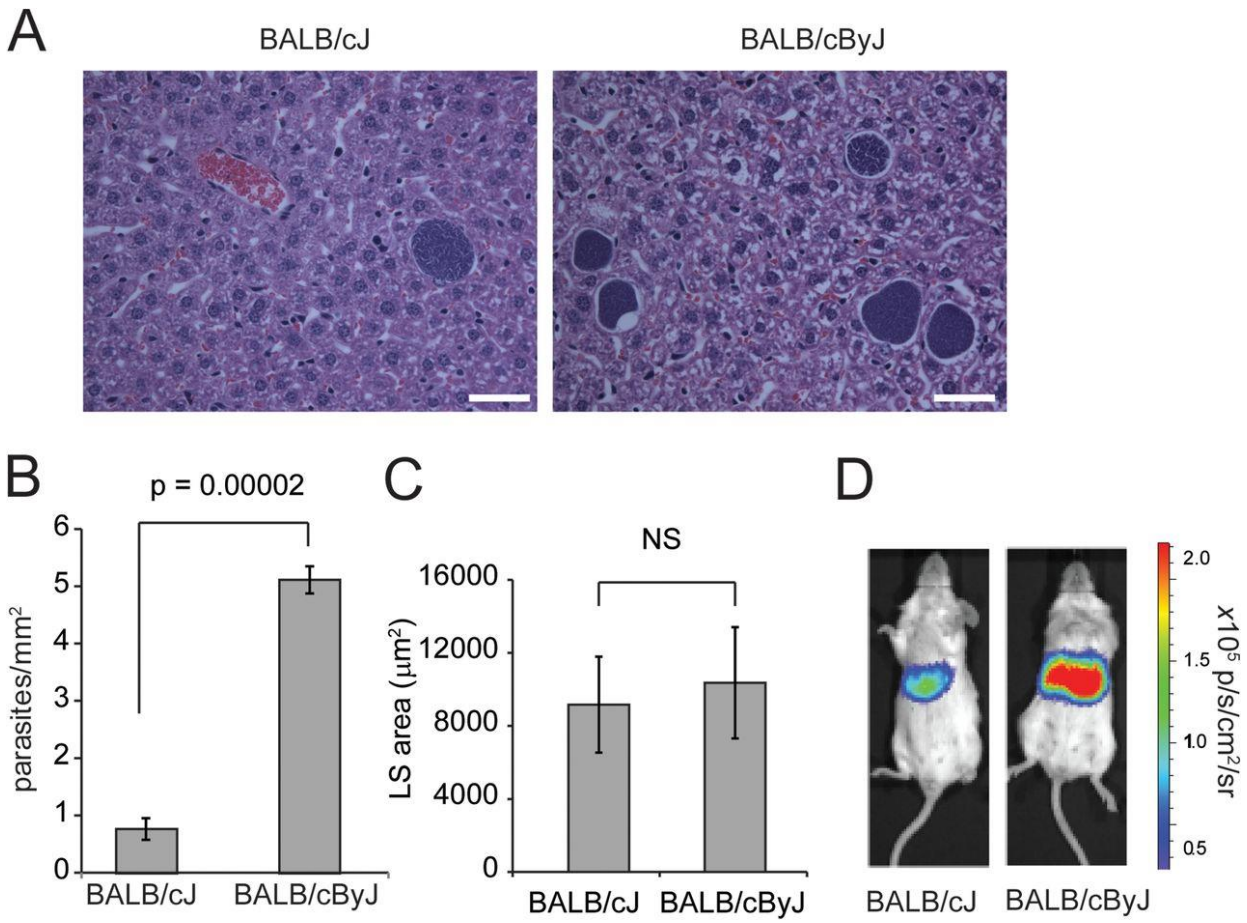

Figure 3.1: BALB/c mouse substrains show increased susceptibility to *P. yoelii* pre-erythrocytic infection

(A) Five mice were infected with 1 M *P. yoelii* sporozoites by intravenous injection. Livers were excised at 44 h post infection. LS parasites were visualized by hematoxylin and eosin (H&E) staining. (B) Density of LSs in tissue quantified by microscopic counting. Data are representative of those from at least three independent experiments. (C) LS size does not vary by strain. Three BALB/cByJ or BALB/cJ mice were infected with 1 M *P. yoelii* sporozoites and stained with H&E as illustrated in panel A. The size of 10 LS parasites was quantified in each sample. (D) LS burden in mice infected with 100,000 *P. yoelii* sporozoites. Parasites expressed GFP-luciferase fusion protein, and burden was quantified by light output using an *in vivo* imagining system (IVIS) 24 h after infection.

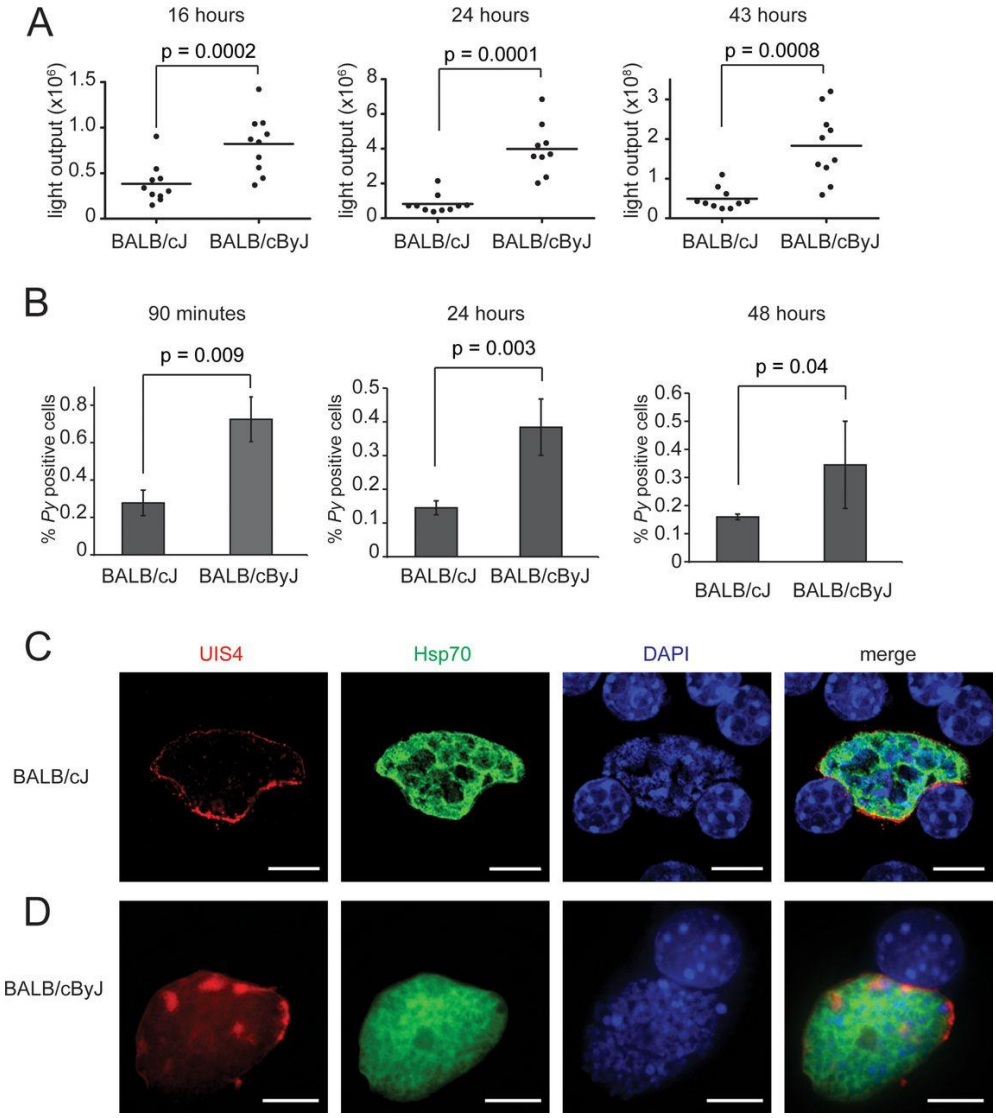

Figure 3.2: Differences in susceptibilities of BALB/c mice to sporozoite infection are largely explained by differences in hepatocyte infection

(A) BALB/cJ or BALB/cByJ mice (n = 10 for each substrain) were injected with 100,000 P. *yoelii* GFP-Luc sporozoites. Total LS burden was quantified by IVIS 16, 24, or 43 h after infection. (B) Primary hepatocytes were isolated from BALB/cByJ or BALB/cJ mice. Plated hepatocytes from three separate animals were infected with 50,000 P. *yoelii* sporozoites in culture. Ninety minutes post infection, hepatocytes already displayed distinct differences in susceptibility to infection, which was maintained throughout *in vitro* LS development. (C and D) LS parasites 48 h after infection do not appear morphologically different in their *in vitro* development between infected BALB/cJ (C) and BALB/cByJ (D) mice. All data are representative of those from three independent experiments.

development) suggests that once infection is established, mice do not differ in their capacity to support further LS development.

Differential susceptibility is established during hepatocyte invasion

Genetic variation of the host could lead to a variant phenotype in multiple cell types, which might impact pre-erythrocytic infection. To evaluate if host hepatocytes are responsible for the difference in susceptibility to infection *in vivo*, we isolated primary hepatocytes from BALB/cByJ and BALB/cJ mice via collagenase-mediated perfusion and infected them *in vitro* with *P. yoelii* sporozoites. We then assessed infection levels using flow cytometry [18]. Since hepatocytes are isolated from nonparenchymal cells by Percoll gradient, this experiment strictly assessed hepatocyte susceptibility to infection without the potential impact of other cell types. We found that BALB/cByJ mouse hepatocytes were dramatically more susceptible to *P. yoelii* pre-erythrocytic infection as early as 90 min after sporozoite infection (Fig. 3.2B). This suggests that differences in hepatocytes are the primary cause of the differential susceptibility to pre-erythrocytic infection observed *in vivo*. Neither rates of LS development nor their size varied between BALB/cJ and BALB/cByJ mouse primary hepatocyte cultures (Fig. 3.2C, D). This supports the notion that the difference in *in vivo* infection arises from differential susceptibility of hepatocytes to initial sporozoite infection.

Both CD81 [10] and scavenger receptor B1 (SR-B1) [11], [12] have been implicated in facilitating sporozoite infection of hepatocytes. Furthermore, p53 levels are suppressed in infected hepatocytes, and boosting p53 levels reduced pre-erythrocytic infection [19]. We asked if differences in BALB/cByJ and BALB/cJ mouse hepatocyte susceptibility to infection would be explained by differences in the basal levels of one of these factors. Expression of CD81 on

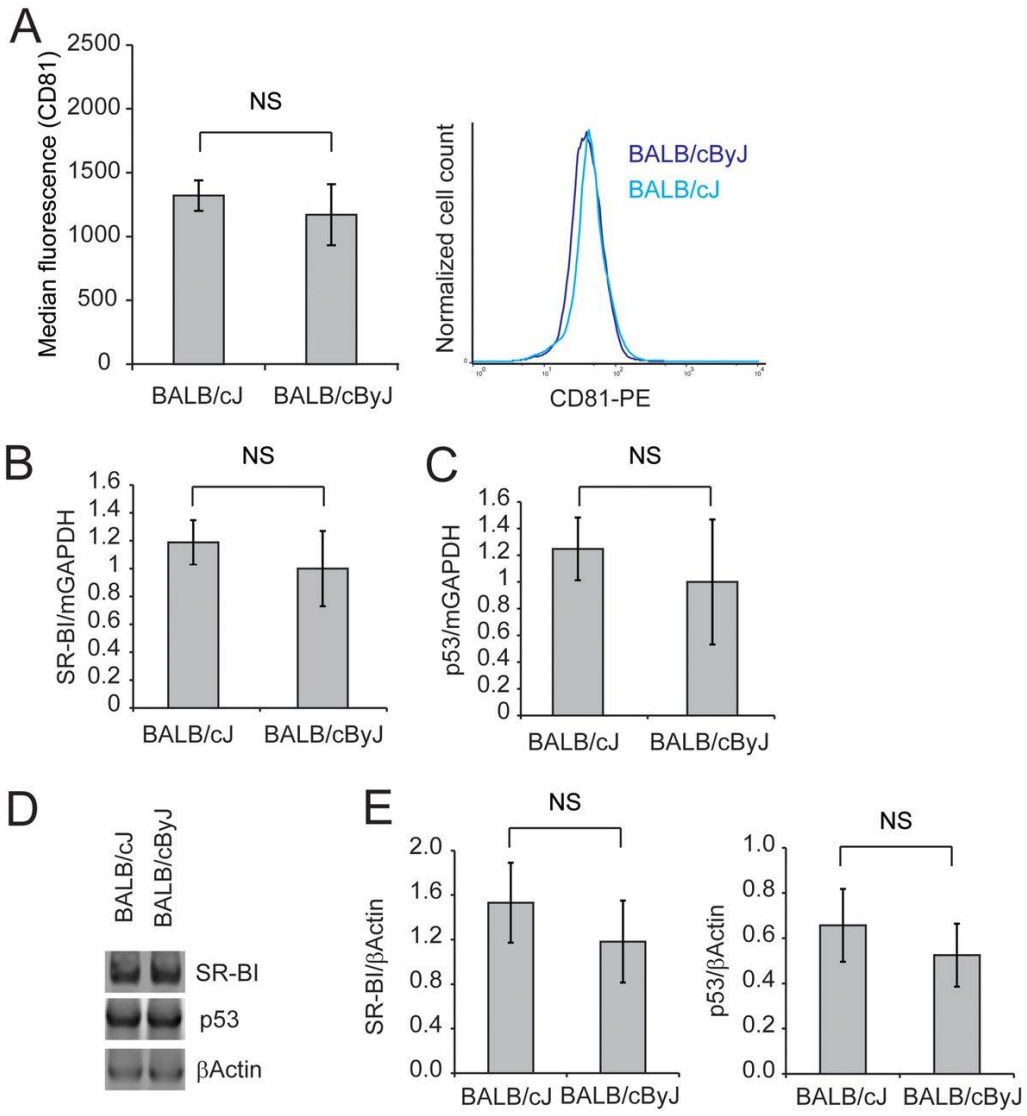

Figure 3.3: Differences in hepatocyte susceptibility cannot be explained by known infection-related host factors

(A) CD81 surface expression levels are similar in BALB/cJ and BALB/cByJ mouse hepatocytes, as assessed by surface staining of CD81 followed by flow cytometry. (B and C) Transcript levels of scavenger receptor B1 (B) and p53 (C) do not vary between hepatocytes of BALB/cJ and BALB/cByJ mice. (D and E) Protein levels of SR-BI and p53 do not vary in hepatocytes between BALB/cJ and BALB/cByJ mice. Transcript levels were determined by qPCR and compared to the transcript of the given gene and normalized to mouse GAPDH. Protein levels were assessed by immunoblotting, and quantities were normalized to mouse β-actin. Data are representative of those from three independent experiments.

hepatocytes derived from BALB/cJ or BALB/cByJ mice showed no significant difference (Fig. 3.3A). Furthermore, no significant differences in SR-BI and p53 levels of mRNA (Fig. 3.3B, C) or protein (Fig. 3.3D, E) was observed in BALB/cJ and BALB/cByJ mouse hepatocytes. Therefore, differential expression of previously identified host hepatocyte factors did not account for the distinct BALB/cJ and BALB/cByJ mouse susceptibilities to pre-erythrocytic infection.

Known factors of hepatocyte infection do not explain phenotype

Recently, an association between high hepatocyte ploidy and increased susceptibility to pre-erythrocytic infection was uncovered [20]. Thus, we asked if there was a difference in the frequency of hepatocyte polyploidy between BALB/cJ and BALB/cByJ mice. We collagenase-perfused livers and then stained hepatocytes with a DNA dye, treated them with RNase, and assessed DNA content by flow cytometry. BALB/cByJ mouse hepatocytes exhibited a greater frequency of the polyploid 8n and 16n states than did BALB/cJ mouse hepatocytes (Fig. 3.4).

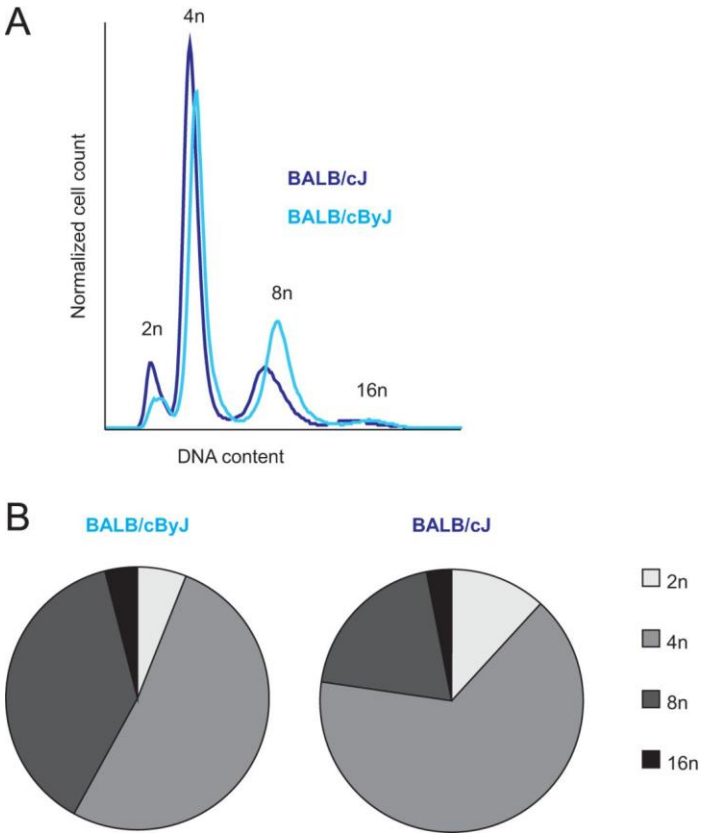

Figure 3. 4: BALB/cByJ mice have an increased frequency of polyploid hepatocytes

(A) Histograms monitor DNA content in hepatocytes in BALB/cJ and BALB/cByJ mouse hepatocytes. (B) Pie charts depict the distribution of hepatocyte ploidy in BALB/cJ and BALB/cByJ mouse hepatocytes. BALB/cByJ mouse hepatocytes are more enriched for 8n and 16n cells than are BALB/cJ mouse hepatocytes.

