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Plasmodium Pre-Erythrocytic Stages: Biology, Whole Parasite Vaccines and Transgenic Models

[1]Kota Arun Kumar and [2]Satish Mishra

[1]Department of Animal Sciences,
School of Life Sciences, University of Hyderabad, Hyderabad-500046, India
[2]Department of Molecular Microbiology and Immunology,
Johns Hopkins Bloomberg School of Public Health, Johns Hopkins University, Baltimore, MD 21205, USA

ABSTRACT

Malaria remains one of the world's worst health problems, which causes 216 million new cases and approximately 655,000 deaths every year WHO World Malaria Report, 2011. Malaria transmission to the mammalian host is initiated through a mosquito bite that delivers sporozoites into the vertebrate host. The injected sporozoites are selectively targeted to liver which is the first obligatory step in infection thus making this stage an attractive target for both drug and vaccine development. Research using rodent models of malaria has greatly facilitated the understanding of several aspects of pre-erythrocytic parasite biology and immunology. However, translation of this knowledge to combat *Plasmodium falciparum* infections still offers several challenges. We highlight in this review some of the recent advances in the field of *Plasmodium* sporozoite and liver stage biology and in the generation of whole organism attenuated vaccines. We also comment on the application of transgenic models central to Circumsporozoite Protein (CSP) in understanding the mechanism of pre-erythrocytic immunity.

Keywords: *Plasmodium*, Sporozoites, Circumsporozoite Protein, Liver Stages, Malaria Vaccine

1. INTRODUCTION

1.1. The Sporozoite Stage, Molecular Motor and Host Cell Invasion

Plasmodium sporozoites are the infective forms of parasite to the vertebrate host and infection is initiated when female *Anopheles* mosquitoes inject these parasites in the avascular portion of the dermis while probing for a blood meal (Vanderberg and Frevert, 2004). Within the dermal layer, the sporozoites glide through extracellular spaces as well as several cellular barriers (Amino *et al*., 2006) utilizing a molecular motor present underneath the plasma membrane. The components of the molecular motor are located within the cortical space present between the sporozoite plasma membrane and the inner membrane complex (Kappe *et al*., 2004). A sporozoite specific type 1 transmembrane protein called as Thrombospondin Related Anonymous Protein (TRAP) has been shown to be essential for parasite gliding motility and host cell invasion (Sultan *et al*., 1997). While the cytoplasmic tail of TRAP connects itself to the actin-myosin motor through a glycolytic enzyme aldolase (Buscaglia *et al*., 2003), an extracellular domain of TRAP consisting of integrin like a domain and TSR (Thrombospondin Type 1 repeat) likely attaches itself either to the cellular receptors or solid substrates. When the molecular motor engages TRAP in a backward direction, the extracellular domain is fixed to substrate or cellular receptors generates a traction that allows the sporozoites to glide forward, a movement referred to as gliding motility (Kappe *et al*., 2004). Another related protein belonging to TRAP family referred to as TRAP-Like Protein (TLP) was found to be exclusively expressed in salivary gland sporozoites. Like TRAP, the cytoplasmic tail of TLP also binds to aldolase via its penultimate tryptophan residue and the absence of TLP was associated with partial defect in gliding motility (Heiss *et al*., 2008). However, an independent study reported a deficiency of TLP knock out parasites in cell

Corresponding Author: Satish Mishra, Department of Molecular Microbiology and Immunology,
Johns Hopkins Bloomberg School of Public Health, Johns Hopkins University, Baltimore,
MD 21205, USA

traversal with no defect in gliding (Moreira *et al*., 2008). More recent studies have implicated the role of Heat Shock Protein 20 (HSP20) in regulating the function of cell traction and motility of sporozoites and ablation of HSP20 impaired migration in the host cells (Montagna *et al*., 2012).

While establishing infection, sporozoites exhibit two modes of cellular invasions: one referred to as breaching where sporozoites randomly enter and exit cells (Mota *et al*., 2001). During this process, sporozoites induce a transient pore in the plasma membrane of the host cells that is resealed eventually. In the alternate mode referred to as productive invasion, the sporozoites form an invagination in the hepatocyte plasma membrane by formation of a moving junction. This movement results in the formation of a vacuolar structure, derived primarily from hepatocyte plasma membrane (Bano *et al*., 2007) that completely subsumes enclosing the invaded sporozoite, thus initiating the process of sporozoite transformation into Exo-Erythrocytic Forms (EEFs) or liver stages. A role of host cell F-actin has been implicated in the formation of moving junction while the sporozoites are productively invading. This event appears to be an active process that recruits actin related protein 2/3 complex (Apr2/3) at the site of moving junction and the process was severely impaired in the presence of actin-filament destablising agent-jasplakinolide (Gonzalez *et al*., 2009).

