Role of the Histamine Releasing Factor (HRF) in Plasmodium parasite transmission and disease pathogenesis

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Role of the Histamine Releasing Factor (HRF) in *Plasmodium* parasite transmission and disease pathogenesis

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Alla mia nonna Cecchina perché ci sei sempre stata, anche ora,

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« La science, comme la poésie, se trouve, on le sait, à un pas de la folie. »

Alexandre Yersin

Summary

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Abbreviations

Ab	Antibody
ADCC	Antibody-dependent cellular cytotoxicity
ADCI	Antibody-dependent cellular inhibition
AID	Activation-induced cytidine deaminase
AMA	Apical membrane protein
APC	Antigen presenting cell
BBB	Blood-brain barrier
CD	Cluster of differentiation
CM	Cerebral malaria
COX	Cyclooxygenase
CS	Circumsporozoite
CSP	Chemoprophylaxis
DC	Dendritic cell
ECM	Experimental cerebral malaria
EMP	Erythrocyte membrane protein
GAP	Genetically attenuated parasite
GPI	Glycosylphosphatidylinositol
HDC	Histidine decarboxylase
HRF	Histamine releasing factor
hrfΔ	Genetically HRF-deficient parasites
ICAM	Intracellular adhesion molecule
IFA	Immune fluorescence assay
IFN	Interferon
Ig	Immunoglobulin
LAP	Live attenuated parasite
IL	Interlukin
IRBCs	Infected red blood cells
LAG	Lymphocyte-activation gene
MIF	Macrophage migration inhibitory factor

MIP	Macrophage inflammatory protein
MSP	Merozite surface protein
NK	Natural killer
NKT	Natural killer T
NO	Nitric oxide
PAMPs	Pathogen-associated molecular patterns
PD	Programmed death
PD-L	Programmed death ligand
p.i.	Post infection
PRRs	Pattern-recognition receptors
PV	Parasitophorous vacuole
RAS	Radiation-attenuated sporozite
RBCs	Red blood cells
SERA	Serin repeat antigen
SMA	Severe malarial anaemia
TCR	T cell receptor
Th	T helper cell
TLR	Toll like receptor
TNF	Tumor necrosis factor
TSAP	Tumor suppressor activated pathway
uRBCs	Uninfected red blood cells
VCAM	Vascular cellular adhesion molecule Plamsodium
P.	*Plasmodium*
Pf	*P. falciparum*
Pb	*P. berghei*
Pc	*P. chabaudi*
Py	*P. yoelii*
Pv	*P.vinckei*
kDa	Kilo Dalton
Å	Ångström

Résumé en Française

Au seuil du troisième millénaire, le paludisme constitue et demeure un problème majeur de santé publique. Avec le sida (VIH) et la tuberculose, le paludisme fait partie des trois maladies infectieuses les plus mortelles au monde. Bien qu'en diminution, il s'agit de la maladie parasitaire la plus dévastatrice car elle concerne 95 pays, avec environ la moitié de la population mondiale exposée au risque d'infection. En 2015, les estimations font état d'environ 500'000 décès en Afrique sub-saharienne dont 65% étant des enfants de moins de 5 ans (WHO, 2015). Outre son impact sanitaire, dans les régions hautement endémiques, le paludisme représente un sérieux obstacle au développement socio-économique avec une diminution de près de 1,3% du produit intérieur brut (Sachs and Malaney, 2002). Si la maladie est essentiellement retrouvée dans la plupart des régions tropicales et subtropicales de la planète, avec un risque particulièrement élevé en Afrique, des cas d'importation ont également été recensés en Europe.

Le paludisme est du à une infection par un protozoaire intracellulaire du genre *Plasmodium*, transmis entre individus par la piqûre de certaines espèces de moustique femelles du genre *Anopheles*. Dans la nature il existe plus de 250 espèces de *Plasmodium,* mais seulement cinq espèces infectent naturellement l'homme (*Plasmodium malariae, P. vivax, P. ovale, P. knowlesi* et *P. falciparum),* dont *P. falciparum* est responsable de la majorité des décès et des formes cliniques sévères du paludisme. D'autres espèces plasmodiales qui infectent les singes (par exemple *P. knowlesi*, *P. cynomolgi*, etc.) ou les rongeurs (*P. yoelii*, *P. berghei*, *P. chabaudi* et *P. vinckei*) sont étudiées comme modèles expérimentaux.