This suggests that a hepatocyte factor upregulated on both polyploid hepatocytes and BALB/cByJ mouse hepatocytes might associate with increased pre-erythrocytic infection.

RNA-Seq analysis uncovers transcriptional differences between substrains

To uncover novel host factors that associate with increased susceptibility to pre-erythrocytic infection, we next conducted an unbiased interrogation of differences in target organ gene expression between BALB/cJ and BALB/cByJ mice. We harvested hepatocytes and total livers from three mice of each mouse substrain, isolated RNA from each sample, pooled RNA for each sample type, and performed RNA-Seq. Sequencing reads were mapped by Tophat2 [21], and differential RNA abundance was determined by Cuffdiff2 [22]. We identified 129 genes differentially expressed between BALB/cByJ and BALB/cJ mouse livers and 53 genes differentially expressed in hepatocytes only (corrected P value ≤ 0.05; log2 ratio ≥ 0.95) (Fig. 3.5A; see also Table 1). Eighteen of these transcripts were detected as differentially expressed in the total liver samples and purified hepatocyte samples.

We prioritized quantitative RT-PCR (qRT-PCR) validation of genes that showed differences in mRNA abundance in both total liver and isolated hepatocytes of substrains. Minor differences in transcript abundance between BALB/cJ and BALB/cByJ hepatocytes might be masked by nonparenchymal cell transcripts, but the most substantial changes are likely to be detected in both hepatocyte and total liver samples. A majority of transcripts showed differences in mRNA abundance between BALB/cJ and BALB/cByJ livers by qPCR that were consistent with the changes observed via RNA-Seq (Fig. 3.5A). Taken together, these data provide a compelling set of gene products which warrant examination, as host factors that might be associated with susceptibility to pre-erythrocytic infection. Interestingly, transcript expression for five cell surface receptors showed differences between BALB/cJ and BALB/cByJ

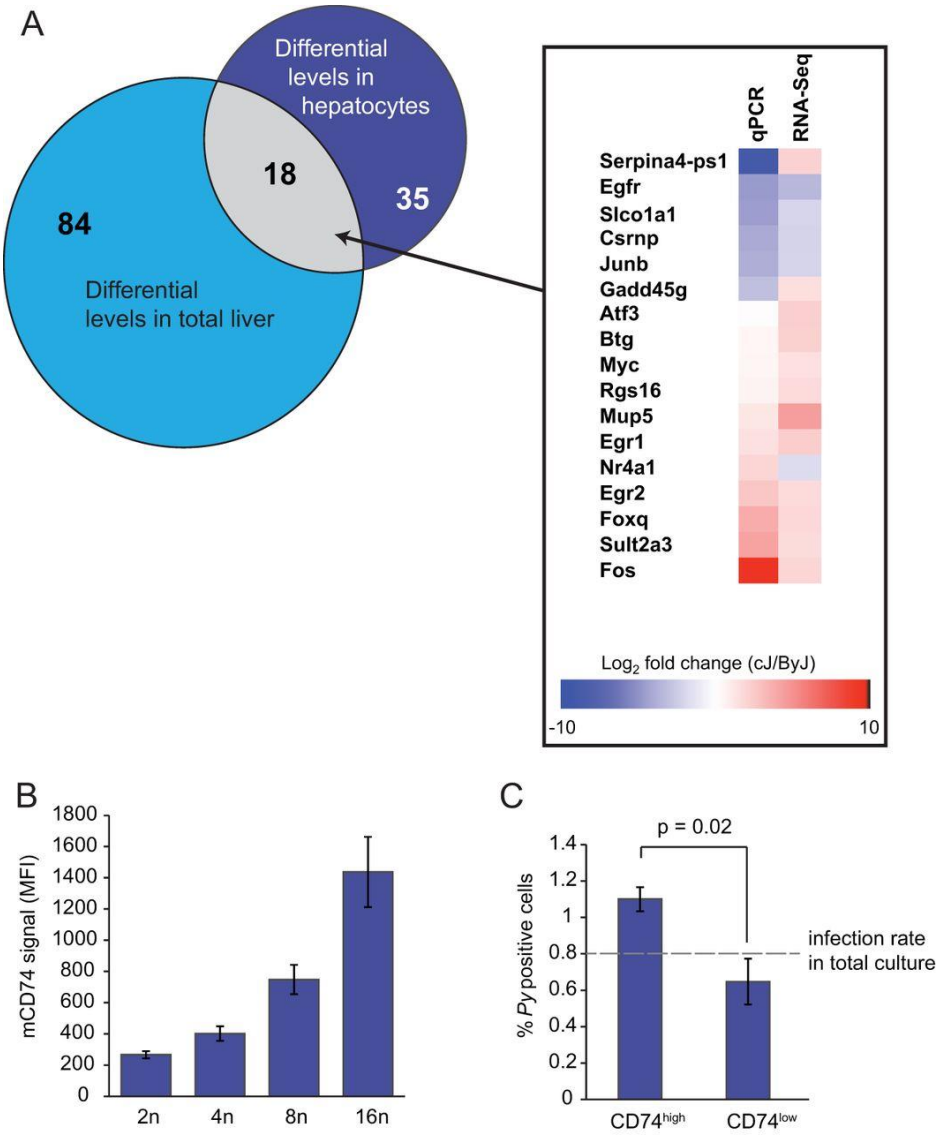

Figure 3.5: RNA-Seq analysis reveals differential gene expression in livers and hepatocytes of BALB/c substrains

(A) Sequencing reads were mapped by Tophat2 [21], and differential gene regulation was determined by Cuffdiff2 [22]. This identified 53 genes differentially expressed between BALB/cByJ and BALB/cJ mouse hepatocytes (corrected p-value ≤ 0.05; log2 ratio ≥ 0.95). Genes also differentially regulated in total liver (18) were assessed by qPCR. A comparison of transcript levels by RNA-Seq and qPCR is shown. For the complete differential regulated RNA-Seq data set, see Table S2 in the supplemental material. (B) Hepatocytes from naive BALB/c mice were isolated and stained for DNA content and CD74. Cells with higher DNA content also exhibited higher levels of CD74. (C) A total of 300,000 Hepa 1-6 cells were infected with 100,000 P. *yoelii* sporozoites. Infection rates (1.5 h after infection) within the highest and lowest 25% of CD74-expressing cells (designated $CD74^{high}$ and $CD74^{low}$, respectively) are illustrated. Infection rate in the total culture is depicted with a dashed line.

hepatocytes. Levels of epidermal growth factor receptor (EGFR) transcript were elevated in BALB/cByJ hepatocytes and total liver. In addition, neurotrophic tyrosine kinase receptor 2 (NTRK2) transcripts were increased in BALB/cJ mouse hepatocytes, and CD74 transcripts were increased in BALB/cByJ mouse hepatocytes. Because it was exclusively upregulated in susceptible BALB/cByJ mouse hepatocytes, we chose to perform subsequent studies on CD74. We first assessed the levels of CD74 protein in hepatocytes, which varied in DNA content. We found that polyploid hepatocytes had substantially higher levels of CD74 than those with lower DNA content (Fig. 3.5B). We reasoned that this might make CD74 a prime candidate to facilitate increased infection in both BALB/cByJ hepatocytes and hepatocytes with high ploidy. To validate this finding, we infected Hepa1-6 cells with *P. yoelii* sporozoites and assessed the infection rate by flow cytometry. We found that the frequency of sporozoite infection in the top 25% of CD74-expressing cells ($CD74^{high}$) was significantly higher than the infection frequency in cells with the lowest 25% of CD74 levels ($CD74^{low}$) (Fig. 3.5C). These data suggest that sporozoites preferentially infect hepatocytes with increased levels of CD74. Thus, CD74 is an intriguing hepatocyte receptor worthy of additional investigation for its potential role in *Plasmodium* sporozoite infection.

DISCUSSION

The bite of an infected *Anopheles* mosquito transmits tens to hundreds of *Plasmodium* sporozoites [16], infecting the liver at extraordinarily low multiplicities of infection. Unlike the related apicomplexan *Toxoplasma*, the sporozoite of *Plasmodium* species that infect mammals specifically targets hepatocytes as a host cell. The motile sporozoite has the capacity to traverse multiple cell types [23], [24], including hepatocytes [24], before committing to

intracellular residence. This allows the parasite to select its optimal host cell at the point of invasion. From the time of sporozoite transmission to the point of parasite egress from

Table 3.1: RNA-Seq data from BALB/cJ and BALB/cByJ mice

Differentially regulated genes in total liver and hepatocytes					**Differentially regulated genes in heptocytes only**			
gene	cByJ/cJ value	p value	q value		gene	cByJ/cJ value	p value	q value
Atf3	0.330964317	5.00E-05	0.013219		A1bg	0.367029005	5.00E-05	0.013219
Btg2	0.418945282	5.00E-05	0.013219		Bcl6	0.347129246	5.00E-05	0.013219
Csrnp1	0.391713823	5.00E-05	0.013219		Cd74	2.373338537	5.00E-05	0.013219
Egfr	2.683460886	5.00E-05	0.013219		Cxcl1	0.349649118	5.00E-05	0.013219
Egr1	0.438158662	5.00E-05	0.013219		Cyp26a1	2.916332934	5.00E-05	0.013219
Fos	0.367991221	5.00E-05	0.013219		Cyp2c67	2.030855707	5.00E-05	0.013219
Foxq1	0.344433104	5.00E-05	0.013219		Cyp3a41a	6.54349978	5.00E-05	0.013219
Gadd45g	0.323294124	5.00E-05	0.013219		Dmbt1	0.106291641	5.00E-05	0.013219
Junb	0.482596762	5.00E-05	0.013219		Egr2	0.191283705	5.00E-05	0.013219
Mt1	0.483181855	5.00E-05	0.013219		Elovl3	2.531269706	5.00E-05	0.013219
Mt2	0.429210587	5.00E-05	0.013219		Fosb	0.160786716	5.00E-05	0.013219
Myc	0.111360325	5.00E-05	0.013219		G0s2	2.309776698	5.00E-05	0.013219
Rgs16	0.308798636	5.00E-05	0.013219		Gadd45a	0.320086278	5.00E-05	0.013219
Serpina4-ps1	5.289766399	5.00E-05	0.013219		Gbp10	2.174734792	5.00E-05	0.013219
Slco1a1	2.835253501	5.00E-05	0.013219		Gm4841	3.334954163	5.00E-05	0.013219
Sult2a3	0.451303688	5.00E-05	0.013219		Hspa1b	2.974799987	5.00E-05	0.013219
Mup5	2.281854099	1.00E-04	0.022736		Klf6	0.367337512	5.00E-05	0.013219
Nr4a1	0.41537142	1.00E-04	0.022736		Mup1	2.610600845	5.00E-05	0.013219
					Mup16	5.394852185	5.00E-05	0.013219
					Mup7	3.611360556	5.00E-05	0.013219
					Mup9	3.148307951	5.00E-05	0.013219
					Ndrg1	2.951088548	5.00E-05	0.013219
					Neat1	0.466473595	5.00E-05	0.013219
					Nr1d1	0.428219467	5.00E-05	0.013219
					Ntrk2	0.150143256	5.00E-05	0.013219
					Per3	0.34848693	5.00E-05	0.013219
					Pfkfb3	2.215384365	5.00E-05	0.013219
					Amd1	11.76299684	1.00E-04	0.022736
					Bag3	2.018691016	1.00E-04	0.022736
					H2-Ab1	2.978440438	1.00E-04	0.022736
					Rhob	0.477193113	1.00E-04	0.022736
					Upp2	0.4979545	1.00E-04	0.022736
					Hspb1	3.940002754	0.00015	0.033435

					Fmo3	0.507046637	2.00E-04	0.042898
					H2-Aa	3.29116151	2.00E-04	0.042898

hepatocytes into the bloodstream, a diversity of host defenses can curtail pre-erythrocytic infection. These factors include but are not limited to (i) inhibition of sporozoite motility in the skin by immune responses [25], [26], (ii) intracellular trapping of sporozoites during traversal of the sinusoidal endothelium [23] or Kupffer cells [27], [28], and (iii) innate immune responses which curtail infection during LS development [29], [30]. While mouse models that differ in their innate and adaptive immune responses to *Plasmodium* are plentiful, we report the first example of genetically similar hepatocytes derived from two substrains of BALB/c mice with dramatically different susceptibilities to initial sporozoite infection.

The hepatocyte-intrinsic differential susceptibility to sporozoite infection between BALB/cJ and BALB/cByJ mice is unexpected since differences in susceptibility between these BALB/c substrains to diseases such as hyperthyroidism and encephalomyelitis are mediated by the immune system [31], [32]. Furthermore, differences in susceptibility to pre-erythrocytic infection between BALB/c and C57BL/6 mice have been mapped to the expression of TREM2 in macrophages [33], [34]. These data indicate that genetic differences that give rise to molecular changes in immune components can alter susceptibility to pre-erythrocytic infection. However, these studies do not implicate specific hepatocyte factors. Interestingly, it has been shown that *P. berghei* infection is curtailed more potently by innate immune responses during liver stage development than *P. yoelii* infection. This suggests that when comparing liver stage development between different rodent malarias and different mouse strains, one must take into account not only the initial susceptibility of the host hepatocyte to sporozoite infection but also the innate immune response that is engendered by the infection [35].

Candidate-based approaches have provided insights into a subset of hepatocyte receptors which are important for sporozoite infection. These investigations demonstrate partial overlap with host entry requirements of hepatitis C virus (HCV). Specifically, *Plasmodium* species and HCV both utilize CD81 [10], [36] and SR-BI [11], [37]. Furthermore, heparan sulfate proteoglycan (HSPGs) [14] play a role in switching between the migratory state and the invasive state of sporozoites [13]. Interestingly, HSPGs also play a role in HCV infection [36], [38]. Yet the dramatic difference in susceptibility to sporozoite infection between BALB/cJ and BALB/cByJ hepatocytes cannot be explained by either CD81 or SR-BI expression, suggesting that other hepatocyte factors involved in sporozoite infection remain to be discovered.

Plasmodium sporozoites that infect mammals (with the exception of *P. berghei*) are highly specific for their hepatocyte host cell *in vivo*, yet no single described host factor is completely essential for infection. Small interfering RNA screens have identified a small number of hepatocyte molecules which impact sporozoite invasion and/or development [39] *in vitro*, but inefficient knockdown in hepatocytes has limited the utility of this approach. Genetically similar mice with variable susceptibilities to pre-erythrocytic infection might thus provide a useful tool for linking specific genetic changes to differences in host susceptibility. Our data suggest that the same factor(s) might explain the differential susceptibility to sporozoite infection between substrains of BALB/c mice and hepatocytes with low and high ploidy [20].

Several genes that are differentially expressed between BALB/cJ and BALB/cByJ mice might play a role in facilitating or inhibiting sporozoite invasion of hepatocytes. The receptor tyrosine kinase (EGFR) is more highly expressed in BALB/cByJ mouse hepatocytes, making it an enticing candidate receptor for sporozoite entry. EGFR is also involved in HCV infection, where its signaling properties facilitate the clustering of the critical entry molecules CD81 and

occludin [40]. Since CD81 is also important for *P. yoelii* and *P. falciparum* invasion, it is possible that EGFR functions in a similar way for sporozoite infection. Alternatively, a direct interaction between the sporozoite and EGFR might facilitate hepatocyte entry, and the expression of proteins containing the epidermal growth factor (EGF) domain in sporozoites [41] might provide the potential for such an interaction.

The increase of CD74 expression in BALB/cByJ hepatocytes, as well as the preference of *P. yoelii* sporozoites for $CD74^{high}$ hepatocytes, presents a tantalizing clue that warrants further study. It has also been shown that *P. falciparum* and *P. berghei* macrophage inhibitory factor [42] bind host CD74. Additionally, *P. yoelii* MIF is important for LS development in BALB/c mice [43]. Taken together, these data suggest that an interaction between CD74 and *Plasmodium* MIF might facilitate effective pre-erythrocytic infection. The relationship between parasite MIF and host CD74 as well as their role in sporozoite invasion of hepatocytes is an important future area of investigation. In contrast to EGFR and CD74, the receptor tyrosine kinase NTRK2 is expressed at higher levels in less susceptible BALB/cJ hepatocytes, suggesting that this receptor might play an inhibitory role in sporozoite invasion. Unlike the well-described EGF-like domains, to our knowledge, no *Plasmodium* protein is similar to the NTRK2 ligand neurotrophin-3.

The impact of host genetics on hepatocyte susceptibility to infection is not restricted to rodent *Plasmodium* parasites. Hepatocyte donor origin also impacts *P. falciparum* pre-erythrocytic infection *in vitro* [9]. Although host factors which facilitate sporozoite hepatocyte entry for *Plasmodium* spp. vary [10], [44], factors that mediate differential susceptibility to rodent malaria parasites could still be prime targets to study as factors for susceptibility of humans to malaria parasite infection. The dramatic difference in susceptibility between BALB/cJ

and BALB/cByJ hepatocytes to sporozoite infection might assist in identifying novel host receptors, which facilitate the initiation of *Plasmodium* pre-erythrocytic infection. This could guide novel approaches to prevent human malaria parasite infection.