1.2. Commitment of Sporozoite Infection to Hepatocytes: The Role of CSP

The commitment of sporozoite infection to liver is a complex process with a definitive evidence for the major sporozoite surface protein called CSP in this process. CSP plays pleiotropic roles in biology of the sporozoites and liver stages and being extensively accentuated on the sporozoite surface, it is not surprising that there are several functional domains in this protein that facilitate the navigation of sporozoites either to salivary gland or to hepatocytes. The CSP from different *Plasmodium* species exhibit the same structural features that include a signal peptide, a central domain mainly consisting of amino acids repeats and a C-terminal hydrophobic sequence (McCutchan *et al*., 1996). In addition there are conserved motifs among CSP proteins referred as region I, II-plus and III. Region I is a pentapepide sequence represented by KLKQP and is known to facilitate the attachment of sporozoites to salivary glands (Sidjanski *et al*., 1997). Region I also serve as a substrate for the parasite cysteine protease required for CSP processing (Coppi *et al*., 2011). Region II-plus resides in the proximal region of Thrombospondin Repeat (TSR) type I domain present in both, CSP and TRAP and extensive evidence for its role as an adhesive motif for hepatocyte attachment is well known (see below). A likely role of region III has been proposed in providing structural organization and framework for the neighboring region II-plus motif.

The function of CSP protein begins to appear in the mosquito stages called oocyst where sporogony is initiated and in oocyst derived from CS knock out parasites, the sporozoites formation is impaired (Menard *et al*., 1997). Further, the exit of sporozoites from oocyst is also dependent on CSP and is mediated by region II-plus, a conserved motif of positively charged residues of arginines and lysines and mutation of these critical residues to alanines prevents this process (Wang *et al*., 2005). Alternatively, egress of the sporozoites from oocyst has also been assigned to an oocyst specific cysteine protease Egress Cysteine Protease (ECP1) (Aly and Matuschewski, 2005), however whether CSP is a likely substrate for ECP1 remains to be elucidated. Several lines of evidence also indicate that Region II-plus is crucial for the initial attachment of sporozoites to hepatocytes. These observations were evident from the studies that recombinant CSP protein binds to HSPGs in the human liver sections and this binding is dependent on the presence of region II-plus motif (Cerami *et al*., 1992; Frevert *et al*., 1993). Further the binding of sporozoites or recombinant CSP to HepG2 cells, a cell line that favors complete development of liver stages is inhibited in the presence of synthetic inhibitors representing region II-plus (Sinnis *et al*., 1994). A likely explanation for specificity in binding is attributed to the ionic interactions between positively charged residues of region II-plus and the sulphated HSPG glycosaminoglycan chains (Pinzon-Ortiz *et al*., 2001). Accumulating evidence also suggests that processing of CSP by parasite specific cysteine protease is required during hepatocye invasion and the event is initiated when sporozoites come in contact with hepatocytes (Coppi *et al*., 2005). A more recent study revealed the occurrence of two conformational states for CSP that determines the migratory state of the sporozoites. One of the conformations is where the C-terminal cell adhesive domain is not masked by the N-terminal region of the protein and this form functions during sporozoite development in oocyst and hepatocyte invasion. The second conformation masks the C-terminal region and maintains the sporozoites in the migratory state. This study demonstrates that proteolytic cleavage of CSP facilitates the switching to adhesive conformation and that the conserved region I motif (KLKQP) bears cleavage site (Coppi *et al*., 2011).

1.3. Migration Facilitates Activation of Sporozoites for Hepatocyte Infection

Sporozoites need to make tortuous journey from the site of inoculation to hepatocytes in order to continue