Le cycle de vie de l'agent causal du paludisme est complexe et implique un hôte intermédiaire vertébré et un hôte définitif (vecteur). Chez l'hôte vertébré, l'infection par *Plasmodium* débute avec la phase pré-érythrocytaire au cours de laquelle la forme sporozoite déposée dans la peau par le moustique rejoint le foie *via* la circulation sanguine afin de se différencier en une forme infectant les globules rouges. Lors de sa phase érythrocytaire, le parasite s'engage dans un cycle de maturation et multiplication, c'est alors que les symptômes de la maladie vont apparaître. Les premiers symptômes (fièvre, maux de tête, frissons et vomissements) apparaissent au bout de 10 à 15 jours après la piqûre de

moustique infectante. Dans des cas plus graves, la maladie peut se développer en neuropaludisme (NP) où les érythrocytes infectés ainsi que des leucocytes sont séquestrés au niveau des veinules post-capillaires du cerveau. Les patients atteints de cette forme sévère de la maladie présentent des taux élevés de cytokines pro-inflammatoires (IFNγ, TNFα, IL-1, IL-6) dans de nombreux tissus (Perlmann et al., 1999; Stevenson and Riley, 2004).

Le diagnostic peut se faire par évaluation de la parasitémie au microscope ou l'utilisation de tests de diagnostic rapide. Le traitement de choix pour le paludisme réside en une association médicamenteuse contenant de l'artémisinine. Mais au cours des dernières années, l'augmentation des phénomènes de résistance associés à l'utilisation d'antipaludiques de synthèse a conduit à réexaminer les stratégies de lutte pour réduire la mortalité, la morbidité et la transmission vectorielle imputées à la maladie. Dans ce contexte, la mise au point d'un vaccin contre le paludisme est l'approche la plus prometteuse pour parvenir à contrôler de manière significative le paludisme, en particulier en Afrique, où l'habitat écologique est tel que le contrôle des moustiques est difficile, voire impossible à réaliser. En outre, le programme de vaccination lancé par l'Organisation Mondiale de la Santé (OMS) en 1974 a démontré que le contrôle des principales maladies infectieuses est réalisable à l'échelle mondiale grâce aux vaccins. Actuellement, aucun vaccin contre le paludisme n'existe sur le marché. La complexité du parasite qui inclut trois stades de développement (stade pré-érythrocytaire, stade sanguin asexué et stade sanguin sexué) chez l'homme et le choix d'un antigène capable d'induire une réponse immunitaire durable sont des obstacles à la sélection des cibles vaccinales. En effet, plusieurs milliers de protéines (plus des glucides et des lipides) sont synthétisées par les parasites au cours de l'infection. Ces composés peuvent servir de cibles antigéniques pour deux types de réponses immunitaires: humorale avec la sécrétion d'anticorps (IgG) contre les parasites et cellulaire avec l'activation des cellules T qui peuvent attaquer les cellules infectées et stimuler la production d'anticorps. De plus, de nombreuses protéines antigéniques varient entre les différentes souches de *Plasmodium* pendant l'infection. Pour rendre les choses encore plus complexes, le parasite peut également commuter la sélection de protéines qui apparaissent à la surface des globules rouges infectés et échapper ainsi aux anticorps de l'hôte. Le paludisme, par conséquent, constitue un défi complexe pour le système immunitaire humain

et la compréhension des mécanismes régissant la pathogenèse et les réponses immunitaires de l'hôte contre les parasites *Plasmodium* représente un défi majeur. Dans ce contexte, un vaccin efficace peut sembler utopique, malgré le fait qu'une immunité naturelle est de facto développée au fil des ans chez les personnes constamment exposées au parasite.