MATERIALS AND METHODS

Mosquito rearing and sporozoite production

For *P. yoelii* sporozoite production, female 6- to 8-week-old Swiss Webster (SW) mice (Harlan, Indianapolis, IN) were injected with blood-stage *P. yoelii* (17XNL) parasites to begin the growth cycle. Animal handling was conducted according to Institutional Animal Care and Use Committee-approved protocols. Infected mice were used to feed female *Anopheles stephensi* mosquitoes after gametocyte exflagellation was observed. Salivary gland sporozoites were isolated according to standard procedures at day 14 or 15 post-blood meal. For each experiment, salivary glands were isolated in parallel in order to ensure that sporozoites were extracted under identical conditions.

Quantification of liver burden by real-time RT-PCR

Total RNA was extracted using TRIzol reagent (Invitrogen). cDNA synthesis was performed using the Super Script III Platinum two-step quantitative reverse transcription-PCR (qRT-PCR) kit according to the manufacturer's instructions (Invitrogen). All PCR amplification cycles were performed at 95°C for 30 s for DNA denaturation and 60°C for 4 min for primer annealing and DNA strand extension. Parasite 18S was amplified using primers with sequences 5′-GGGGATTGGTTTTGACGTTTTTGCG-3′ and 5′-AAGCATTAAATAAAGCGAATACATCCTTAT-3′. Mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified using sequences 5′-CCTCAACTACATGGTTTACAT-

3′ and 5′-GCTCCTGGAAGATGGTGATG-3′. For quantitative PCR (qPCR), a standard curve was generated using 1:4 dilutions of a reference cDNA sample for PCR amplification of all target PCR products. Experimental samples were compared to this standard curve to give a relative abundance of transcript.

Real-time *in vivo* imaging of LS development in whole bodies of live mice

BALB/cJ or BALB/cByJ mice were infected with Py-GFP-luc salivary gland sporozoites by intravenous (i.v.) tail vein injection. For i.v. injections, salivary gland sporozoites were enumerated and suspended in RPMI medium prior to injection of 50,000 sporozoites per mouse. Mice were injected with 100 µl of RediJect D-luciferin (PerkinElmer) intraperitoneally prior to being anesthetized using the isoflurane anesthesia system (XGI-8; Caliper Life Sciences, USA). Luciferase activity in animals was visualized through imaging of whole bodies using the IVIS Lumina II animal imager (Caliper Life Sciences). Animals were kept anesthetized during the measurements, which were performed within 5 to 10 min after the injection of D-luciferin. Bioluminescence imaging was acquired with a 10-cm field-of-view (FOV), medium binning factor, and an exposure time of 1 to 5 min. Quantitative analysis of bioluminescence was performed by measuring the luminescence signal intensity using the region-of-interest (ROI) settings of the Living Image 3.0 software. ROIs were placed around the abdominal area at the location of the liver. ROI measurements are expressed as total flux (photons/s).

Hepatocyte isolation

Hepatocytes were isolated from mice using a two-step perfusion *in situ*. Portal veins of anesthetized mice were cannulated with warmed perfusion buffer (1× Hanks balanced salt solution [HBSS] without calcium, magnesium supplemented with 8 mM HEPES buffer and 0.5 mM EDTA) at low flow rate. Once cannulated, the inferior vena cava was severed to blanch the

liver. The buffer flow rate was increased to 5 ml/min and allowed to perfuse for 5 min, with occasional clamping and releasing of the vena cava to inflate the liver. The liver was then perfused with 2% collagenase II (Worthington) in collagenase buffer (HBSS supplemented with 8 mM HEPES and 0.5 mM $CaCl_2$) for 5 min at 5 ml/min. The liver was then removed from the abdominal cavity and the gallbladder excised. The liver was placed in a dish of Dulbecco modified Eagle medium (DMEM) and gently pushed through a 100-μm cell strainer to dissociate the hepatocytes, which were then collected with a wide-bore syringe.

Hepatocyte processing

Hepatocytes processed for flow cytometry were spun by benchtop centrifuge at 50 × *g* for 3 min to pellet hepatocytes. The supernatant, containing nonparenchymal cells and lymphocytes, was aspirated, and the remaining hepatocytes were washed in DMEM. The spins and washes were repeated until the supernatant was clear, usually 3 washes. Cells were then fixed and stained as described below. Hepatocytes processed for plating or RNA extraction were spun and washed once as described above and then resuspended in DMEM at a volume of 10.4 ml. To this was added 10 ml of 90% Percoll (GE Health Sciences) in phosphate-buffered saline (PBS), followed by spinning at 50 ×*g* for 10 min with no brake. Pelleted cells were given a final wash with DMEM, then resuspended in warm InvitroGro HI hepatocyte medium (Bioreclamation IVT), and checked for density and viability using trypan blue staining.

Immunofluorescence microscopy

A total of 1.5×10^5 primary hepatocytes were seeded as described above in each well of a type I collagen-coated glass slide (BioCoat; BD Biosciences). Cells were infected with 5×10^4 *P. yoelii* sporozoites. Slides were centrifuged for 3 min at 515 × *g* in a hanging-bucket centrifuge to aid in sporozoite invasion. After 90 min, medium containing extracellular sporozoites was

aspirated, and fresh medium was added. LSs developed for 48 h, at which time cells were fixed with 4% paraformaldehyde, blocked, and permeabilized for 1 h in PBS with the addition of 0.1% Triton X-100 and 2% bovine serum albumin (BSA). Staining steps were performed in PBS supplemented with 2% BSA. Cells were stained using antisera to *Plasmodium* heat shock protein 70 (HSP70), upregulated in infectious sporozoites 4 protein (UIS4) 4°C overnight, and then visualized with the use of Alexa Fluor-488 goat anti-mouse and Alexa Fluor-594 goat anti-rabbit secondary antibodies (Invitrogen). Cells were stained with 4′,6-diamidino-2-phenylindole (DAPI) to visualize both hepatocyte and parasite nuclei. Sporozoites that had not invaded and/or developed in hepatoma cells were distinguished by UIS4 noncircumferential staining and morphology.

Quantification of LSs by flow cytometry

Cells were cultured as described above. A total of 1.5×10^5 cells were plated in each well of a 24-well plate and infected with 10^5 *P. yoelii* sporozoites per well. At the desired time point, cells were trypsinized and fixed with Perm/Fix buffer (BD Biosciences). Cells were blocked in Perm/Wash buffer (BD Biosciences) supplemented with 2% BSA. Additional staining steps were also performed in Perm/Wash buffer supplemented with 2% BSA. Cells were stained with monoclonal antibody to circumsporozoite protein (CSP) conjugated to Alexa Fluor-488 or Alexa Fluor-647 at 25°C. Parasitized hepatocytes were identified by flow cytometry performed on a BD LSRII. Flow cytometric analysis was performed using FlowJo software (TreeStar). All experimental conditions were tested in biological triplicate.

Analysis of DNA content

Mouse hepatocytes were isolated and processed for flow cytometry as described above. After blocking, cells were resuspended in a mixture containing PBS, 5 mM EDTA, 0.1 mg/ml of

RNase A, and SYTOX blue DNA dye (Invitrogen). DNA content was measured by flow cytometry on an LSRII and analyzed by FlowJo (Tree Star).

In vivo and *in vitro* analysis of CD74 expression

For analysis of CD74 expression by ploidy, mouse hepatocytes were isolated and processed for flow cytometry as described above. Cells were fixed in 1% paraformaldehyde and then washed and blocked in PBS plus 2% BSA. Cells were stained with anti-mouse CD74 conjugated to fluorescein isothiocyanate (FITC; BD Pharmingen) at a concentration of 5 μg/ml overnight at 4°C and then stained for DNA analysis as described above. Cells were gated on ploidy state by DNA dye fluorescence, and then the median fluorescence of FITC was measured for each ploidy state. To analyze CD74 expression in infection, Hepa1-6 cells were infected as described above for quantification of LSs by flow cytometry. After blocking, cells were stained with anti-*P. yoelii* CSP conjugated to Alexa Fluor-647 as well as anti-mouse CD74 conjugated to FITC. Flow cytometric analysis was performed using FlowJo software (TreeStar). All experimental conditions were tested in biological triplicate.

Animal handling and infection

All animal protocols were approved by the Cener for Infectious Disease Research IACUC.

Transcriptome sequencing (RNA-Seq) analysis

We used clipped fastq files provided by Expression Analysis. These reads were subjected to quality control (QC) based on the following criteria as provided by Expression Analysis: (i) average Q score across all bases of >25, (ii) removal of any single base with a quality of less than 7 (if enough bases are removed that the sequence is less than 25 bases in length, the read is removed), (iii) removal of homopolymers, (iv) removal of Illumina adapters (if the cleaned sequence is less than 25 bases in total length, the whole read is removed), and (v) the presence of

no more than 4 N′s in any given sequence. These reads were then mapped to the reference genome using Tophat2; then differential gene expression was calculated using Cuffdiff2. Genes with a Benjamini-Hochberg corrected *P* value of ≤ 0.05 and absolute log2 ratio of ≥0.95 were considered significantly differentially expressed between the BALB/cByJ and BALB/cJ mice.

Validation of differentially transcribed genes by qPCR

RNA was extracted from whole BALB/cJ and BALB/cByJ mouse livers using TRIzol reagent (Invitrogen) and a Direct-Zol RNA extraction kit (Zymo Research). cDNA was generated using the QuantiTect reverse transcription kit (Qiagen) and then used as the template for quantitative PCR. Primers for genes tested are listed in Table S1 in the supplemental material. For qPCR, a standard curve was generated using 1:4 dilutions of a reference cDNA sample for PCR amplification of all target PCR products. Experimental samples were compared to this standard curve to give a relative abundance of transcript. Abundances of genes of interest were standardized to the GAPDH abundance from the same sample. The relative measurements from five mice were averaged for each strain, and the ratio of BALB/cJ to BALB/cByJ mice was calculated for each gene tested.

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CHAPTER FOUR:

PHOSPHORYLATION OF RIBOSOMAL PROTEIN S6 IN HEPATOCYTES BOOSTS DEVELOPMENT OF PLASMODIUM LIVER STAGES

OVERVIEW

In this chapter I describe a specific host factor that impacts infection of hepatocytes by *Plasmodium yoelii*. Previous chapters identified two host states that associate with differences in susceptibility to infection, namely polyploidy and mouse substrains. Here, I interrogate the intracellular environments of these two phenotypes using reverse phase protein microarray technology, which enables broad but targeted proteomic investigations, to compare host cells of higher susceptibility to those of lower susceptibility. At the intersection of these phenotypes, I identify one post-translationally modified protein, phosphorylated Ribosomal Protein S6 (RPS6). I show that not only is phospho-RPS6 upregulated in highly susceptible BALB/cByJ hepatocytes and polyploid (8n) hepatocytes but also in infected Hepa1-6 cells.

Using small molecule inhibitors of the primary RPS6 kinases, I show that inhibition of phosphorylation correlates with lowered parasite liver stage burden both *in vitro* and in a mouse model of infection. This reduction of parasite burden is not apparent early in infection but rather at later liver stage developmental timepoints.

INTRODUCTION

Malaria remains one of the most harmful infectious diseases in the world, despite a dramatic drop in yearly cases in the past decade. The disease is caused by the intraerythrocytic form of parasites in the genus *Plasmodium*, which multiply rapidly in the patient's blood and cause fever, anemia, and complications in multiple organs [1]. Previous to this intraerythrocytic disease state, the parasite must undergo obligate intracellular development within the liver. This liver stage is clinically silent and involves orders of magnitude fewer parasites [2], and so represents a bottleneck in the *Plasmodium* life cycle that is an attractive target for intervention. However, liver stage biology remains difficult to study and therefore relatively undescribed.

Intracellular pathogens of all kinds require their host cell to be a welcoming environment, and *Plasmodium* is no exception. Hepatocytes have numerous ways of dealing with the parasite, including apoptosis [3], autophagy [4], and innate immune responses [5]. On the other hand, hepatocytes also represent a relatively immunologically privileged hiding place [6], richly stocked with amino acids, lipids, and nucleotides the parasite needs to grow and replicate. The balance between these positive and negative environments likely determines the ultimate fate of the intrahepatocytic *Plasmodium* parasite. Moreover, there appears to be considerable hepatocytes heterogeneity in terms of providing beneficial environments for the parasite. The host factors which tip the balance in the parasite's favor are not yet fully understood, but it has been shown that several host membrane proteins play a significant role in determining which cell a parasite may enter [7], [8]. Most notably, the amount of the receptor EphA2 on the hepatocyte membrane helps to not only affect the entry of the parasite but also its successful progression through liver stage development [9]. Once inside, parasite survival is also influenced by the levels of the tumor suppressor p53 [10], which negatively impacts parasite development. DNA

content of the hepatocyte has also been shown to impact *Plasmodium* entry of hepatocytes [Chapter 2, 11], with more highly polyploid cells showing increased susceptibility to infection. Likewise, genetic background of the hepatocyte affects susceptibility to parasite infection, as mice of dissimilar [12], [13] or even highly genetically similar [Chapter 3, 14] substrains contain hepatocytes of different susceptibility, and human hepatocytes from different donors show differential susceptibility to human parasite infection *in vitro* [15].

Ribosomal protein S6 (RPS6) is an essential part of the 40S subunit of the eukaryotic ribosome [16]. It carries five serine residues as potential phosphorylation sites [17], [18], and is downstream of two central cell signaling pathways—the Akt/mTOR pathway via the p70 Ribosomal S6 kinases (S6Ks) [19], and the MEK/ERK MAP kinase pathway via the p90 Ribosomal S6 Kinases (RSKs) [20]. Phosphorylation of RPS6 was long thought to be linked to protein biosynthesis [21], [22], but subsequent reports show protein synthesis is independent of RPS6 phosphorylation [23], [24], and may actually lower biosynthesis in mammals [25]. RPS6 phosphorylation has also been linked to cell size and proliferation [25], [26], glucose homeostasis and intolerance [25], [26], muscle functioning [27], and clearance of apoptotic cells [28], and may be directly protective from DNA damage in cancerous cells [29], [30].

The host cellular environment can greatly influence the survival and development of intrahepatocytic *Plasmodium* [10], [31], [32]. Here, we compare the protein expression and post-translational modification profiles of two sets of susceptible hepatocytes to discover potential host proteins that may boost *Plasmodium* liver stage infection. We demonstrate that phosphorylation of RPS6 is beneficial to pre-erythrocytic infection, and that prevention of phosphorylation negatively impacts parasite numbers and development in hepatocytes.

RESULTS

Protein Lysate Microarrays identify differential signaling in susceptible hepatocytes

Previous work has identified transcriptional differences between hepatocytes of different susceptibilities, both polyploid [33] and BALB/c substrain [14]. However, measuring transcription does not fully reflect changes in translation or post-translational modification, which may have profound effects on the intracellular environment. In *Plasmodium* infection, several intracellular signaling pathways have been shown to be perturbed on transcriptional, protein, and post-translational levels [10], [34]. Reverse phase protein microarray technology, which enables broad but targeted proteomic investigations [35], uses cellular lysates deposited in picoliter droplets on nitrocellulose-coated glass slides. Levels of specific proteins or their post-translational modifications can be detected by probing the lysates with appropriate antibodies [10], [35] allowing the same samples to be tested against hundreds of antibodies in parallel. We assembled a diverse set of antibodies which recognize host proteins involved in numerous cellular outcomes previously implicated in *Plasmodium* infection, including survival, apoptosis, proliferation, cell-cycle control, and autophagy, and probed protein microarrays from high- and low-susceptibility hepatocytes to investigate the molecular differences between highly polyploid and diploid hepatocytes, and between BALB/cByJ and BALB/cJ livers.

Because cellular differences between cells are not necessarily biologically relevant to *Plasmodium* infection, we reasoned that candidates identified in both sets of susceptible cells were more likely to be valid candidates for follow-up experiments (Figure 4.1A). We isolated primary mouse hepatocytes of different ploidy states by fluorescence-activated cell sorting (FACS). Protein extracts from each sample were prepared and printed in triplicate on separate nitrocellulose pads followed by probing the arrays with antibodies to obtain quantitative

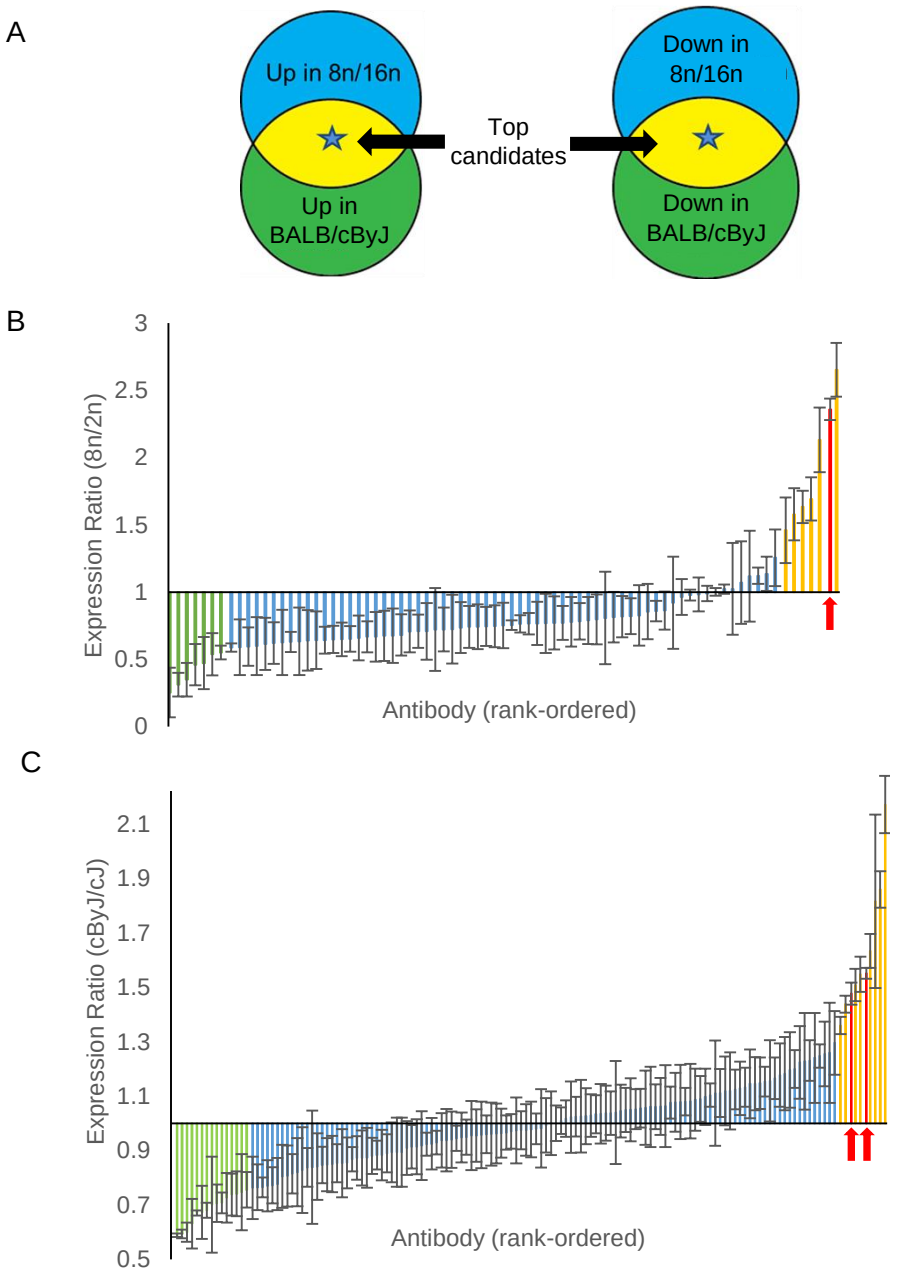

Figure 4. 1: Reverse-phase protein microarrays identify host cellular pathway disruptions in highly susceptible hepatocytes

Candidates identified as upregulated in highly-susceptible hepatocytes, or downregulated in less susceptible cells, are more likely to be biologically relevant to *Plasmodium* infection (A). Comparison of signaling pathway expression between more susceptible cells [8n (B), BALB/cByJ (C)] to less susceptible cells [2n (B), BALB/cJ (C)] identifies most upregulated (Ratio >1.2 fold, in orange) and most downregulated proteins (Ratio<0.8 fold, in green). Phosphorylated RPS6 is upregulated in both sets of susceptible cells (in red, marked with arrows). Error bars represent the S.E.M. of 5 (B) or 10 (C) biological replicates.