their life cycle. During this sojourn, they pass through dermal cells of skin, endothelial cells of blood vessel; reach the sinusoidal lumen from where they enter into the liver parenchyma passing through the Kupffer cells, the resident macrophages of the liver (Frevert *et al*., 2005; Baer *et al*., 2007). The major challenges sporozoites face are to retain infectivity for hepatocytes and to safe guard themselves from the destruction of immune attack during their progression from skin to the liver. While migration endows the sporozoites with the unique capacity to remain intracellular thereby avoiding destruction by $CD11b^+$ phagocytic cells (Amino *et al*., 2008), it also facilitates the contact of sporozoites with several host factors that influence sporozoite infectivity to hepatocytes. For instance, high levels of hepatocyte specific sulphated HSPGs provide a cue to the sporozoites to switch from migratory to invasive mode and chemical modification of host cells leading to depletion of HSPG sulphation levels keeps the sporozoites in migratory state. This switch is likely mediated by calcium dependent protein kinase CDPK-6 and is accompanied by proteolytic cleavage of CSP (Coppi *et al*., 2007). The ability of sporozoites to regulate Ca^{2+} dependent exocytosis of micronemes has been reported during migration through cells (Mota *et al*., 2002). This could additional facilitate a timely release of surface proteins like TRAP and CSP required for motility, invasion and commitment to hepatocyte infection. A seminal finding implicated the role of hepatocyte growth factor released during sporozoite mediated cell wounding in enhancing the hepatocyte responsiveness to infection by signaling through a met tyrosine kinase receptor (Carrolo *et al*., 2003). However the relevance of HGF mediated signaling in increasing the infectivity of neighboring hepatocytes is highly debatable. One study reported the activation of NF-kappaB (NF-κB) signaling following exposure of cytosolic contents released from sporozoite wounded cells in primary hepatocytes and cultured HepG2 cells (Torgler *et al*., 2008). A role of Toll/IL-1 receptor and MyD88 has been implicated in mediating this signaling resulting in the induction of inducible NO synthase and limiting the efficiency of *Plasmodium* infection of hepatocytes. Importantly, this effect was observed only in Wild Type (WT) sporozoites but not with SPECT knock out parasites (Ishino *et al*., 2004) that are deficient in cell traversal activity. Attempts to characterize other host signaling proteins using a kinome wide RNAi screen identified five host hepatocyte kinases implicated in sporozoite infection. Functional *in vivo* validation of one of these kinases PKC zeta showed significant inhibition of *Plasmodium berghei* (*P.berghei*) infection, when liposome-formulated PKC zeta-targeting siRNA was administered systematically to mice (Prudencio *et al*., 2008).

Search for other host factors that lead to sporozoite activation have identified the role of intracellular concentrations of potassium [142mM K^+] in enhancing the sporozoite infectivity with a concomitant decrease in the migration (Kumar *et al*., 2007). In these studies the effect of 142mM [K^+] on sporozoite infectivity was readily reversed in the presence of potassium channel inhibitors, likely raising the possibility that influx of K^+ ions alter sporozoites infectivity patterns. A role of bifunctional gene with adenylyl cyclase activity and K^+ channel function has been formally demonstrated in the sporozoite stages and silencing the expression of this gene product resulted in altered exocytosis in sporozoite with a dramatic decrease in their ability to infect hepatocytes (Ono *et al*., 2008). Though host factors likely play a crucial role in sporozoite activation, there is evidence that in instances where sporozoites are compromised in their cell traversal activity, lack of exposure to host factors have no effect on their ability to efficiently transform into liver stages (Ishino *et al*., 2004). Whether there exists any redundant mechanism of sporozoite activation that may be turned on by default, in the absence of exposure to host factors is speculative and needs further investigation.

The role of sporozoite surface proteins like Membrane Attack Complex/Perforin (MACPF)-related domain protein (Ishino *et al*., 2005) and *P. berghei* phospholipase (*Pb* PL) have been shown to influence the sporozoite migration under *in vivo* conditions. While MACPF knock out parasites revealed an essential role of this protein in crossing sinusoidal cell layer for successful invasion into hepatocytes, the *Pb* PL parasites bearing mutations in the catalytic domain were unable to reach the liver as efficiently as WT sporozoites from the site of inoculation (Bhanot *et al*., 2005). A role for TLP has also been assigned in cell traversal activity. Attenuation of TLP expression yielded a phenotype where sporozoites were rendered deficient in cell traversal *in vitro* and with a striking defect in infectivity to mice when the mutants were delivered intradermally (Moreira *et al*., 2008). The defect, however, was less severe when the infection was done by intravenous injection. The role of TLP in cell traversal was further confirmed using Madin-Darby Canine Kidney (MDCK) cells monolayer assay. MDCK cells form tight junctions and sectioning the monolayes to detect parasites inside and outside the cells revealed greater retention of the TLP knock out sporozoites in the cytoplasm of the MDCK cells as compared to WT sporozoites (Mishra *et al*., 2012).

1.4. Tight Regulation of Salivary Gland Sporozoite Developmental Process Allows their Differentiation only in Hepatocytes

Sporozoite transformation into EEFs occurs only in the vertebrate host. This implies that salivary gland sporozoites

need to have a tight control over their development process so that the sporozoites remain quiescent while in salivary gland and start to differentiate only inside vertebrate host. A recent study has demonstrated that this is an active process regulated by phosphorylation of eukaryotic initiation factor-2alpha (eIF2α) by a protein kinase IK2, a global inhibitor of protein synthesis that leads to arrest of translation and stalling of mRNA into granules (Zhang *et al*., 2010). Further two independent studies have implicated the role of the RNA binding protein, Puf2 in translational regulation (Gomes-Santos *et al*., 2011; Mueller *et al*., 2011) and one of these studies report the direct role of Puf2 in regulation of IK2. In the absence of both IK2 and Puf2, the sporozoites transform into liver stages prematurely while in salivary gland and lose their infectivity. An emerging scenario from these studies is that *Plasmodium* utilizes the same mechanism as mammalian cells to inhibit global protein synthesis. While mammalian serine threonine kinases like PERK, GCN2, HRI and PKR phosphorylate the serine 59 of eIF2α in achieving this inhibition during environmental stress, the *Plasmodium* IK1, IK2 and PK4 seem to be the mammalian counter parts to achieve the same effect both during the progression from sporozoite to EEF stages and while transition during erythrocytic stages. Infact, phosphorylation of the regulatory serine 59 of *Plasmodium* eIF2α by PK4 has been shown to be essential for completion of erythrocytic cycle (Zhang *et al*., 2012). The implication of these observations is that drugs that target PK4 activity can alleviate the disease and inhibit transmission of malaria.