Des études récentes suggèrent une forte relation entre susceptibilité au paludisme de forme sévère et réponse de type allergique. En effet, des niveaux plasmatiques et tissulaires élevés d'histamine, un amine vaso-active connue pour ses propriétés pro-inflammatoires et immuno-régulatrices, ont été associés à la sévérité de la maladie chez l'homme infecté par *P. falciparum* et dans de nombreux modèles animaux (MacDonald et al., 1995). Récemment, notre laboratoire a mis en évidence le rôle crucial de l'histamine dans la pathogénèse de la maladie chez le rongeur (Beghdadi et al., 2008). L'utilisation de souris traitées avec des antihistaminiques ou génétiquement déficientes en histamine ont montré une résistance au NP, suggérant un rôle important de la voie de signalisation de l'histamine pendant l'infection palustre (Beghdadi et al., 2008). Cette résistance est caractérisée par une préservation de l'intégrité de la barrière hémato-encéphalique avec une diminution de l'expression d'ICAM-1 par les cellules endothéliales et une absence de séquestration d'érythrocytes infectés et d'adhésion de leucocytes au niveau des vaisseaux sanguins cérébraux (Beghdadi et al., 2008). Au cours de l'infection, l'histamine peut être libérée par les basophiles et les mastocytes via le complexe IgE-FceRI (Perlmann et al., 1999) ou via l'activation des cellules par le facteur de libération de l'histamine (HRF), une protéine ubiquitaire multifonctionnelle hautement conservée chez les eucaryotes (Bommer and Thiele, 2004a), y compris *Plasmodium* (MacDonald et al., 2001). Impliquée dans la réversion tumorale et dans l'allergie, HRF a également été décrite comme cible d'antipaludiques tels que l'artémisinine (Bhisutthibhan et al., 1998). En outre, une étude réalisée au Mali a montré qu'une concentration élevée de la protéine parasitaire HRF dans le sérum est liée au développement d'une forme sévère de paludisme, mettant ainsi en évidence l'importance de cette protéine dans le développement de la maladie (MacDonald et al., 2001). D'après les observations précédentes, il est raisonnable de postuler que la libération d'histamine induite par le parasite depuis son entrée dans la peau de l'hôte suivie par son développement dans le foie et, finalement, dans le sang périphérique, peut contribuer au développement des propriétés pathogènes du parasite en modulant la réponse immunitaire de l'hôte.

Pour évaluer l'importance de la protéine parasitaire HRF pendant le développement de la réponse immunitaire et son impact sur la sévérité de la maladie associée à la production d'histamine, il était nécessaire de générer des lignées de parasite déficientes en *hrf*. Contrairement à la souris chez qui la délétion du gène est létale au stade embryonnaire, chez *P. berghei* (*Pb*), nous avons réussi à générer deux parasites murins déficients pour la protéine HRF (*hrfΔ*): *Pb*ANKA GFP et *Pb*NK65 GFP, une souche mortelle du paludisme murin qui, contrairement à *Pb*ANKA, n'engendre pas de NP, mais provoque une forte anémie et nous permet de suivre la réponse immunitaire sur une plus longue période.

Tout d'abord nous avons étudié le rôle de la protéine parasitaire pendant le cycle de vie et surtout dans le développement du parasite chez le moustique en déterminant par qRT-PCR ou par comptage le nombre de parasites (ookinète et sporozoites) au cours du temps. Les résultats obtenus ont démontré que la délétion du gène *hrf* chez *Plasmodium* n'affecte pas le développement du parasite en soi, ce qui signifie que la suppression n'est pas létale pour le parasite. En outre, nous avons observé que la mutation ne montre aucune anomalie significative dans la motilité et dans la formation des formes exo-érythrocytaires *in vitro* et dans la traversée cellulaire *in vivo*.