Upregulated in BALB/cByJ				Downregulated in BALB/cByJ			
Antibody	Description	Fold change	p-value	Antibody	Description	Fold change	p-value
FoxA2	Forkhead Box A2	1.8604	0.037	Rab5	Ras-related protein	0.5883	0.011
pRPS6 (S240/4)	Phospho-Ribosomal Protein S6	1.5513	0.012	Stat1	Signal transducer and activator of transcription	0.5931	0.026
SOD2	Superoxide Dismutase 2	1.5483	0.041	Par1	Protease-activated Receptor 1	0.5994	0.035
pRPS6 (S235/6)	Phospho-Ribosomal Protein S6	1.4773	0.027	Mili	Mouse homolog of Piwi	0.6703	0.013
p-4EBP	Initiation Factor 4E-Binding Protein	1.4382	0.021	IKKa	Inhibitor of NF-kB	0.6801	0.040
SR-B1	Scavenger Receptor B1	1.3597	0.023				

Upregulated in 8n cells				Downregulated in 8n cells			
Antibody	Description	Fold change	p-value	Antibody	Description	Fold change	p-value
p90RSK	MAPK-activated S6 kinase	2.6531	0.080	EIF4E	Eukaryotic Initiation Factor 4E	0.2533	0.078
pRPS6 (S235/6)	Phospho-Ribosomal Protein S6	2.3591	0.017	ERK1/2 p202/204	p44/p42 MAP kinase	0.3113	0.089
NF-kB	Nuclear Factor kappa B	2.1329	0.264	PTEN	phosphatase and tensin homologue	0.3494	0.101
FASN	Fatty Acid Synthase	1.9274	0.062	Atg7	Autophagy-related 7	0.4599	0.164
FasR	Fas Receptor	1.6927	0.227	MEK1/2 p217/221	MAPK Kinase	0.4723	0.809
Dvl3	Dishevelled Segment Polarity Protein 3	1.6339	0.077	Lck	lymphocyte-specific tyrosine kinase	0.5371	0.215
SOD2	Superoxide Dismutase 2	1.4601	0.085	p-HSP27	Heat shock protein 27	0.5508	0.216

Table 4.1: Quantification of most dysregulated proteins from reverse-phase microarrays

Comparison of signaling pathway expression between more susceptible cells [8n, BALB/cByJ] to less susceptible cells [2n, BALB/cJ] identifies most upregulated and most downregulated proteins. Proteins in BALB/c subset include all with a 1.3 fold change up, or 0.75 fold change down, and a p-value<0.05. Proteins in ploidy subset include all with a 1.3 fold change up, or 0.75 fold change down.

information on changes in host cell protein abundance and/or modifications (Figure 4.1B). We then prepared a matching printing and probing on whole liver lysates from BALB/cJ and BALB/cByJ mice (Figure 4.1C). In both cases, the signal from the antibody was normalized to B-actin, a housekeeping gene whose cellular level stays consistent with cell volume. The normalized value of the most susceptible hepatocytes (8n cells or BALB/cByJ individual antibody has a level of cross-reactivity that has the effect of masking the full extent of differential expression [35]. This cross-reactivity is highly antibody specific and can be minimized by validating individual antibodies technically and biologically. Technical validation uses Western blotting to verify antibody specificity to its target. Biological validation uses microarray analysis of cells treated with numerous well-characterized stimuli to verify that the antibody produces significant signal variance which parallels differences in cellular levels of the antigen [35]. All antibodies chosen for this work were previously validated to minimize cross-reactivity, yet since background signal is not fully eliminated even in validated antibodies, the true fold change of the antigens may well be higher than that revealed in this screening assay.

Several proteins were shown to be significantly upregulated in the BALB/c data set; however only one protein was statistically significant in the ploidy data set—phosphorylated Ribosomal Protein S6 (RPS6, Table 4.1). Moreover, in the BALB/c set, two separate phosphorylation sites were identified as being significantly upregulated, while the total protein level remained approximately equal (Figure 4.1C). We then validated the observed increase in phosphorylated RPS6 (p-RPS6) levels using flow cytometry in the context of polyploidy (Figure 4.2A), and by Western blot for BALB/c substrains (Figure 4.2B). In addition, we found that Hepa1-6 cells containing *Plasmodium* parasites show elevated levels of p-RPS6 at 24 hours post-

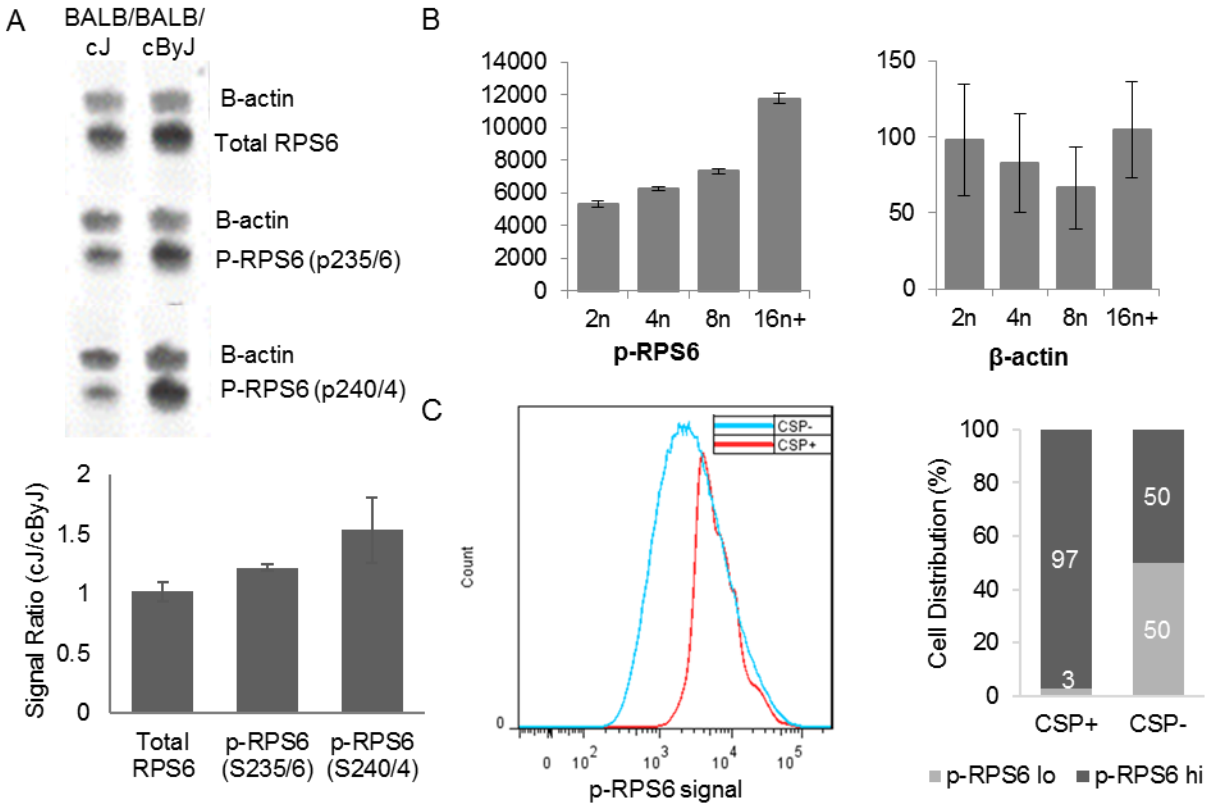

Figure 4.2: Phosphorylated RPS6 is increased in BALB/cByJ, highly polyploid, and *Plasmodium*-infected cells

Western blotting confirms that p-RPS6 is higher at both sites of primary phosphorylation in more susceptible BALB/cByJ livers. Total S6 does not significantly differ. Signal quantification is below blot (A). Increased relative expression of p-RPS6 in higher ploidy hepatocytes is confirmed by flow cytometry. Median Fluorescence Intensity is corrected for cell volume as calculated in Austin, *et al*. [11]. In contrast, corrected beta-actin signal is consistent between ploidy subsets when corrected for cell volume (B). Flow cytometry of *Plasmodium*-infected cells at 24 hpi show higher p-RPS6 signal in infected cells (CSP+). CSP+ cells are primarily in the upper 50th percentile of p-RPS6 expression (C).

infection (Figure 4.2C and D). Thus, we chose this protein to more closely characterize in the context of *Plasmodium* infection.

Small molecule inhibition of p-RPS6 decreases parasite burden *in vitro*

RPS6 is primarily phosphorylated by two families of kinases, the p70 S6 kinases (S6K) and the p90 RSK kinases (Figure 4.3A). S6Ks are the most common pathway [36], [37] but evidence increasingly points to the RSK pathway as leading to important cellular outcomes [38]. In hepatocytes, S6Ks plays a critical response in insulin responses, including lipid biosynthesis and insulin sensitivity [39], [40]. RSKs can act on this pathway in an inhibitory fashion, acting as a mediator of insulin resistance [41]. Both kinases are important for hepatocyte proliferation in response to growth factors or partial hepatectomy [25], [42], [43]. Small molecule inhibitors are able to block the phosphorylation of RPS6 by these kinases (Figure 4.3A). To interrogate the role of p-RPS6 in *Plasmodium* infection, we used small-molecule inhibitors to block the two pathways. BI-D1870 is a specific pan-p90 RSK inhibitor [44], while LY2584702 is a potent inhibitor of all p70 S6K isoforms [45]. Both compounds decreased the phosphorylation of RPS6 in insulin-stimulated Hepa1-6 cells (Figure 4.3B). We treated Hepa1-6 cells for 24 hours with these inhibitors, then infected them with *P. yoelii*, keeping the inhibitors in the cell media. Ninety minutes after infection, there was only a slight, non-significant difference in the percentage of cells containing parasites (Figure 4.3C), showing that the amount of p-RPS6 does not change the rate at which parasites initially infect cells. However, at 24 hours post infection both treatments significantly lowered parasite numbers (Figure 4.3D). This decrease is most apparent between 90 minutes and 24 hours, but additional decline in parasite numbers is seen at 48 hours post infection (Figure 4.3E).

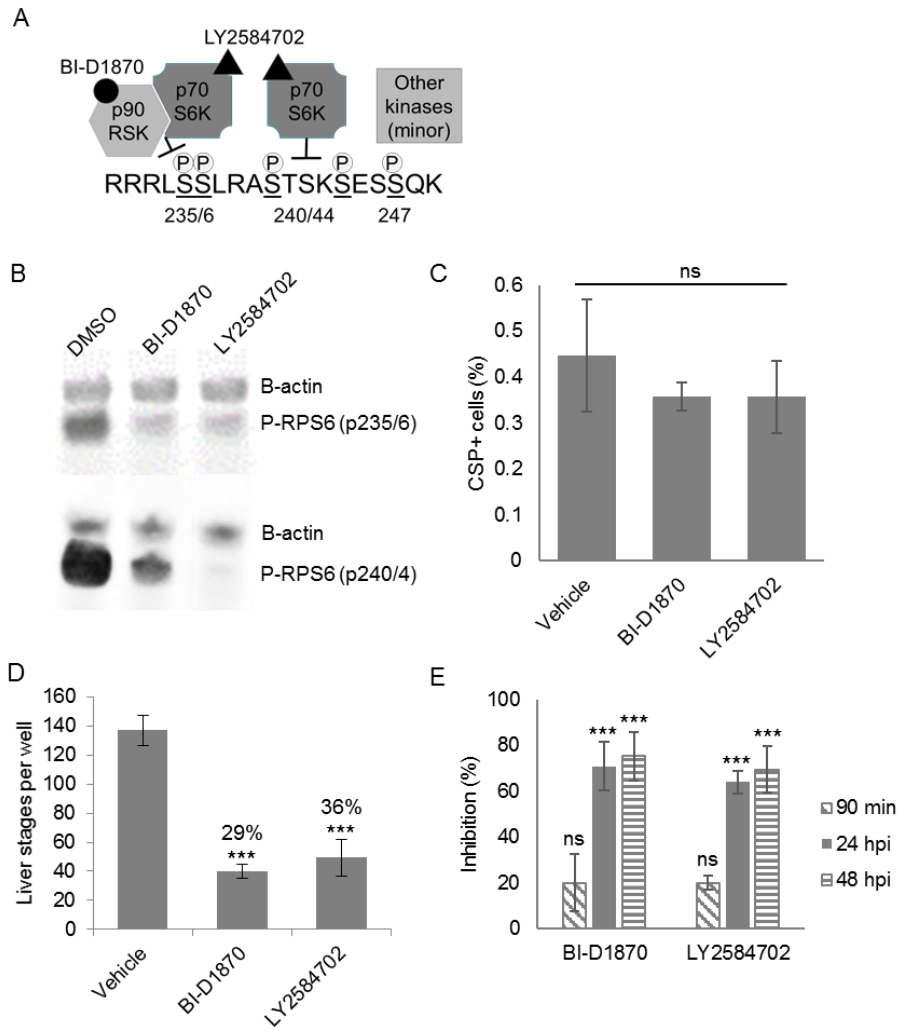

Figure 4.3: Small-molecule inhibition of RPS6 phosphorylation decreases *P. yoelii* infection in vitro

Small-molecule inhibitors block S6K and RSK phosphorylation of RPS6 at two phosphosites, S235/236 and S240/244. A third site, S247, is not thought to be biologically relevant to S6 function (A). Cells were treated for 24 hours with BI-D1870, which inhibits p90RSK, LY2584702, which inhibits p70S6K, or DMSO Vehicle, then stimulated with insulin for 10 minutes before lysis (B). Treatment with inhibitors does not significantly change the amount of CSP+ cells at 90 minutes post infection, as measured by flow cytometry (C). At 24 hours post infection, parasites were counted by microscopy. Cells treated with both inhibitors show significantly fewer parasites than those treated only with DMSO. Numbers represent the percentage of parasites compared to vehicle (D). Parasite inhibition when compared to vehicle-treated cells dramatically increases between 90 minutes and 24 hours post infection, with a slight additional decrease in parasite load between 24 and 48 hpi. Significance is measured compared to vehicle control at each time point (E). (*p<0.05, **p<0.01, ***p<0.001, ns=no significance)

RPS6 phosphorylation inhibition decreases parasite load in mice

In vitro systems are imperfect models for parasite infection. Hepatoma cell lines have significant physiological differences from primary hepatocytes, and parasite development and timing is also quite different *in vitro* than in the host liver. Thus, we moved to an *in vivo* model of infection to confirm the effect of p-RPS6 inhibition. Mice were treated for 24 hours with the inhibitors, then infected with a luciferase-expressing parasite, *PyGFP-Luc*. Inhibitor treatment was continued throughout liver stage development, and liver burden measured using bioluminescent *in vivo* imaging at 24 and 44 hours post infection [46] (Fig. 4.4A).

At 24 hours after infection, all treatments showed similar levels of liver burden (Figure 4.4B, D). However, at late liver stage, 44 hours post infection, both inhibitors show significantly reduced liver stage parasite burden (Figure 4.4C, E). Infected livers were then harvested for histological and fluorescent imaging. Treatments did not show damage to hepatocytes nor significant levels of immune cell infiltrates, indicating that the drop in parasite burden is not directly due to an immune response (not shown). Quantification of liver stage parasites shows that small molecule treatment does not affect the total size of parasites (Figure 4.4F), but does affect the number of parasites in the liver (Figure 4.4G). This suggests that the drop in apparent parasite burden is due to the elimination of parasites, presumably by host cell apoptosis or autophagy.