1.5. The Unconventional EEFs-The Skin Stages

Though hepatocytes are the permissive cells that favor complete development of sporozoites into liver stages, recent observations using intra vital microscopy have demonstrated the existence of EEF like forms in the skin (Gueirard *et al*., 2010). The skin cells supported complete exo-erythrocytic schizogony, albeit skin derived merozoites did not significantly contribute to the erythrocyte infection. Some EEFs were uniquely associated with immune privileged sites of skin hair follicles that are devoid of MHC-Class I antigen presentation and persisted for weeks. Whether these quiescent EEFs are functional equivalents of *Plasmodium vivax* hypnozoites that can cause relapse infections needs further investigation. Using rodent model, the effect of protective immunity generated by irradiated sporozoites was analysed on the persistence of skin EEFs. These studies revealed that skin EEFs were susceptible to immune clearance in immunized mice, nonetheless, unlike liver derived EEFs they were not susceptible to primaquine (Voza *et al*., 2012).

1.6. Development of EEF Inside Hepatocytes

Productively invaded sporozoites undergo transformation into EEFs or liver stages. The gene products of several developmentally regulated transcripts that are up regulated in the salivary gland sporozoites referred to as UIS (up-regulated in infective sporozoites) genes (Matuschewski *et al*., 2002) have been instrumental in complete transformation of the EEFs. Silencing of *UIS-3* by knock out approach has led to the generation of parasites that were attenuated in the early liver stage (Mueller *et al*., 2005a). In addition to its role in parasite development, UIS-3 interacts with the host cell fatty acid binding protein (L-FABP) (Mikolajczak *et al*., 2007) by virtue of its unique localization on Parasitophorous Vacuolar Membrane (PVM) surrounding EEF. The role of L-FABP in trafficking of lipids within the membrane compartments of hepatocytes is well known. Whether L-FABP facilitates a similar role in recruiting host lipids to UIS-3 required for liver stage development is an intriguing hypothesis to test especially in the light of recent crystallographic evidence suggesting its interaction with phospholipid-phosphatidyl ethanolamine (Sharma *et al*., 2008). Depletion of *UIS-4* resulted in a phenotype similar to *UIS-3* knock out with arrested EEFs and impairment in the initiation of blood stage infection (Mueller *et al*., 2005b). The immunolocalization of UIS-4 both on the surface of PVM as well as on the tubulo vesicular extensions, likely suggests its role in trafficking of host molecules.

Developing EEFs must meet the ever demanding requirement of fatty acids needed for membrane biogenesis during cytomere formation. Evidence for metabolic pathways involved in de novo lipid synthesis essential during liver stage development already exists (Tarun *et al*., 2008; Yu *et al*., 2008; Vaughan *et al*., 2009). The FAS-II de novo pathway for synthesis of fatty acids is reported in *Plasmodium* and is known to occur in the apicoplast; a relict plastid organelle of cyanobacterial origin. Several enzymes that catalyse the FAS-II mediated pathway are encoded in *Plasmodium* and two enzymes, *FabB/F* and *FabZ* were found to play a critical role in the liver stage development (Vaughan *et al*., 2009).

1.7. Overcoming the Host Defence Responses

Host defence responses like respiratory burst, inflammation and phagocyte mediated destruction of EEFs may limit the progression of the liver stages. Both sporozoites and liver stages have evolved a plethora of strategies to antagonize the host responses. A role for CSP in preventing the activation of respiratory burst while migrating through the liver resident Kupffer cells was reported (Usynin *et al*., 2007). In this regard, both CSP and sporozoites are capable of inducing the

cAMP production in Kupffer cells that have the ability to inhibit the NADPH oxidase activity necessary for generation of reactive oxygen species. This signaling is mediated by HSPGs and low density lipoprotein receptor related protein LRP-1, expressed on Kupffer cells.

The liver stages are clinically silent harboring few thousands of merozoites yet there is no trace of any inflammatory response generated during their growth in hepatocytes. One study reported the role of CSP protein in this process (Singh *et al*., 2007). CSP has in its N-terminus part an export motif called as Pexel (*Plasmodium* export element) or Vaculolar Transport Signal (VTS) first identified in blood stages and reported to export proteins to infected erythrocyte membrane (Hiller *et al*., 2004; Marti *et al*., 2004). Transfection of *P. berghei* blood stages with GFP fusion constructs containing N-terminus of CSP encompassing Pexel motif resulted in translocation of GFP protein into erythrocyte cytoplasm. Further mutating the critical arginine and lysine residues to alanines or eliminating the Pexel motif of parasite precluded the translocation of CSP in hepatocyte cytoplasm resulting in compromised EEF development. The same study also identified the existence of both a bipartite Nuclear Localization Signal (NLS) as well as Nuclear Export Signal (NES) in CSP. Peptide sequences representing the NLS competed with NF-κB, a proinflammatory transcription factor from translocating into the host nucleus using importin-alpha receptor present on the nuclear lamina. These studies highlight the role of CS in anti-inflammatory responses by preventing the nuclear translocation of NF-κB.

Mitigation of host immune responses is also essential at the point when hepatic merozoites are ready to be released into the blood stream following its complete development in hepatocytes. To safe guard their delivery into the liver sinusoids, specialized membrane bound structures called as merosomes, a vesicle derived from host plasma membrane (Graewe *et al*., 2011), buds from the detached hepatocytes and deliver them in the liver sinusoids (Sturm *et al*., 2006). During this process, the parasites inhibit the exposure of Phosphatidylserine (PS) on the outer leaflet of host plasma membrane that acts as a signal for the phagocytes to engulf the dying infected cells. The prevention of PS exposure is mediated by sequestration of the host Ca^{2+} into the merozoites while the detachment of the dying host cells was inhibited in the presence of general cysteine protease inhibitors likely pointing to the central role of cysteine proteases in this process.

A role for host Heme Oxygenase-1 has been shown to have anti-inflammatory functions and in promoting efficient liver stage infection and mice lacking the expression of heme oxygenase-1 were able to resolve infection (Epiphanio *et al*., 2008). Concomitant with these results were the observations that exogenous over expression of HO-1 in mice liver or treatment of mice with carbon monoxide or biliverdin each of which are enzymatic end products of HO-1 also increased the parasite liver load. Taken together, these studies suggest an elegant interplay of host and parasite factors that play an efficient role in preventing the inflammatory responses thus facilitating a successful establishment of liver stage infection.

1.8. Effect of Concomitant and Super Infections on EEF Development

Though natural infections by *Plasmodium* sporozoites are shown to induce $CD8^+$ T cells, it is unknown whether concomitant infections have any impact on the modulating the $CD8^+$ T cell memory responses generated against pre-erythrocytic stages. One study reported that the blood stage parasites inhibit Dendritic Cells (DC) function by altering its antigen presentation capacity thus precluding the induction of efficient liver stage immunity (Ocana-Morgner *et al*., 2003). Using the P. *yoelli* CSP-TCR transgenic model, an independent study confirmed identical patterns of activation and differentiation of CD8+ T cells following exposure to either normal or radiation attenuated sporozoites (Hafalla *et al*., 2007). Importantly, the effector and recall responses of memory CD8+ T cells were unaltered in the presence or absence of an ongoing blood stage infection.

More recent studies have shown an intricate association of a critical density dependent blood stage parasitaemia in regulating the growth and development of EEFs (Portugal *et al*., 2011). In this study, a model of super infection was recapitulated in mouse by initiating a sporozoite induced blood stage infection with *P. berghei* followed by subsequent infections with same species of parasite expressing luciferase and GFP delivered through mosquito bite. In mice with an ongoing blood stages induced by primary infection, an arrest of EEF development that precluded the initiation of either luciferase or GFP blood stages was observed. The phenomenon of EEF inhibition mediated by super infection was tested in a series of knock out and immune deficient mice models and the contribution of either innate or adaptive immune responses were completely ruled out. The inhibition was strictly dependent of the blood stages and the effect was abrogated when malaria was cured under chloroquine administration. While analyzing if nutritional aspects of the host had any impact on this phenomenon, the authors discovered the role of hepcidin, a host iron regulatory hormone in severely limiting the growth of liver stages during an ongoing blood stage infection. By stimulating the

production of host hepcidin by blood stage parasites, iron was redistributed away from hepatocytes, depriving it to developing EEFs and thus attenuating their growth. From an epidemiological perspective, the importance of this phenomenon was explained in the context of protecting the non-immune individuals from parasite densities below life threatening levels thus benefiting both host survival and parasite transmission. In addition, this mechanism may facilitate the erythrocytic stages to protect its niche from repeated new infections.