Small-molecule treatment causes delay in late-stage development

RPS6 is conserved among Eukaryotes, with a highly conserved sequence between organisms [47]. In fact, the human and mouse proteins are identical, and share a 48% sequence identity with *Plasmodium spp*. despite the evolutionary differences between the taxa (Fig. 4.5A). However, the bulk of the sequence identity is skewed toward the N-terminus of the protein, with

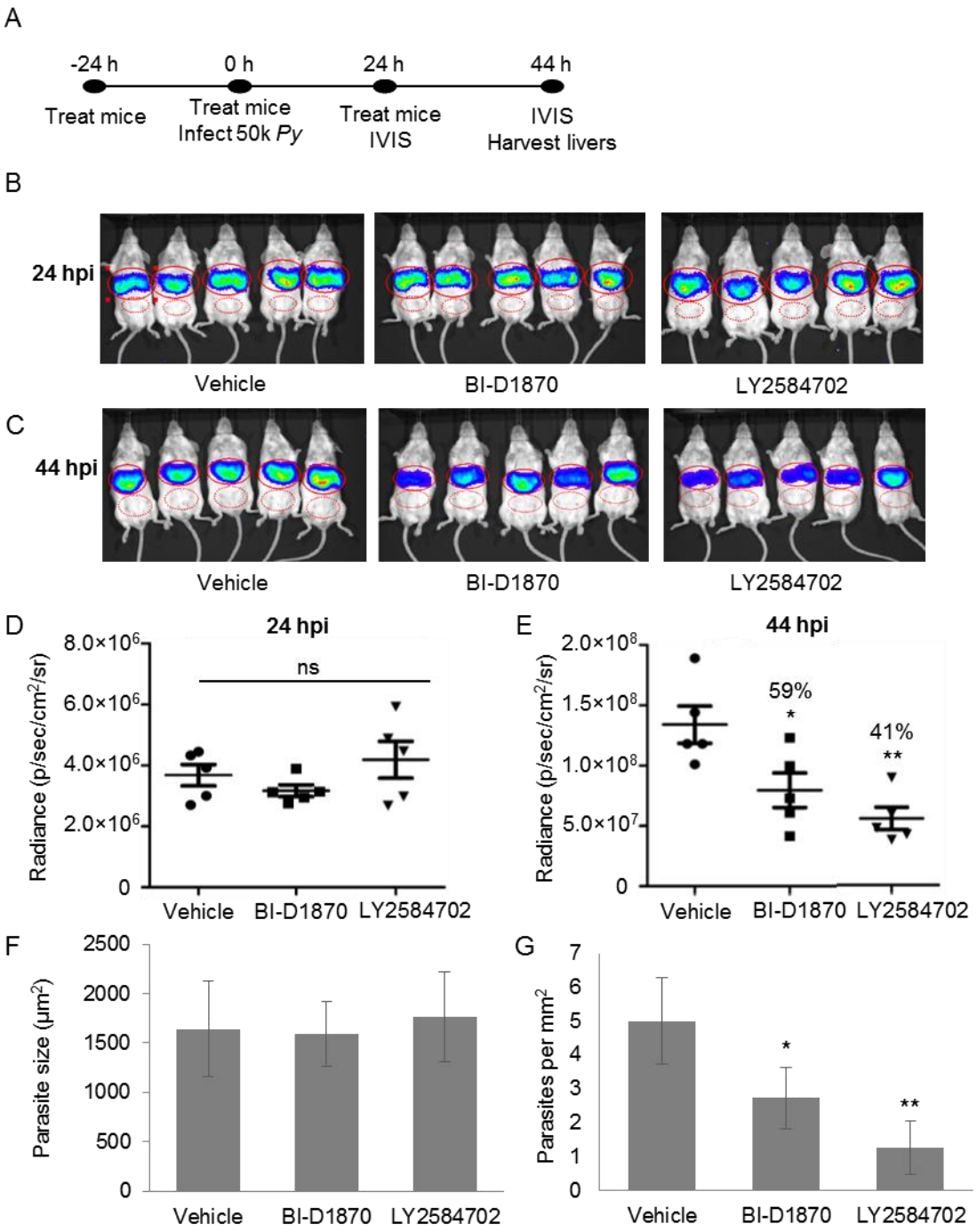

Figure 4.4: Inhibitors of RPS6 phosphorylation decrease late liver burden in mice

Mice were treated with p-RPS6 inhibitors at days -1, 0, and 1. They were infected with 50k luciferase-expressing *P. yoelii* sporozoites at day 0, and liver burden measured at day 1 (24 hpi) and day 2 (44 hpi) (A). At 24 hpi, mice treated with inhibitors showed no significant difference in liver burden using inhibitors (B,C). However, at 44 hpi liver burden was significantly lowered in treated mice (D,E). Size of 44 hour liver stage parasites shows no difference between treatments and vehicle (F), however, parasite number is reduced (G).

much less similarity at the C-terminus where phosphorylation occurs. Though *Plasmodium* RPS6 sequences contain phosphorylatable serines, they differ significantly in motif, most notably lacking the SS235/236 pair that is the site of primary initial phosphorylation in mammals. *Plasmodium* genomes contain orthologs for both p70S6K and p90RSK kinases, but it is unclear whether RPS6 itself is phosphorylated in the parasite. Both kinase orthologs show approximately 40% sequence similarity to mouse kinases; it is likewise unclear whether this similarity would allow our compounds to target parasite kinases.

To explore whether the effect of these kinase inhibitors compounds is due to action in parasites directly rather than in the host cells, we tested their effect on replication of *Plasmodium falciparum* blood stage parasites, which unlike *P. yoelii*, can be grown in culture. After 48 hours of culture, parasites treated with either BI-D1870 or LY2584702 showed a reduction in parasite replication, though the decrease is of lower magnitude than seen in the liver (Fig. 4.5B). This suggests there may be a slight growth defect caused by the inhibitors; however, even this may be partially due to host rather than parasite factors as red blood cells have high levels of p-RPS6 driven by mTOR signaling [50]. Regardless, neither of the inhibitors showed a greater than 20% reduction in parasite growth, compared to a 93% decrease when treated with the anti-parasite drug chloroquine.

The *P. yoelii* liver stage matures from a sporozoite into tens of thousands of progeny, termed merozoites, in approximately 48 hours [51]. The genome and organelles replicate and are then packaged into individual merozoites within the liver form known as a exo-erythrocytic schizont. Early schizogony involves replication of DNA and organelles, as well as creation of cytomeres, or invaginations of the parasite plasma membrane which will eventually make up thousands of individual merozoite surface membranes; however, only in the last few hours of

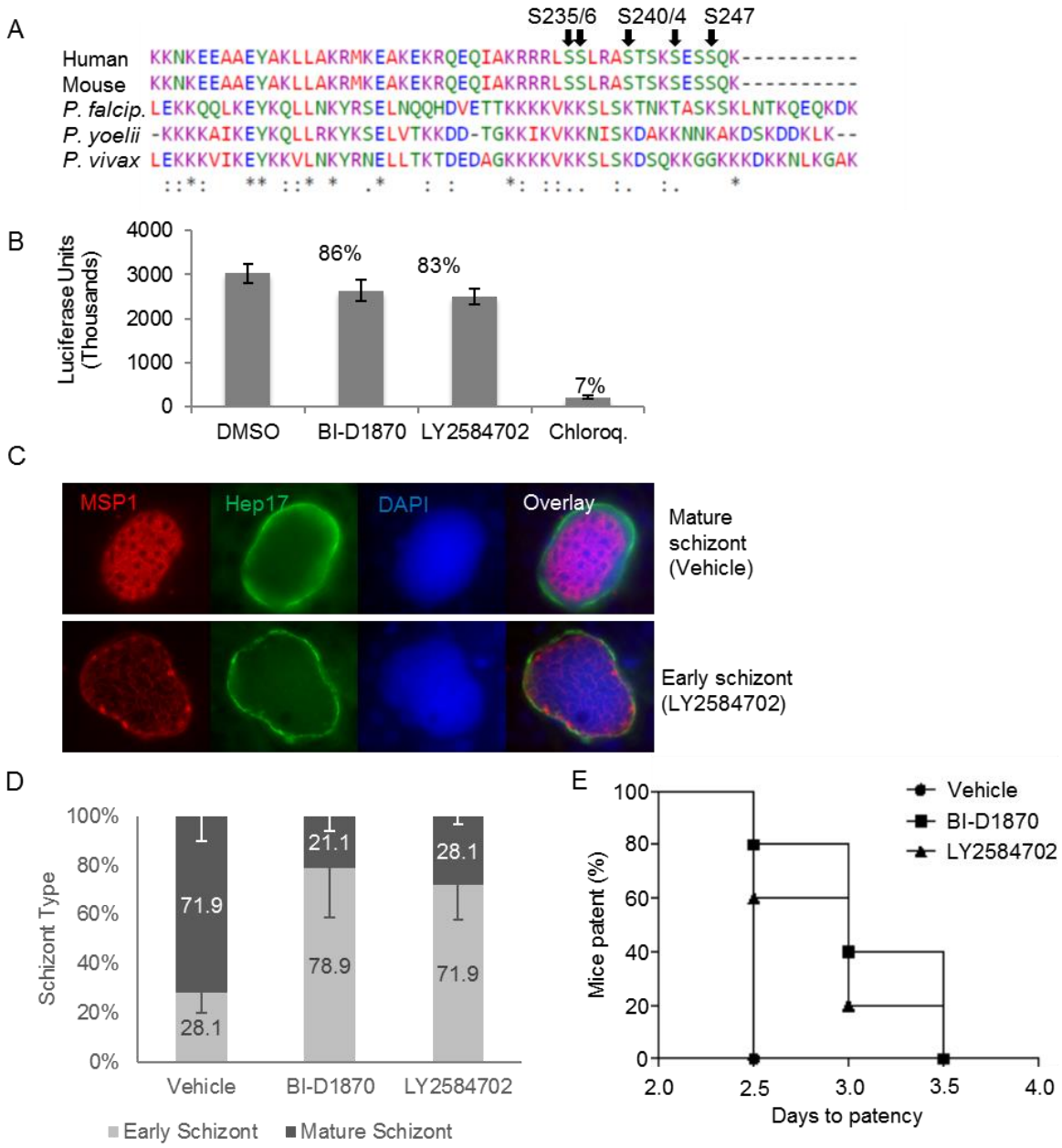

Figure 4.5: Inhibitor compounds cause mild delay in parasite growth

The phosphorylatable C-terminus of mammalian RPS6 differs dramatically with that of *Plasmodium* homolog, which lacks the critical SS motif which is the main site of phosphorylation (at 235/236). Homologs were identified using Protein BLAST [48] and aligned with Clustal Omega [49] (A). Growth of *P. falciparum* blood stages was measured by luciferase expression after 48 hours of incubation with p-RPS6 inhibitors *in vitro*. Inhibition of RPS6 phosphorylation has only a mild effect on blood-stage parasite replication compared to vehicle control. Numbers represent the percentage of parasites compared to vehicle (B). Mice treated with inhibitors show both early and mature schizonts at 44 hpi (C) but have a lower proportion of mature schizonts compared to vehicle-treated mice (D). Inhibitor treatment also caused a half- to one day delay in blood stage patency (E), indicating delayed maturation.

schizont maturation does this segregation into individually membrane-packaged merozoites occur [52]. These membranes, both the cytomere of early schizogony and the individual merozoites of late schizogony, can be visualized microscopically by staining for the Merozoite Surface Protein (MSP1). In both vehicle and inhibitor treated livers, both early schizonts and mature schizonts can be seen at 44 hpi (Fig 4.5C). However, in vehicle-treated livers the majority of schizonts are mature, with individual merozoites packaged within MSP1-containing membranes. In contrast, in both BI-D1870 and LY2584702 treated mice the majority of schizonts showed only early cytomeric membrane staining, indicating that treatment with these small molecules delays schizont maturation (Fig 4.5D). In addition, mice treated with these inhibitors show a half-day to one-day delay in transition from the liver stage to blood stages as seen by microscopy (known as patency) (Fig 4.5E), which directly reflects the number of parasites within the liver and thus the number of infective merozoites produced [53]. A one day delay usually corresponds to a log-fold reduction in liver stage parasitemia [54], which is a larger reduction than shown with either IVIS or parasite counts and supports the hypothesis that small molecule treatment delays schizont maturation into merozoites.

Phosphorylation-null mice trend toward lower liver burden

Both RPS6 kinases, p70 S6K and p90 RSK, have numerous other targets [55], [56], many of which could be beneficial to the parasite. To determine whether the effect on parasite burden and growth is specifically due to p-RPS6, and not another downstream target, we turned to a mouse with a modified RPS6 lacking phosphorylatable residues, termed the $RPS6^{p-/-}$ mouse [25]. These mice have no defect in either S6K or RSK kinases, or in other kinase targets, but all five serines on the RPS6 C-terminus have been mutated to alanines and thus cannot be phosphorylated.

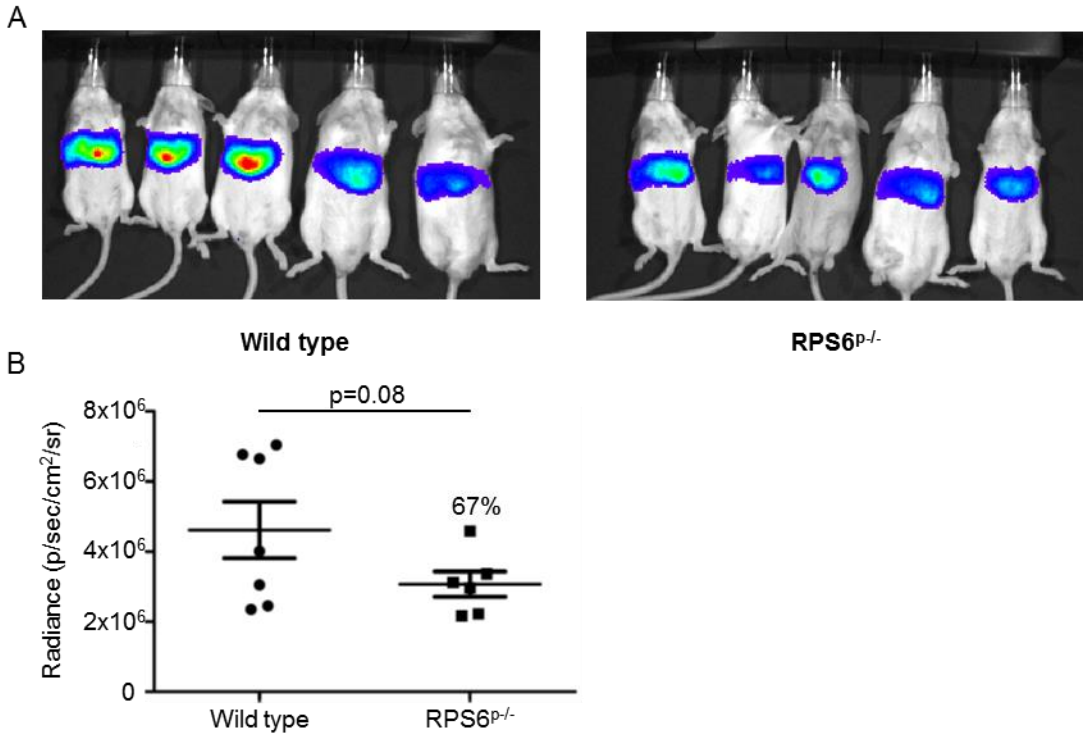

Figure 4.6: Phosphorylation-null RPS6$^{p-/-}$ mice show lowered liver burden

At 44 hours post infection, RPS6$^{p-/-}$ bear fewer parasites than littermate wild type mice, as measured by luciferase activity. Mice shown include three females (left) and two males (right) in each group (A). The median luciferase activity in the phospho-null mutants is two-thirds that of wild type mice, though the wild type mice show a large range of parasite burden and the statistical analysis does not reach significance (B). A total of seven wild type and six mutant mice were analyzed by IVIS and paired by sex and coat color for statistical analysis using a paired student's t-test.

We infected phospho-null RPS6$^{p-/-}$ with *PyGFP-Luc* in parallel with wild-type littermates. As with the inhibitor treatment, no difference in luciferase expression was seen at 24 hours. However, at 44 hours after infection, the mice overall showed an observable, though not statistically significant, trend toward lowered liver stage burden (Fig. 4.6). Unlike with inhibitors, no difference in parasite maturation, nor a delay in patency, was observed.

DISCUSSION

In this study, we have identified phosphorylation of Ribosomal Protein S6 as a factor in hepatocyte infection by *Plasmodium yoelii*. Interrogation of two models of differential hepatocyte susceptibility to infection by reverse-phase protein microarray revealed p-RPS6 as the only antigen which was significantly increased in both susceptible hepatocyte phenotypes, polyploid (8n) hepatocytes and BALB/cByJ mouse livers. Inhibition of both major kinases of RPS6 lowers parasite survival both *in vitro* and *in vivo* and appears to delay maturation of the exo-erythrocytic schizont into merozoites. Likewise, mice lacking phosphorylation sites on RPS6 display a lowered liver stage parasite burden, though not delayed schizont maturation.

Hepatocyte phosphorylation of RPS6 could benefit the parasite in numerous ways. Phospho-RPS6 has been shown to decrease protein translation, as mouse fibroblasts with the $RPS6^{p-/-}$ mutation, which are incapable of phosphorylation, show greater global protein synthesis than wild type cells [24]. It would appear, then, that RPS6 phosphorylation decreases the incorporation of amino acids into proteins, and thereby increase the relative amount of free amino acids in the cytoplasm. As growth of liver stage parasites from entry until exit involves an increase in size on the order of magnitude [57], parasites require a great deal of cellular building blocks, including amino acids. While *Plasmodium* is capable of some amino acid biosynthesis, it lacks the genes necessary for biosynthesis of several essential amino acids [58]. Therefore it seems logical to assume that cellular availability of free amino acids would be of benefit to the developing parasite. In fact, it has been demonstrated that the increase of amino acids due to the autophagic breakdown of host proteins increases robust parasite development [4]. Intriguingly, it has been demonstrated that parasite circumsporozoite protein (CSP) also decreases protein translation *in vitro* [59], possibly by directly interacting with ribosomes [60]. Biochemical work

to show whether CSP and p-RPS6 have direct interactions may be a worthwhile direction for future studies.