1.9. Whole Attenuated Parasites as Pre-erythrocytic Vaccines and Mechanism of Immunity

Evidence for the feasibility of a pre-erythrocytic vaccine came from the very early discovery that experimental vaccination of mouse with Radiation Attenuated Sporozoites (RAS) generated protective immunity (Nussenzweig *et al*., 1967). The RAS immunized mice developed complete resistance to blood stage infection following challenge with viable infectious sporozoites, a condition referred to as sterile immunity. Soon these studies were extended to humans, who received several hundred bites of irradiated *Plasmodium falciparum* (*P. falciparum*) infected mosquitoes and showed complete protection (Clyde *et al*., 1973). The basis of protection was attributed to both neutralizing antibodies that effectively block hepatocyte infection of sporozoites and also to αβT cells that recognize and eliminate infected hepatocyte (Nardin *et al*., 1999; Tsuji and Zavala, 2003; Hafalla *et al*., 2006). Though central role of CD8+ T cells as primary cytotoxic T cells in RAS induced immunity is well known (Romero *et al*., 1989; Weiss *et al*., 1990; Rodrigues *et al*., 1991), sterile immunity could also be achieved in the complete absence of Class I antigen presentation and was shown to be mediated by CD4+T cells, Interferon-Gamma (IFN-γ) and anti-sporozoite neutralizing antibodies [Abs] (Oliveira *et al*., 2008). Infact CD4+ T cell have shown to be capable of cytolytic function (Tsuji *et al*., 1990) and immunization of humans with RAS induces CSP specific CD4+ T cells that were capable of lysing autologous B cells pulsed with CSP peptide (Frevert *et al*., 2009). More complex mechanism of RAS induced immunity is also associated with interleukin-12 (IL), inducible Nitric Oxide synthase (iNOs) and Natural Killer (NK) cells (Hafalla *et al*., 2011).

Advances in reverse genetics have allowed the generation of a battery of Genetically Attenuated Parasites (GAPs) that expose uniform antigenic repertoire to host immune system due of their defined, precise and early developmental arrest in liver (Mueller *et al*., 2005a; 2005b; VanBuskirk *et al*., 2009). This is in sharp contrast to asynchronous EEFs derived from RAS that exhibit a broader antigenic repertoire. Persistence of CSP antigen for several weeks following RAS immunization and its ability to stimulate CSP-specific CD8+ T cell responses have been documented recently (Cockburn *et al*., 2010). Whether such paradigms are conserved also in GAP mediated immunity is an interesting question to analyze because systematic comparison of correlates of protection induced by RAS and GAPs revealed several parallels in their mechanism of protection mediated by CSP (Kumar *et al*., 2009). These findings have important implications for pre-erythrocytic vaccine development because sporozoite and early liver stage antigens alone could be sufficient components for induction of sterile immunity.

In contrast to early arrested GAPS, attenuation of EEFs has also been possible at late liver stages (Butler *et al*., 2011; Haussig *et al*., 2011; Falae *et al*., 2010). Considering that late liver stages have a subset of antigens common to blood stages, these late liver stage arrested GAPs may have superior efficacy to develop protective effector mechanism against blood stage infection. Like RAS, the general protective mechanisms in GAPs have been attributed to CD8+ T cells and IFN-γ (Jobe *et al*., 2007; Tarun *et al*., 2007; Mueller *et al*., 2007).

Translating the success of obtaining GAPs from rodents parasites to humans has been a reality as evident by attenuation of *P. falciparum* liver stage development both by targeted disruption of p52 (Schaijk *et al*., 2008) and by simultaneously double deletion of p52/p36 (VanBuskirk *et al*., 2009). Morphological assessment of sporozoite development of p52-/p36- in primary human hepatocytes transplanted in SCID Alb-uPA immunodeficient mice revealed a severe growth defect and loss of persistence. These studies provide rational and feasibility for generation of a safe and efficacious *P. falciparum* GAP based vaccine in near future.

Protective T cell immunity was also shown to be induced in rodents models infected with live *Plasmodium* sporozoites kept under chloroquine cover, the drug used for curing the blood stages of the parasite. Interestingly, immunity persisted only in the presence of the liver stage parasites and was abrogated when the mice were treated with primaquine suggesting that active antigen presentation by live parasites was essential for the effective T cell immunity (Belnoue *et al*., 2004). Similar protective efficacy has been observed recently in studies where mice infected with sporozoites and kept on antibiotic exposure of azithromycin and clindamycin showed developmental arrest in liver stage and the mechanism of inhibition was attributed to their effect on the apicoplast functions and biogenesis in EEFs (Friesen *et al*., 2010).