Phospho-RPS6 is also linked to increased glucose availability in both pancreatic B-cells [25], [26] as well as in muscle cells [25], [27], apparently through promotion of insulin secretion. Insulin signaling induces a number of cellular pathways in the hepatocyte, including import of glucose and lipids from the blood. Glucose concentration plays a large role in *P. berghei* development *in vitro* [61]. Optimum development seen with at least 2 mg/mL glucose concentration, which is about double the average blood glucose concentration; thus, parasites may have an advantage *in vivo* if they infect hepatocytes with higher glucose biosynthesis or uptake. In addition, RPS6 phosphorylation has been shown to increase lipid biosynthesis in cancer, and has been linked to ATP levels in muscle cells [27]. Thus, phospho-RPS6 may increase nutrient availability to the rapidly developing parasite.

It is also critical for the parasite to avoid death of the host cell. Apoptosis has been shown to decrease parasite load, and high levels of the pro-apoptotic p53 is refractory to successful parasite development [10]. Conversely, blocking the Bcl-xL apoptosis pathway increases parasite load in the liver [62]. Phosphorylation of RPS6 has been shown to be partially protective in cancer models—$RPS6^{p-/-}$ cells show a dramatic increase of cleaved caspase-3 in response to stress in Kras-related pancreatic cancer [29], although this increase is not found in noncancerous cells. Tellingly, these phospho-null mutants show three-fold higher p53 levels. In addition, phospho-null mutants are more susceptible to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), while a mutant RPS6 that mimics constitutive phosphorylation was more resistant [28]. This function of p-RPS6 would obviously be a benefit to the parasite.

Phosphorylated RPS6 was the only protein found to be upregulated to a statistically significant level; however, one other intriguing lead was found in the susceptibility screen. Superoxide dismutase 2 (SOD2) is a mitochondrial protein which converts the toxic reactive oxygen species (ROS) superoxide into diatomic oxygen (O_2) and hydrogen peroxide, and thus forms a critical step in the prevention of oxidative stress [63]. *Plasmodium* species thrive in a hypoxic environment [64], but hypoxia is known to create ROS as a byproduct [65], [66] and high levels of ROS can trigger intrinsic apoptosis, to which the parasite is vulnerable. Previous evidence suggests that the parasite has ways of preventing apoptosis of its host cell [67]; perhaps the increase in SOD2 levels is a feature which makes both BALB/cByJ and highly polyploid hepatocytes beneficial to the parasite and contributing to their increased susceptibility to infection. Future work interrogating the role of host SOD2 would be an intriguing line of inquiry.

This work has shown that inhibition of phosphorylation of RPS6 decreases infection with *Plasmodium* liver stage parasites. However, several questions remain. First, it appears that despite differences in parasite and host RPS6 sequences, the kinase inhibitors used do delay parasite growth to a degree. The extent of inhibition seen in blood stage growth does not appear to fully explain the phenotype of lowered infection; however, we cannot rule out that the delay in parasite growth may be directly causing parasite clearance during the course of liver infection. In addition, both RPS6 kinases, p70S6K and p90RSK, have numerous other targets [55], [56], many of which could be beneficial to the parasite. While the microarray data show only significant changes in levels of p-RPS6 rather than other kinase targets, we cannot rule out off-target effects of the inhibitors. In fact, the delay in schizont maturation and delay in patency in inhibitor-treated mice, but not in $RPS6^{p-/-}$ mice, suggests this may be the case. This question

could be resolved by testing the effect of the inhibitors in the RPS6$^{p-/-}$ mouse [25]. If an additional drop in liver burden is seen with the inhibitors despite the absence of p-RPS6, it will be clear that off-target action of the inhibitors at least contributes to the phenotype.

The results of infection in RPS6p-/- mice at least suggests that p-RPS6 is important in liver stage infection. However, the data is difficult to interpret. Part of the difficulty stems from the fact that these mice are not fully inbred [25] and have some degree of genetic diversity even among littermates. All mice used in this study were derived from the same individual grandparents but a diversity of coat color shows that the mice contain multiple alleles. This can be somewhat minimized by matching the mice by litter and coat color, but the mice still may differ genetically. We have seen in the BALB/c model that even small differences in the genome can have dramatic effects on susceptibility [14], so even the differences between siblings may be enough to change infectivity regardless of RPS6 phosphorylation. This can be addressed through back-crossing, but this was outside the scope of this work. Nevertheless, additional mouse numbers in future experiments will make clear whether the effect of the RPS6$^{p-/-}$ mutation is due directly to RPS6 phosphorylation.

MATERIALS AND METHODS

Cell lines, cell culture, and experimental animals

In vitro, Hepa1-6 cells were used for *P. yoelii* infections and Western Blots. Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) complete media (Cellgro), supplemented with 10% FBS (Sigma-Aldrich), 100IU/ml penicillin (Cellgro), 100mg/ml streptomycin (Cellgro), 2.5mg/ml Fungizone (HyClone/Thermo Fisher) and GlutaMAX (Thermo Fisher), and split 1–2 times weekly. Female BALB/cAnN mice (6-8 weeks old) were purchased

from Harlan Laboratories. Female BALB/cJ and BALB/cByJ (6-8 weeks old) were purchased from Jackson Laboratories. RPS6$^{p+/+}$ and RPS6$^{p-/-}$ mice were developed by Oded Meyuhas [25] and kindly provided by Mario Pende (INSERM, France). All mice were maintained in accordance with protocols approved by Center for Infectious Disease Research Institutional Animal Control and Use Committee (IACUC).

RPS6 phospho-null mice breeding and genotyping

RPS6$^{p+/+}$ and RPS6$^{p-/-}$ mice were bred from a mixed genetic background with variable coat colors. Female RPS6$^{p+/+}$ and male RPS6$^{p-/-}$ mice were bred to create heterozygotes. F1 mice were paired by coat color and bred to create RPS6$^{p+/+}$ and RPS6$^{p-/-}$ littermates. Pups were genotyped via automated qRT-PCR (Transnetyx) and confirmed with qPCR using a SYBR green assay (Invitrogen). Primers tested for presence of the neomycin resistance gene cassette (5'- CTTGGGTGGAGAGGCTATTC and 3'- AGGTGAGATGACAGGAGATC) and normalized to an internal positive control (T-cell receptor, 5'- CAAATGTTGCTTGTCTGGTG and 3'- GTCAGTCGAGTGCACAGTTT).

Mosquito rearing and sporozoite production

For *Plasmodium* sporozoite production, female 6–8-week-old Swiss Webster mice (Harlan) were injected with blood stage *P. yoelii* 17XNL parasites to begin the growth cycle. Animal handling was conducted according to the Institutional Animal Care and Use Committee-approved protocols. Infected mice were used to feed female *Anopheles stephensi* mosquitoes after gametocyte exflagellation was observed. Salivary gland sporozoites were isolated according to the standard procedures at days 14 or 15 post blood meal. Strains used include wild-type *Py*17XNL, *Py*UIS4-Myc, and *Py*GFP-Luc.

Hepatocyte isolation and processing

Hepatocytes were isolated from mice using a two-step perfusion *in situ*. Portal veins of anesthetized mice were cannulated with warmed perfusion buffer (1x HBSS w/o calcium, magnesium supplemented with 8 mM HEPES buffer and 0.5 mM EDTA) at low flow rate. Once cannulated, the inferior vena cava was severed to blanch the liver. The buffer flow rate was increased to 5 mL/min and allowed to perfuse for five minutes with occasional clamping and releasing of the vena cava to inflate the liver. The liver was then perfused with 2% collagenase II (Worthington) in collagenase buffer (HBSS supplemented with 8 mM HEPES and 0.5 mM CaCl2) for five minutes at 5 mL/min. The liver was then removed from the abdominal cavity and the gall bladder excised. The liver was placed in a dish of DMEM without serum and gently pushed through a 100-micron cell strainer to dissociate the hepatocytes, which were then collected with a wide-bore syringe. The liver cell suspension was centrifuged at 50x g, and the remaining pellet washed in DMEM without serum. This step was repeated twice until the supernatant was clear, to isolate hepatocytes from cell debris and non-parenchymal cells.

Protein lysate microarray

For BALB/c substrains, whole livers from BALB/cJ and BALB/cByJ mice (n=10 per strain) were snap frozen and ground, then reconstituted in SDS Lysis Buffer SDS lysis buffer (2% SDS, 50 mM Tris-HCl, 5% glycerol, 5 mM EDTA, 1 mM NaF, 10 mM β-glycerophosphate, 1 mM PMSF, 1 mM activated Na_3VO_4, 1 mM DTT, 1% phosphatase inhibitor cocktail 2 and 1% phosphatase inhibitor cocktail 3 (Sigma-Aldrich), 1% PhosSTOP Phosphatase Inhibitor Cocktail Tablet (Roche), and stored at −80°C. For ploidy, livers of BALB/cAnN mice were perfused and processed as described above. Cells were stained for DNA content with Vybrant Orange (Invitrogen) in DMEM without serum, then sorted on a BD Influx (BD Biosciences).

Custom lysate microarrays were printed in-house using an Aushon Biosystems 2470 arrayer (Aushon Biosystems) on 16-pad nitrocellulose-coated glass slides (Grace Bio-Labs). Lysates were arrayed at 333 μM spacing using solid 110 μM pins, which resulted in an average feature diameter of 170 μM. Lysates were arrayed in quadruplicate technical replicates. Slides were then stored dry, in the dark, and at room temperature until probing. Slides were probed and quantified as previously described by Sevecka *et al*. [35].

In vitro immunofluorescence and counting

1.5×10^5 Hepa1-6 cells were seeded in each well of an 8-well Permanox chamber slide (NUNC Inc.). Cells were infected with 5×10^4 *P. yoelii* UIS4-Myc sporozoites. Slides were centrifuged for 3 min at 515*g* in a hanging-bucket centrifuge to aid in sporozoite invasion. After 90 min, media containing extracellular sporozoites was aspirated, and fresh media containing, drug or vehicle as noted, was added. Media and drugs were changed every 24 hours. LSs developed for 24 or 48 hours, at which time cells were fixed with 10% formalin, blocked and permeabilized for 1 hour in PBS with the addition of 0.1% TritonX-100 and 2% bovine serum albumin (BSA). Staining steps were performed in PBS supplemented with 2% BSA. Cells were stained using anti-sera to *Plasmodium* heat shock protein 70 (HSP70) and anti-myc monoclonal antibody (Abcam) 4^{o}C overnight, then visualized with the use of AlexaFluor-488 donkey anti-mouse and AlexaFluor-594 donkey anti-goat secondary antibodies (Invitrogen). Cells were stained with DAPI to visualize both hepatocyte and parasite nuclei. Sporozoites that had not invaded and/or developed in hepatoma cells were distinguished by UIS4 non-circumferential staining and morphology.

Flow cytometry of infected and uninfected cells

Hepa1-6 cells were harvested with 0.25%Trypsin-EDTA and fixed with Cytoperm/Cytofix (BD Biosciences). The cells were blocked with Perm/Wash (BD Biosciences) +2% BSA for one hour at room temperature, then stained for overnight at 4C with primary antibodies. Cells were washed three times with PBS, then stained for one hour at room temperature with secondary antibodies (donkey anti-mouse or anti-rabbit AlexaFluor 488). Infected cells were also incubated with monoclonal antibody to P. *yoelii* Circumsporozoite protein conjugated to AlexaFluor 647 (Life Technologies). The cells were washed and suspended in PBS+5 mM EDTA. For ploidy measurements, RNAse A (0.1 mg/mL) and SYTOX Blue (Invitrogen) were added to the resuspension medium. Infection rate, protein signal, and DNA content were measured by flow cytometry on an LSRII (Becton-Dickson) and analyzed by FlowJo (Tree Star). Primary antibodies used include rabbit anti-p-S6 (S235/6) at 1:50 and mouse anti-beta actin at 1:200 (Cell Signaling Technologies).

Western blotting

For BALB/c blots, whole liver lysate was gathered as described above. For drug treatments, $5x10^5$ Hepa1-6 cells were seeded in each well of a 12-well plate. Drugs or DMSO control were added to the medium, and cells were incubated for 24 hours. Insulin-transferrin-sodium selenite (ITSS) was then added to the medium for 10 minutes to stimulate cells. Media was aspirated and the cells were lysed *in situ* by SDS lysis buffer at 4C for 20 minutes. Lysate was harvested and filtered using filter plates spun for 5 minutes at 1500 rpm. 4x Western Loading Dye (Invitrogen) was added and lysates were boiled for 5 minutes. iBlot gel system was used to run proteins, then transferred using the ePAGE transfer system (Invitrogen).

Membranes were briefly rinsed in methanol and ddH_2O, then blocked for at least 2 hours at room temperature in TBS+0.5% Tween-20+5% BSA. Primary antibodies were added and

incubated at 4C overnight. All primary antibodies are all from Cell Signaling Technologies, and were used at 1:1000. Blots were then washed 5x 5 minutes in TBS-Tween, then incubated in TBS-Tween-BSA with secondary antibodies (anti-mouse 800, 1:12,000, and anti-rabbit 647, 1:10,000; Licor). Blots were washed a second time, allowed to dry, and visualized and analyzed via Odyssey IR scanner (Licor).

Blood stage luciferase assay

In vitro P. falciparum NF54 GFP-Luc blood-stage cultures were maintained in RPMI 1640 (25 mM HEPES, 2 mM l-glutamine) supplemented with 50 μm hypoxanthine and 10% A^+ human serum in an atmosphere of 5% CO_2, 5% O_2, and 90% N_2. Cells were subcultured into O^+ erythrocytes to a level of 2.5% parasitemia and 50% hematocrit. Blood culture was put into wells of a 96-well plate, and treated with drugs at noted levels in triplicate, and incubated as above. 72 hours after culture, culture was resuspended and mixed with Bright-Glo Luciferase (Promega) and luminescence measured using a Centro XS^3 plate reader (Berthold).

Small molecule treatment

BI-D1870 and LY2584702 (SelleckChem) were resuspended in DMSO at a stock solution of 10 mM (BI-D1870) or 1 mM (LY2584702). Stocks were diluted into cell media using serial dilutions to 1 μM (BI-D1870) or 100 nM (LY2584702) and added to Hepa1-6 cells. Cells were treated 24 hours previous to infection, and media and treatment refreshed every 24 hours thereafter.

***In vivo* treatment and infection**

Drugs were prepared by initial suspending in DMSO to a stock solution, then diluting the stock in warmed PBS and Tween-80, gently pipetting to avoid precipitation. Final vehicle was

2% DMSO+5% Tween-80+drug: BI-D1840 (50 mg/kg) or LY2584702 tosylate (12.5 mg/kg). Drug was injected intraperitoneally every 24 hours at days -1, 0, and 1. At day 0, $5x10^4$ *P. yoelii* GFP-Luc sporozoites were injected via tail vein. Liver stage burden of *PyGFP-luc* development was monitored by *in vivo* bioluminescence imaging (IVIS) as described below. Liver stage burden was also monitored by determining the day of onset of blood-stage parasitemia by analyzing Giemsa-stained blood smears twice daily.

For $RPS6^{p-/-}$ infection, wild type and phospho-null littermates were infected in parallel with $5x10^4$ *PyGFP-Luc* sporozoites injected via tail vein. Liver stage burden was determined by IVIS and blood-stage parasitemia. Data was analyzed by matching mice by sex and coat color using a paired t-test.

***In vivo* Bioluminescent Imaging of Liver-Stage Development**

Luciferase activity in animals was visualized through imaging of whole bodies using the *in vivo* Imaging System (Caliper Life Sciences) as previously described [46]. Mice were injected intraperitoneally with 100 μl of RediJect D-Luciferin (Perkin Elmer) prior to being anesthetized using the isoflurane-anesthesia system (XGI-8, Caliper Life Sciences). Bioluminescence images were acquired with a 10 cm field of view, medium binning factor, and an exposure time of 3–5 min. Quantitative analysis of bioluminescence was performed by measuring the luminescence signal intensity using the region of interest (ROI) settings of the Living Image 3.0 software. ROIs were placed around the abdominal area at the location of the liver and background luminescence subtracted based on Background ROI measurements on the lower abdomen.

Immunofluorescence staining

Livers were harvested at 44 hpi, rinsed thoroughly in PBS, then fixed in 10% formalin for 48-72 hours at 4C. Liver lobes were cut into 50 μm sections using a Vibratome apparatus (Ted Pella Inc.). Sections were permeabilized in Tris buffered saline (TBS) containing 3% H_2O_2 and 0.25% Triton X-100 for 30 min at room temperature, then blocked in TBS containing 5% dried milk (TBS-M) at least 1 h and incubated with primary antibody in TBS-M at 4°C overnight. Primary antibodies used were mouse monoclonal anti-Hep17 (a PVM marker) and rabbit polyclonal anti-MSP1 (merozoite surface membrane marker). After washing in TBS, secondary antibody was added in TBS-M for 2 h at room temperature in a similar manner as above. After further washing, the section was incubated in 0.06% $KMnO_4$ briefly to quench background fluorescence. The section was then washed with TBS and cells were stained with DAPI to visualize the DNA and mounted. Liver stages were visualized, measured, and counted using a Nikon upright fluorescence scope and MetaMorph software.