1.10. Transgenic Models to Study the Pre-Erythrocytic Immune Correlates of CSP

Development of transgenic animal and parasite models central to the CSP have enabled the precise understanding of several aspects of protective immune responses elicited by attenuated sporozoites. These models have been used to assess the both correlates of humoral and cell mediated immunity in addition to unraveling the early events of T cell responses and antigen presentation mechanisms. Using a TCR transgenic mice specific for the H-2k^d restricted CD8+ T cell epitope of *P. yoelli* CS, several events associated with early activation of naïve-antigen specific CD8+ T cells were deciphered (Sano *et al*., 2001). Following 24 h of exposure to sporozoite antigens, the naïve antigen specific CD8+ T cells achieved effector functions *in vivo* as evinced by up regulation of IFN-γ and perforin mRNA expression, while *ex vivo* cytotoxic activity was detectable by 48 h post immunization. Importantly, a strong inhibition of parasite development mediated by these CSP-TCR CD8+ T cells was observed in mice that were challenged with live sporozoites within 24 h following immunization with attenuated parasites.

Utilizing the same transgenic system, another study demonstrated that priming of naive antigen specific T cells occur in the lymph node that drain the site of sporozoite injections (Chakravarty *et al*., 2007). Dendritic cells played an important role in this process as protective immunity was abrogated by elimination of the lymph nodes. The acquisition of antigens by DCs released during the process of cellular migration by sporozoites (Mota *et al*., 2001) can explain the effective priming of these naïve T cells. The antigen stimulated naïve T cells home to the liver and upon encountering processed malaria epitope initiates an effector function that eliminates the infected hepatocytes in a cytolytic manner.

Understanding the mechanism of antigen presentation occurring on DCs and hepatocytes following immunization with RAS has important implications for development of pre-erythrocytic vaccines. By generating a *P. berghei* CS mutant parasite carrying a model H-2k^b epitope, the cellular processing and presentation of antigens in DC and hepatocytes has been elucidated in detail (Cockburn *et al*., 2011). While presentation of antigen on both DC and hepatocytes was TAP (Transporter of Antigen Peptide) dependent, the acquisition of sporozoite antigens in the cytoplasm of these cell types was reported to occur distinctly. A likely explanation for the sporozoite antigens to enter cytoplasm of DCs is when CSP antigen is cross presented via endosome to cytosol pathway following phagocytosis and retro-translocation in the cytosol. A definitive role of endosomes in this process was implicated using mouse model (3d mice) that are defective in endosomal TLR function and cross presentation. DCs obtained from immunized 3d mouse failed to prime the H-2k^b epitope specific T cells, a function that was unaffected when the DC were coated with exogenous peptide. In contrast, hepatocytes present sporozoite antigens by directly secreting into the cytosol. Though the exact mechanism of antigen delivery into the hepatocyte cytoplasm remains unknown, a role for *Plasmodium* export element that was earlier implicated essential for the parasite proteins to cross PVM (Singh *et al*., 2007) did not seem to be critical for this function.

Plasmodium sporozoite and liver stages express few thousands of gene products (Tarun *et al*., 2008) and identification of immune targets that contribute to sterile immunity mediated by RAS poses a great challenge for development of an effective pre-erythrocytic vaccine. The precise number of immune targets and their relevance in induction of protective immune responses remain elusive. Addressing this issue, one study demonstrated the predominance of the CSP based immune response contributing to the pre-erythrocytic immunity following RAS immunization using a transgenic mice model expressing *P. yoelli* CSP (Kumar *et al*., 2006). These transgenic mice were completely tolerant for both CD4+ and CD8+ T cell responses for CSP and by further crossing the mice in antibody deficient (JhT(−/−), lacking J_H gene) background an immunological setting (CSP-Tg JhT) was obtained that facilitated evaluating the protective potency of non-CSP T-cell antigens following RAS immunization. A prime boost regiment of 10^5 irradiated Sporozoites (IrSp) followed by challenge with $2x10^4$ live infectious sporozoites reversed the parasite liver stage burden by 3 and half logs in CSP-Tg JhT mice as compared to JhT mice alone. This study was first of its kind to demonstrate that tolerance of mice to single parasite antigen greatly abolishes the generation of protective immune responses thus signifying the immunodominant role of CSP. However following multiple immunizations (more than two) of CSP-Tg JhT mice, complete sterile immunity was obtained. The basis of such protection was principally mediated by CD8+ T cells as depletion of this subset of cells followed by challenge with live sporozoites led to blood stage infection. These results were consistent with an independent study where *P.berghei* CS locus was swapped with *P.falciparum* CSP to generate a *PbPf* transgenic parasite. Immunization of mice with these transgenic parasites followed by challenge with live *P.berghei* sporozoites resulted in generation of sterile immunity. These results suggest that immunity generated in the absence of exposure to *P.berghei* CSP antigen was sufficient for obtaining sterile protection (Gruner *et al*., 2007).

The role of non-CS CD8+ antigens in sterile immunity was though unequivocally proven using the CSP-Tg JhT model, this scenario may be relevant only in the complete absence of the immunodominant responses against CSP, an instance that is hard to expect under natural settings. Immunization of animals and humans with RAS induces high levels of antibodies and effector T cells against CSP. Memory B cells specific for *P. falciparum* CSP has been detected in individual living in endemic areas (Wipasa *et al*., 2010). Further, in individual who are naturally infected with *P. falciparum*, presence of IFN-γ secreting CD4+ T cells that recognize a universal epitope in CSP have been associated with resistance to reinfection (Reece *et al*., 2004). Given that *P. falciparum* CS based immune responses preponderate following immunization with RAS (Doolan *et al*., 2008); it will be difficult to predict the how immunogenicity for non-CS antigens would build following repeated immunization. Importantly when an ongoing immune response against an immunodominant antigen has been initiated, the kinetics of CD8+ T cell epitope competition between CS and non-CS antigens may be hard to predict in the absence of any measurable correlates of protection for non-CS liver stage antigens. One major limitation in studying contribution of immunity against non-CS antigens is that *Plasmodium* sporozoites, where CS has been silenced cannot be obtained. Nonetheless, search for other liver stage antigens based on in silico predictions have been undertaken in *P. falciparum* (Doolan *et al*., 1997; 2003). In a recent study, 34 *P.yoelli* sporozoite antigens were identified based on having strong H-2K^d restricted epitopes and on their ability to sort antigens into the secretory pathway in the sporozoites stages. Synthetic peptides corresponding to these epitopes were obtained to screen for the presence of peptide-specific CD8$^+$ T cells secreting IFN-γ in splenocytes from CSP-Tg/JhT(−/−) BALB/c mice hyper immunized with RAS (Mishra *et al*., 2011). These studies revealed that the numbers of IFN-γ-secreting splenocytes specific for the non-CSP antigen-derived peptides were 20-100 times lower than those specific for the CSP-specific peptide. When mice were immunized with recombinant adenoviruses expressing selected non-CSP antigens, the animals were not protected against challenge with *P. yoelii* sporozoites although large numbers of CD8$^+$ specific T cells were generated. These studies may reiterate the fact that endogenous non-CS T cell epitopes derived from sporozoite may have much superior efficacy for antigen processing and presentation that may not be recapitulated in subunit delivery platforms. The possible presence of moieties like GPI and membrane lipids in sporozoites that provide an adjuvant like effect in boosting the immune responses following multiple immunizations cannot be ruled out.

Antibodies against CSP block the entry of the sporozoites into hepatocytes (Yoshida *et al*., 1980; Potocnjak *et al*., 1980). The inhibition is visibly apparent when they cross link to CSP present on the surface of the sporozoite and generates a characteristic precipitin reaction that appears like a thread extending from the posterior end of the sporozoite (Vanderberg *et al*., 1969). Though CSP reactions and antibody titers measured by ELISA forms the basis for evaluating the anti-CSP humoral responses, a quantitative measurement of sporozoite inhibition is highly essential to assess the neutralization potential of the several CSP based vaccines (Stoute *et al*., 1997; Nardin *et al*., 2000) that aim towards inducing strong anti-CSP antibodies. The feasibility of such measurement was evident by obtaining transgenic parasite line where the repeats of the *P.berghei* CSP were replaced by the repeats of the *P. falciparum* (Persson *et al*., 2002). These parasites defined as biologically rodent and antigenically human, could readily assess the neutralizing ability of CSP-based antibodies generated in humans following $(TIB)_4$ MAP (Pf multiple antigen peptide containing the T1 epitope in combination with the $(NANP)_3$ B cell epitope) vaccination (Nardin *et al*., 2000). A direct comparison of the antibody titers generated in response to $(TIB)_4$ MAP vaccination in a limited number of humans have revealed no correlation with their ability to neutralize the sporozoites, thus reiterating the fact that antibody titers alone may not be a true correlate to assess the protective efficacy of CSP based subunit vaccines (Kumar *et al*., 2004). In this regard, the PbPf transgenic parasites serve as invaluable tools in assessing the neutralizing potency of CSP based vaccines that are currently undergoing human trials.

2. CONCLUSION

At the start of the new millennium, malaria still poses the greatest threat of all parasites to human health and the scenario represents no change from many years ago. The recent rapid and spectacular developments in molecular biology and allied biological sciences raise hopes that new antimalarial vaccines will be developed. Many bemused gaps in our understanding of liver stage biology remain to be addressed. The clandestine nature of the pre-erythrocytic life cycle has not made the study any easier. Radically different tools will be required to address fundamental questions and to devise an effective intervention strategy against liver stages. *Plasmodium* is a master of disguise and researchers have to try a diverse range of tactics to target the parasites in both human and mosquito host. Vaccine for malaria has been a research goal for more than a half a decade now. Indeed, understanding human immunity to malaria and identifying novel pre-erythrocytic antigens are two top research priorities.

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