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CHAPTER FIVE:

THESIS RECAP AND FUTURE DIRECTIONS

RECAP OF THESIS

The preceding chapters presented data regarding types of hepatocytes showing a phenotype of increased susceptibility to *Plasmodium* infection. Work done previous to this thesis [1], [2, Appendix A] showed upregulation in cell cycling and proliferation signaling pathways in *Plasmodium*-infected hepatocytes. In Chapter Two, I demonstrated that cell cycling plays no role *in vivo* infection (Figure 2.2), which was later confirmed by others [3]. Rather, the ploidy state of hepatocytes influences the susceptibility to infection (Figures 2.1 and 2.4). The increase in susceptibility of highly polyploid hepatocytes is at independent of cell size and liver zonation (Figures 2.6 and 2.7). As the difference in infection occurs at or soon after the point of infection, it is unlikely that the parasite directly senses the ploidy state of the hepatocyte; rather, the increased infectivity seems at least partially due to changes in the complement of hepatocyte surface proteins (Figure 2.8). Importantly, this work determines that hepatocyte polyploidy is one of very few known host factors of susceptibility that is conserved between parasite species (Figure 2.5).

Chapter Three describes the quantification of a second phenotypic variability in infection, between the two closely related mouse substrains BALB/cJ and BALB/cByJ. While the differential liver stage susceptibility of these two substrains had been informally known, this paper was the first rigorous description of this difference. We describe a consistent five-fold increase in liver stage burden in BALB/cByJ mice when compared to BALB/cJ (Figure 3.1), which appears to be due to increased hepatocyte infection rather than immune system clearance or differential development in the liver (Figure 3.2). The known host protein factors of infection CD81, SR-B1, and p53 are not differentially expressed in the two substrains (Figure 3.3) but BALB/cByJ mice show higher polyploidy (Figure 3.4) as well as increased EphA2 [4, Appendix

C]. Because the genetic background is so similar between the two strains, as they only diverged less than 75 years ago [5], we conducted a broad, unbiased search for host candidates of susceptibility using RNA-Seq transcriptomics. We identified a total of 137 total candidates including several known host membrane proteins (Figure 3.5) for potential future work.

In Chapter Four, I again chose a partially unbiased approach, the reverse-phase protein microarray, to more closely interrogate the phenotypes described in the previous two chapters. In order to more likely identify true factors of infection, I compared the hits from each set of arrays to find candidates present in both phenotypes (Figure 4.1). In this screen, I identified the phosphorylated version of Ribosomal Protein S6 (RPS6) as being significantly and strongly upregulated in both susceptible cell sets (Figure 4.2). Blocking RPS6 phosphorylation by small molecule kinase inhibitors led to a drop in *Plasmodium* infection both *in vitro* and *in vivo* (Figures 4.3 and 4.4). Unlike the phenotypes seen in the first two chapters, phosphorylated RPS6 does not seem to affect susceptibility at the point of infection; rather it affects persistence of the parasite over the course of development.

Taken together, this body of work shows that *Plasmodium* parasites do not find hepatocytes to be equal. Certain hepatocytes are clearly preferred as host cells. Numerous lines of inquiry, including those presented in this thesis, have led to the hypothesis that parasite selection of hepatocytes for productive invasion, rather than traversal, allows establishment of the rapidly growing and reproducing parasite in a supportive environment. Even more fascinating is the mounting evidence that the hepatocyte environment is drastically changed by parasite infection in ways that support development [1], [2], [6], and some evidence pointing to active manipulation of host systems for its benefit [7]–[10]. Understanding the needs of the

parasite during liver stage infection opens up a world of new interventional strategies [11, Appendix B] critical for the disruption, and eventual elimination, of malaria.

FUTURE DIRECTIONS

Looking beyond candidate-based approaches

The hepatocyte is highly complex, with thousands of surface proteins alone and webs of signaling pathways contributing to highly precise and varying cellular responses; thus, identification of host factors of susceptibility cannot be a matter of testing individual candidates. Some success in identifying *Plasmodium* hepatocyte entry factors has been achieved by examining host factors needed by other hepatotropic pathogens, particularly the Hepatitis C virus [12], [13], even though the mechanism of entry is known to differ significantly from these viruses [13]–[15]. Similarly, though the parasite mechanism of entry is likely to be similar to other Apicomplexa, such as *Toxoplasma*, the specific tropism and low infection rates of *Plasmodium* sporozoites differs significantly from *Toxoplasma,* which infects multiple cell types at very high rates [16], [17]. Therefore, while these approaches have led to some important insights regarding the way which the parasite and host engage, fundamental differences between these homologous systems and *Plasmodium* will inevitably miss some of the interactions that drive the exquisite selectivity that the malaria sporozoite has for its hepatocyte host.

The complex biology of both mammalian hepatocytes and Plasmodium mean that candidate-based approaches, while powerful, simply cannot fully capture the interplay between host and parasite. While all of the individual parasite and host factors discovered for liver stage invasion, growth, and development are clearly important, there are no "magic bullets" that will explain any given interaction on its own. It seems likely from current data that no single hepatocyte surface protein is "the" parasite entry factor, but rather that entry of the parasite and creation of the PVM requires a multi-factorial and spatially and temporally arranged set of interactions between host and parasite proteins [18], [19]. Likewise, no single host response is

likely to be crucial for either parasite development or clearance by the host; rather, parasite survival likely depends on a finely tuned intracellular environment consisting of numerous cellular pathways working in concert [1], [2], [9].

Fortunately, newly developed but underutilized tools, publically available data, and new high-throughput techniques are on the cusp of revolutionizing the study of liver stage malaria. Large scale '-omics' technologies have the ability to approach the complex and multi-faceted interactions between the *Plasmodium* parasite and its hepatocyte host by generating large amounts of relatively unbiased data about both parasite and host cells, individually and simultaneously, driving discovery down to the molecular level. Many of these large datasets have already been generated; other datasets can be generated using increasingly inexpensive and precise technologies that can provide unbiased '-omics' on small and rare sample quantities.

In this thesis I have shown the usefulness of this approach, as a relatively small-scale proteomic approach has identified a candidate, p-RPS6, which has proved to play a role in liver stage infection. Comparison of larger datasets, or of more phenotypes, may even more powerful analyses. Using an iterative, cross-disciplinary approach (Figure 5.1), computational systems biology tools can analyze these unbiased datasets with the aim of driving hypothesis generation for targeted experimentation, which can, in turn, generate more data to inform more accurate computer models. These computer models can identify multiple broad host responses and correlate them to parasite molecular biology, giving researchers a more complete picture of molecular behavior during liver stage infection.

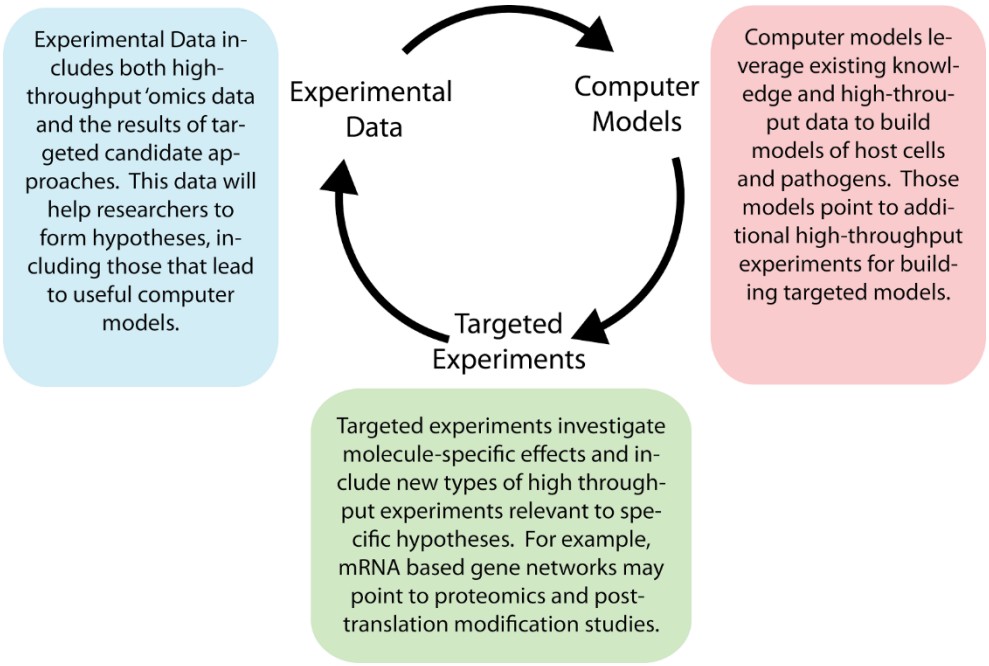

Figure 5.1: Systems biology approaches to liver-stage malaria

In this model, malaria researchers partner with computational biologists and high-throughput experimentalists to allow new approaches to solve key questions in liver stage malaria.

Global data generation and machine learning

Plasmodium species contain between 4000 and 7000 genes [20]–[22]; the human genome is estimated to have roughly 20-30,000 ORFs [23]. However, transcriptional profiling of *P. berghei*-infected hepatocytes [1] has shown that only a subset of 1064 hepatocyte genes are dysregulated in the first 24 hours of infection. Likewise, a similar approach looking at the P. yoelii parasite transcriptional profile [24] showed only about 2000 of 7700, or roughly 25%, of parasite genes are expressed during the various stages of liver development. This greatly reduces the number of genes which must be considered. However, a non-targeted approach would require as many experiments as genes in order to set the coefficients in an analytical equation where each gene was a variable. In other words, analyzing a system with a combined 3,000 host

and parasite genes would require a minimum of 3,000 separate microarray or RNA-Seq experiments to solve the equations, and would ideally have ten times that amount, or 30,000 separate experiments, to properly deal with experimental noise.

This complexity means that in the search for new drugs and vaccine candidates, a "guess and check" approach to candidates will never realistically be able to exhaust all possible targets. As such, in order to develop a holistic picture of liver stage infection, we need the capability to organize and analyze large amounts of data. Computing and statistical tools are required to extract the underlying genetics and molecular biology that drive *Plasmodium*'s development in the liver. To this end, it will be critical to turn to the powerful machine learning tools developed for computational and systems biology. These tools, backed by high-throughput data sets, make it possible to rank candidates and systematically inform the best genes or proteins to investigate.

Machine learning refers to a body of computational techniques that use algorithms that can learn from a set of structured input and select which measurements best differentiate between examples. By entering the biological data that result from known inputs (e.g. a cellular response to, for example, insulin), specific outputs can be mapped to these inputs, allowing the computer to learn how cellular systems react. The computer can then learn to map data inputs to outputs and determine which cellular changes are related to which inputs (e.g. infection), and whether they are statistically significant. Using these algorithms, it will become easier to quickly determine between cellular disruptions critical to parasite development and those which are unimportant.

Like other methodologies that rely on statistics, machine learning tools become more powerful as more data is available. Larger datasets allow for better training of the machine tools, which leads to more robust signals and less statistical noise. Large scale, high-throughput "-

omics" technologies offer the ability to measure many biological perturbations at once, generating input to train models to predict important outcomes in Plasmodium liver stage infection. Currently, the bulk of this data comes from either mRNA transcriptomics or total proteomics, although some surface proteomes have been gathered. High-throughput technology can also track global changes of lipid and metabolic processes, as well as small regulatory RNAs and post-translational modification of proteins. Many of the generated data are accessible at public databases such as the Gene Expression Omnibus (GEO) [25], [26], PlasmoDB, a database of *Plasmodium* gene information [27], or the Protein Atlas [28]. These technologies and the data they generate can provide the platforms to answer the major questions in the field of liver stage biology; namely, how the parasite interacts with the hepatocyte during the process of invasion, and how the parasite interacts with the hepatocyte during development through liver stages.

Using phenotypic intersections in high-throughput data

To exhaustively interrogate all possible host-parasite interactions using a candidate-based approach would be extremely costly in both time and resources. Therefore, it is critical to use strategies to narrow down lists of candidates by identifying which host factors correlate to *Plasmodium* infection. Much high-throughput data has already been extracted from both *Plasmodium* and its mammalian host cells. This data can and should be analyzed by machine learning tools in order to extract levels of knowledge that may currently be hidden. However, as powerful as the deep analysis of individual datasets may be, critical knowledge will likely be best found by integrating sets of both parasite and host-based data in order to correlate features found in multiple desired phenotypes, and identify those most likely to be biologically relevant (Figure 5.2). One strategy could be the comparison of the surface complement of hepatocytes to the complement of cells unable to support *Plasmodium* sporozoite infection. Recently, a large-

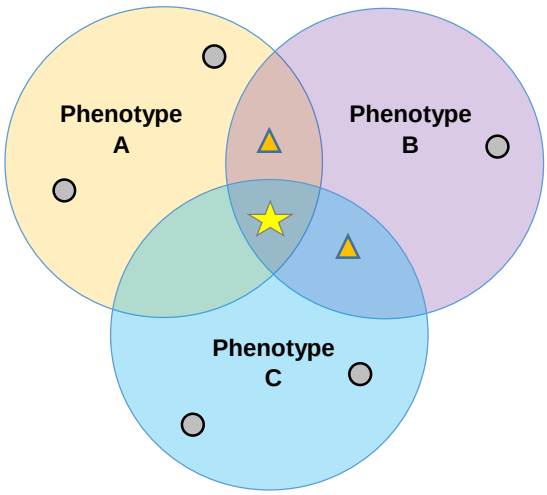

Figure 5.2: Intersections of phenotypes can inform selections for further inquiry

Analyzing phenotypes using high-throughput techniques will likely identify a number of differential cellular responses, many of which will be unrelated to infection (grey circles). By looking at hits that are differentially expressed in multiple phenotypes (orange triangles), it is more likely that any given response is significant to infection. The most relevant candidates, and the most likely to provide useful data in follow-up candidate based approaches, will be those that show up in all relevant phenotypes (yellow star).

scale interrogation of primary human hepatocytes discovered 32 "hepatocyte-specific" surface proteins; that is, proteins found only on the surface of hepatocytes and not on lung fibroblasts or epithelial cells [29]. This approach to identify receptors unique to the hepatocyte has the potential to discover what might be driving *Plasmodium*'s host cell specificity; however, comparison of the hepatocyte to other liver cells may narrow the search even further. Moreover, we can additionally identify hepatocyte factors of infection using comparison of phenotypic differences between hepatocytes.

In this endeavor, it will be crucial to directly interrogate infected cells and compare them to uninfected cells. Interpreting the differences found between infected and uninfected states in the liver can be difficult, particularly in determining the cause and effect of each change. Every measurement leads to a new unknown: the upregulation of a transcript may be due to parasite manipulation, a general host response to infection, or a preselection by the parasite of host cells with this upregulation already in place. Following hepatocytes over the course of infection may provide some clues—transcripts unchanged at early timepoints but dysregulated later could imply direct manipulation by the liver stage parasite. Even this tactic must be used carefully, as cellular responses that are actively disrupted by parasite products may be confounded with those that are general responses to infection. One possible way around this difficulty may be by using a subtractive approach, comparing the transcriptome or proteome of other liver infections such as the Hepatitis C Virus [30], [31] and noting which responses change only in response to *Plasmodium*. As this approach necessarily requires the comparison of large numbers of cellular signaling cascades, immune responses, and feedback loops, it will be critical to have appropriate tools with which to organize and analyze the data.

Likewise, a direct comparison of transcriptional changes within infected hepatocytes over time (for example, in Albuquerque, *et al.* [1]) could be mapped together with the transcriptional changes of liver stage parasites, (as in Tarun, *et al.* [24]). Gene clustering analysis may allow the emergence of temporally-aligned regulatory network changes within host and parasite cells, pointing toward a putative causation and direct host-parasite interaction. Moreover, the different transcripts found in these examples can be analyzed for co-regulatory relationships, pointing toward possible regulatory proteins (e.g. transcription factors), which may show other possible ways the host may be changed by liver stage infection (or vice versa). This can then be followed

up by researchers using traditional techniques to determine whether the predictions of the models are valid. Whether predicted molecular candidates are or are not valid (that is, whether they actively impact a given phenotype or not), the data gathered by these experiments can further train the models to further improve the predictive power of the algorithms. This cycle can undergo multiple iterations, each time allowing the development of more and more accurate and powerful tools. Ultimately, all high-throughput data can be integrated to create hepatocyte and parasite gene regulatory network maps which would serve as a map for understanding the outstanding questions in the field of liver stage biology.

Computational tools provide powerful analysis

Gene regulatory networks (GRNs) include putative regulators, their targets, and clusters of co-regulated genes as detected from gene expression data, augmented by additional information such as binding motifs. A particularly powerful GRN modeling tool, EGRIN (Environment and Gene Regulatory Influence Network) [32], [33] makes it possible to analyze all experiments on the same organism at once, determine under what conditions (e.g. parasite life cycle stage) genes are co-regulated, and detect probable regulators of those genes. These GRNs, gathered separately from parasite and hepatocyte data, can provide a background of known regulators which can inform further experimental data generation. For example, suppose a well-defined *Plasmodium* EGRIN predicts that a set of genes will be co-expressed based on robust blood stage data. This EGRIN can be mapped onto less-robust liver stage data sets, and co-expression phenotype can be measured. If the genes are likewise co-expressed in liver stage, then researchers know that blood stage models are valid and these more accessible stages can be used to study these genes and their regulators. If, on the other hand, they are not co-expressed in liver stage, the EGRIN will point to upstream regulatory elements (e.g. transcription factors) that

are likely to be driving the changes between blood and liver stages, and further study of these elements may illuminate liver stage biology.

Systems analysis involves three interrelated processes that can be leveraged to analyze high-throughput data and build hypothetical biological interactions driving parasite infection and survival. These are statistical tests for differential transcript expression, module analysis to identify specific pathways and pathologically relevant gene sets, and machine learning-based biomarker selection processes. Taken together, these tools make it possible to turn a data table into a list of comprehensible biological hypotheses. These hypotheses will typically have the following form: "Factor A is significantly different in samples with phenotype X compared to samples with phenotype Y." Phenotypes can have a broad range of definitions, such as the expression level of an individual gene, relative susceptibility of an individual to infection, or even a binary classifier such as infected or uninfected. With any given phenotype, especially binary phenotypes, factors can be extracted and ranked by predictive relevance while training the model, then applied during analysis to identify which molecular changes between the phenotypes are most likely to be biologically relevant. Techniques for ranking these factors and testing the rankings are well defined.

Module analysis looks at sets of factors that are often co-regulated, allowing individual measurements to be statistically linked, lowering the amount of necessary correction. Module gene analysis relies on the many well-annotated pathway and gene sets available in publically available databases, and computational tools with which to analyze them. Significant enrichment of sets of genes within an experimentally generated dataset is calculated using algorithms such as the Gene Set Analysis (GSA) [34] or the Gene Set Enrichment Algorithm (GSEA) [35], [36]. These algorithms look for multiple genes within a set that are differentially expressed. As the

gene sets used for input are well annotated, gene set enrichment can be more easily and robustly interpreted than simple individual gene expression. One limitation of this approach is that existing gene set annotations will be based on pre-existing experiments and putative gene homologies that may not be relevant to novel malaria experiments. While human, and to a lesser extent mouse, gene sets are robustly annotated, fewer Plasmodium gene sets have been studied and even fewer have been made easily and publically accessible. The algorithm cMonkey2 [37] addresses this by determining de novo gene clusters using both gene expression and sample conditions. For example, cMonkey2 can be trained with datasets from both mosquito stage sporozoites and human blood stage data, the algorithm will note which genes are co-regulated only in sporozoites, which are co-regulated only in blood stage, and those that are co-regulated in both stages. The trained algorithm can then generate a robust Plasmodium gene set that can be used for analysis on liver stage and other parasite life cycle forms.

Another tool for interrogating host-parasite interactions is biomarker selection and interpretation. Biomarkers are characteristics which can be objectively measured and used to predict biological functions, such as susceptibility to *Plasmodium* infection or severity of clinical disease. Machine learning biomarker techniques are even more powerful (and complicated) than either statistical testing or module analysis [38], [39]. Biomarker selection powerfully extracts features from high-dimensional data (i.e. multiple experiments, outputs, and conditions) in order to predict which input features will most closely correlate with a given phenotype. For example, comparison of allelic variation in patients with severe malaria versus patients with mild or moderate disease could identify specific biomarkers which would allow prediction of severe disease before a person was even infected. Techniques such as these make it possible to determine which molecular changes drive, or are at least highly correlated with, an observed

phenotype, which will then allow researchers to rank and prioritize experiments most likely to discover interesting and meaningful host-parasite interactions.

Using these machine learning tools, high-throughput, large scale datasets can be increased in analytic power tremendously. This, in turn, can increase the likelihood that any individual candidate identified in these large screening experiments will actually be a valid player in liver stage infection. Traditional, candidate-based experiments will provide more specific data about cellular responses and host-pathogen interactions. This specific data can then be re-input into the machine learning algorithms to better train new predictive models, which can then better inform the next round of high-throughput screens. If attempted thoughtfully, this iterative approach can even benefit from the models which have informed the candidate-based approaches. For instance, while blood stage parasites differ significantly from the liver stages, they are still the same organism, and have many essential pathways in common. The plethora of material available in blood stage, therefore, can generate critical data to overlay on the less accessible liver stage biology. Likewise, data from virology can inform our models of hepatocyte functioning, giving us a better picture of how the host responds to infection.

Most powerfully, the data which currently exists can be used to train even more powerful analytic models. As candidates are identified by models, they can be followed up by researchers using traditional techniques to determine whether the predictions of the models are valid. Whether predicted molecular candidates are or are not valid (that is, whether they actively impact a given phenotype or not), the data gathered by these experiments can further train the models to further improve the predictive power of the algorithms. This cycle can undergo multiple iterations, each time allowing the development of more and more accurate and powerful tools. Ultimately, all high-throughput data can be integrated to create hepatocyte and parasite

GRNs which would serve as a map for understanding the outstanding questions in the field of liver stage biology.

A call to arms

Malaria parasites inflict an enormous health burden worldwide. Yet, the scientific resources dedicated to their study has not matched the potential impact of a comprehensive understanding of the interaction between the malaria parasite and its human host. The study of these complex, dynamic, and adaptable parasites is heavily biased toward a small subset of transcripts and proteins, which cannot fully explain the activities of the sporozoite as a whole. The candidate-based approach has provided some beautiful mechanistic detail surrounding a small number of parasite proteins. Yet, it has stopped short of providing a comprehensive understanding of the specific interactions which allow the malaria parasite to shape and refine its host cell. Technology has given researchers the ability to both gather tremendous amounts of useful data, as well as the tools needed to pull relevant patterns out of the data to inform new and powerful hypotheses. Recently, such a pattern was detected in a large-scale study of blood stage malaria, leading to the discovery of critical host-parasite interactions that contribute to deadly severe disease in adults [40]. Using similar techniques could likewise provide powerful new information about the liver stage of the disease.

Malaria parasites survive in the presence of evolutionary pressure in multiple environments, from mammalian hepatocytes and erythrocytes to mosquito gut tracts and salivary glands. They exhibit a diversity of amazing biological properties: rapid growth that surpasses all other eukaryotes, the capacity to stretch their host cell to 50-100 times its normal volume, remarkable remodeling of the host RBC in blood stages, and in the sporozoite form, the ability to survive extracellularly. The investigation into questions that have fascinated biologists for

centuries—questions around cell size, growth, division, death, and interaction with environmental cues—are all paramount to a better understanding of the malaria parasite and its interaction with its host. These questions have, fortunately, attracted brilliant minds. In recent years, these minds have utilized emerging technology to create large datasets to address these fascinating questions. It is the job now of the malaria community to integrate novel technological and computational tools to both exploit these data to their fullest extent, and to generate new robust datasets which can build on the knowledge. The integration of experiment and analysis represented by these tools is a tremendous opportunity for substantial scientific contribution that can be translated into improvements in human health. Let us not pass it by.

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APPENDIX A: SUPPRESSION OF HOST P53 IS CRITICAL FOR *PLASMODIUM* LIVER-STAGE INFECTION

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Suppression of Host p53 Is Critical for *Plasmodium* Liver-Stage Infection

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SUMMARY

Plasmodium parasites infect the liver and replicate inside hepatocytes before they invade erythrocytes and trigger clinical malaria. Analysis of host signaling pathways affected by liver-stage infection could provide critical insights into host–pathogen interactions and reveal targets for intervention. Using protein lysate microarrays, we found that *Plasmodium yoelii* rodent malaria parasites perturb hepatocyte regulatory pathways involved in cell survival, proliferation, and autophagy. Notably, the prodeath protein p53 was substantially decreased in infected hepatocytes, suggesting that it could be targeted by the parasite to foster survival. Indeed, mice that express increased levels of p53 showed reduced liver-stage parasite burden, whereas p53 knockout mice suffered increased liver-stage burden. Furthermore, boosting p53 levels with the use of the small molecule Nutlin-3 dramatically reduced liver-stage burden in vitro and in vivo. We conclude that perturbation of the hepatocyte p53 pathway critically impacts parasite survival. Thus, host pathways might constitute potential targets for host-based antimalarial prophylaxis.

INTRODUCTION

Parasites of the genus *Plasmodium* are the causative agents of the deadly disease malaria, afflicting 350–500 million people annually and causing 800,000 deaths worldwide (Snow et al., 2005). After transmission by an infected *Anopheles* mosquito, the parasite travels quickly through the bloodstream to the liver and infects hepatocytes. The parasite then grows and replicates within hepatocytes, presumably evading detection by the host, and ultimately spawns tens of thousands of daughter merozoites, which are released into the bloodstream and infect red blood cells, leading to symptomatic infection (Vaughan et al., 2008).

One feature of host manipulation that has been previously suggested is the ability of *Plasmodium berghei* rodent malaria parasites to render their host hepatocyte partially resistant to artificial induction of apoptosis in vitro, both early (van de Sand et al., 2005) and late (Leirião et al., 2005) during liver-stage development. It remains unclear, however, how broadly the host hepatocyte responds to infection and if the parasite attempts to counteract responses that might impact its survival. One potential mechanism that could explain how parasitized hepatocytes become resistant to apoptosis involves activation of the hepatocyte growth factor receptor, but this mechanism appears unique to the rodent malaria parasite *P. berghei* (Carrolo et al., 2003; Kaushansky and Kappe, 2011). Furthermore, some studies have measured transcriptional changes that occur in *P. yoelii* and *P. berghei*-infected hepatocytes (Albuquerque et al., 2009; Tarun et al., 2008), yet perturbations in the translational and posttranslational host cell environment that occur upon parasite liver-stage infection have not been elucidated.

RESULTS

It remains technically challenging to study protein level cellular responses to liver-stage infection because infection rates are low, and thus, infected cells can only be isolated in limited quantities. To circumvent this roadblock, we used reverse-phase protein microarray technology, which enables broad but targeted proteomic investigations on small sample sizes (Sevecka et al., 2011). The platform uses cellular lysates deposited in nanoliter droplets on nitrocellulose-coated glass slides in which levels of specific proteins or their posttranslational modifications can be detected by probing the lysates with appropriate antibodies (Figure 1A). We assembled a diverse set of antibodies, many of which have been previously validated for use in reverse-phase arrays (Sevecka et al., 2011). These antibodies recognize proteins involved in numerous cellular outcomes, including survival, apoptosis, proliferation, cell-cycle control, and autophagy. Approximately 10,000 parasite-infected HepG2/CD81 hepatoma cells as well as uninfected cells were isolated by fluorescence-activated cell sorting (FACS), making use of GFP-tagged *P. yoelii* parasites (Tarun et al., 2006). Protein extracts from each sample were prepared and printed in quadruplicate on 48 separate nitrocellulose pads followed by probing the arrays with the selected set of antibodies to obtain quantitative information

APPENDIX B: HOST-BASED PROPHYLAXIS SUCCESSFULLY TARGETS LIVER STAGE MALARIA PARASITES

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original article

Host-based Prophylaxis Successfully Targets Liver Stage Malaria Parasites

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Eliminating malaria parasites during the asymptomatic but obligate liver stages (LSs) of infection would stop disease and subsequent transmission. Unfortunately, only a single licensed drug that targets all LSs, Primaquine, is available. Targeting host proteins might significantly expand the repertoire of prophylactic drugs against malaria. Here, we demonstrate that both Bcl-2 inhibitors and P53 agonists dramatically reduce LS burden in a mouse malaria model *in vitro* and *in vivo* by altering the activity of key hepatocyte factors on which the parasite relies. Bcl-2 inhibitors act primarily by inducing apoptosis in infected hepatocytes, whereas P53 agonists eliminate parasites in an apoptosis-independent fashion. In combination, Bcl-2 inhibitors and P53 agonists act synergistically to delay, and in some cases completely prevent, the onset of blood stage disease. Both families of drugs are highly effective at doses that do not cause substantial hepatocyte cell death *in vitro* or liver damage *in vivo*. P53 agonists and Bcl-2 inhibitors were also effective when administered to humanized mice infected with *Plasmodium falciparum*. Our data demonstrate that host-based prophylaxis could be developed into an effective intervention strategy that eliminates LS parasites before the onset of clinical disease and thus opens a new avenue to prevent malaria.

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INTRODUCTION

Plasmodium parasites cause malaria worldwide, infecting 200–500 million and killing nearly over 600,000 people annually. Despite the impact of the disease and efforts over decades to eradicate it, malaria persists worldwide.[1] One of the roadblocks to eradication has been the development of drug-resistant parasites, which often evolve within years of the distribution of new antimalarial drugs.[2] All currently available treatments and prophylactic regimens are thought to directly target parasite proteins. However, the rapid replication of the parasite allows for quick development of mutations that render them resistant to treatment.[3] Although combination therapies based on artemisinin have recently been more effective at circumventing the development of drug resistance,[4] this strategy is beginning to lose potency as the parasite develops resistance to each drug.[1,4–6]

The complex lifecycle of the malaria parasite provides multiple potential points for intervention.[7] *Plasmodium* parasites are deposited in the skin by the bite of a female *Anopheles* mosquito before they travel to the liver. Once in the liver, parasites traverse the sinusoids, enter the parenchyma, and invade hepatocytes. Over the next 2–10 days, the liver stage (LS) parasite exploits the resources of its host hepatocyte to produce 10,000 – 100,000 of red blood cell-infectious progeny. While parasites divide more quickly within the hepatocyte than any other time in their lifecycle, symptomatic disease is only initiated after the LS is complete and the erythrocytic stage begins. The liver also harbors long-lived dormant forms of *Plasmodium vivax* called hypnozoites, which are the source of relapsing infection.[8] Eliminating the LS parasite would prevent initial and relapsing disease and subsequent transmission. Yet there is only a single licensed drug, Primaquine, that targets all LS parasites, and its use is limited by side effects.

The LS parasite relies on a precise intracellular environment that supports growth, as evident in part by the minimal development of axenic parasite culture.[9] Thus, even slight perturbations of key hepatocyte factors using host-based prophylactic (HBP) drugs might completely prevent the parasite from proceeding to blood stage disease. We have shown earlier that *Plasmodium* parasites manipulate several hepatocyte factors involved in cell survival signaling during LS infection.[10,11] Specifically, parasites actively suppress the tumor suppressor P53,[10] which is involved in a variety of cellular outcomes including apoptosis and cell-cycle arrest.[12] Malaria parasites also modulate the mitochondrial apoptotic cascade by increasing levels of the prosurvival Bcl-2 family members, and by suppressing levels of the proapoptotic factor Bad.[10] Reversing either parasite-driven change in the hepatocyte reduces LS burden, indicating that P53 suppression and Bcl-2 family activity are critical for parasite survival.[10,13] Consequently, increasing levels of P53 using genetic or pharmacological approaches reduces LS burden.[10] Similarly, blocking the Bcl-2 family activity eliminates malaria parasites through hepatocyte apoptosis.[13] Here, we test the

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APPENDIX C: MALARIA PARASITES TARGET THE HEPATOCYTE RECEPTOR EPHA2 FOR SUCCESSFUL HOST INFECTION

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cross presentation (*Tapbp*, *Rab27a*, and *Slc11a1*) (*25*–*27*); chemokine-mediated recruitment of immune cells to the tumor microenvironment (*Ccr8*, *Cxcl1*, and *Ccrl4*) (*28*–*30*); and type I interferon signaling (*Irf3*, *Ifnar2*, *Oas2*, *Ifi35*, and *Ifitm1*) (*31*, *32*) (Fig. 4D and fig. S7C). Expression of these genes was also increased in murine bone marrow–derived DCs stimulated with *Bifidobacterium* in vitro (table S6), consistent with previous reports that these species of *Bifidobacterium* can directly elicit DC maturation and cytokine production (*23*).

To test whether functional differences in DCs isolated from TAC, JAX, and *Bifidobacterium*-treated TAC mice could be sufficient to explain the differences in T cell priming observed in vivo, we purified DCs from lymphoid tissues of naïve TAC, JAX, and *Bifidobacterium*-treated TAC mice and tested their ability to induce carboxyfluorescein diacetate succinimidyl ester (CFSE)–labeled CD8$^+$ SIY-specific 2C TCR Tg T cell proliferation and acquisition of IFN-γ production in vitro. DCs purified from JAX and *Bifidobacterium*-treated TAC mice induced 2C T cell proliferation at lower antigen concentration than did DCs purified from naïve TAC mice (fig. S8, A and B). Furthermore, at all antigen concentrations, JAX-derived DCs elicited elevated levels of T cell IFN-γ production (Fig. 4E and fig. S8A). We observed similar effects upon oral administration of *Bifidobacterium* to TAC mice before DC isolation (Fig. 4E and fig. S8A). Taken together, these data suggest that commensal *Bifidobacterium*-derived signals modulate the activation of DCs in the steady state, which in turn supports improved effector function of tumor-specific CD8$^+$ T cells.

Our studies demonstrate an unexpected role for commensal *Bifidobacterium* in enhancing antitumor immunity in vivo. Given that beneficial effects are observed in multiple tumor settings and that alteration of innate immune function is observed, this improved antitumor immunity could be occurring in an antigen-independent fashion. The necessity for live bacteria may imply that *Bifidobacterium* colonizes a specific compartment within the gut that enables it to interact with host cells that are critical for modulating DC function or to release soluble factors that disseminate systemically and lead to improved DC function.

Our results do not rule out a contribution of other commensal bacteria species in having the capability to regulate antitumor immunity, either positively or negatively. Our data support the idea that one source of intersubject heterogeneity with regard to spontaneous antitumor immunity and therapeutic effects of antibodies targeting the PD-1/PD-L1 axis may be the composition of gut microbes, which could be manipulated for therapeutic benefit. These principles could apply to other immunotherapies, such as antibodies targeting the CTLA-4 pathway. Similar analyses can be performed in humans, by using 16*S* rRNA sequencing of stool samples from patients receiving checkpoint blockade or other immunotherapies, to identify commensals associated with clinical benefit.

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/350/6264/1084/suppl/DC1
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MALARIA

Malaria parasites target the hepatocyte receptor EphA2 for successful host infection

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The invasion of a suitable host hepatocyte by mosquito-transmitted *Plasmodium* sporozoites is an essential early step in successful malaria parasite infection. Yet precisely how sporozoites target their host cell and facilitate productive infection remains largely unknown. We found that the hepatocyte EphA2 receptor was critical for establishing a permissive intracellular replication compartment, the parasitophorous vacuole. Sporozoites productively infected hepatocytes with high EphA2 expression, and the deletion of EphA2 protected mice from liver infection. Lack of host EphA2 phenocopied the lack of the sporozoite proteins P52 and P36. Our data suggest that P36 engages EphA2, which is likely to be a key step in establishing the permissive replication compartment.

Malaria infections place a tremendous burden on global health (*1*). Their causative agents, *Plasmodium* parasites, are transmitted to mammals as sporozoites by the bite of *Anopheles* mosquitoes. After entry into a capillary, sporozoites are carried to the liver, where they pass through multiple cells before recognizing and invading hepatocytes. During invasion, the sporozoite forms a protective parasitophorous vacuole made of hepatocyte plasma membrane, which ensconces the parasite, establishes the intrahepatocytic replication

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