Les souris infectées avec des sporozoïtes *Pb*ANKA-*hrfΔ* ont montré une diminution de la fréquence du NP associée à un retard de deux jours entre l'apparition des formes sanguines de *Pb*ANKA-*hrfΔ* par rapport à *Pb*ANKA WT. L'absence de *Plasmodium* HRF induit une diminution de la sévérité de la maladie, ainsi qu'une anomalie au niveau du développement des parasites mutants pendant le stade hépatique qui est associée à une augmentation précoce systémique d'IL-6 (Mathieu et al., 2015), une cytokine qui inhibe fortement le développement des stades hépatiques du *Plasmodium* (Pied et al., 1991). L'effet inhibiteur de *Pb*HRF sur la production de IL-6 induite par LPS souligne le rôle spécifique et l'effet direct de la protéine parasitaire HRF dans l'inhibition d'une cytokine qui présente une activité antiparasitaire (Mathieu et al., 2015). De plus l'augmentation de la population des cellules B dans le foie des souris infectées par des parasites mutants reflète la possibilité que la protéine *Plasmodium* HRF puisse interférer avec la mémoire des lymphocytes B et désactiver le développement des cellules B, possiblement via l'induction d'apoptose.

Après cette première observation, les parasites *Pb*NK65-*hrfΔ* ont été utilisés pour étudier le rôle de HRF dans la pathogenèse. L'infection par les parasites *Pb*NK65-*hrfΔ* est caractérisée par l'élimination du parasite au 12ème jour après l'infection qui conduit à une protection de longue durée et au développement d'une mémoire immunitaire. Contrairement à l'infection par le parasite de type sauvage, la souche atténuée induit une immunité contre différentes souches de *Plasmodium* et aussi contre les différents stades. D'ailleurs, nos données ont montré le rôle protecteur joué par les cellules B et T sachant que les souris dépourvues de ces lymphocytes ont été incapables de contrôler le développement du parasite dans le sang. En outre, nous avons observé que l'absence de la protéine HRF augmente la survie des cellules T par l'inhibition de l'expression du récepteur "programmed cell death 1 (PD1)" sur des cellules de type $CD4^+$ et $CD8^+$. La production de cytokines telles que l'IL-12 et IL-6 sont également augmentée chez les souris infectées par *Pb*NK65-*hrfΔ* et chez les souris déficientes en IL-6, nous avons observé une augmentation de la parasitémie ce qui suggère un rôle important de l'IL-6 dans le contrôle du développent du parasite. Il a été rapporté que l'IL-6 favorise la prolifération des cellules B ainsi que la différenciation et la sécrétion d'anticorps. Avec cette information à l'esprit nous avons testé si des anticorps spécifiques (IgG) pour les antigènes du parasite ont été produits chez les souris protégées. Les analyses sur le sérum de ces souris ont montré qu'en effet la réponse immunitaire contre *Pb*NK65 WT ou d'autres souches de *Plasmodium* est associée à la production d'anticorps spécifiques IgG2c qui reconnaissent des protéines de *Plasmodium* telles que MSP-1, MSP-8, SERA-1, et SERA-2 de façon spécifique. De plus, ces protéines de *Plasmodium* sont connues pour susciter des anticorps associés à la protection chez les personnes vivant dans les zones endémiques (Banic et al., 1998; Okech et al., 2001) et ont déjà été présentées comme des possibles candidats vaccins. En outre, l'importance des anticorps dans la protection contre le paludisme a été clairement démontrée par des études cliniques où le transfert passif de sérum hyper-immunisé ou d'Ig purifiées d'adultes asymptomatiques aux enfants malades peut modifier le cours de l'infection en réduisant la parasitémie et les symptômes cliniques (Bouharoun-Tayoun et al., 1990; Cohen et al., 1961). Enfin, pour déterminer si les anticorps IgG induits par les parasites *Pb*NK65-*hrfΔ* peuvent être des biomarqueurs à l'exposition aux parasites ou constituent des corrélats de protection stérile, des anticorps purifiés provenant de souris protégées ont été transférés passivement à des souris naïves, infectées par la suite

avec des parasites *Pb*NK65 WT. Les souris traitées avec des IgG purifiées à partir d'individus protégés ont développé une parasitémie moindre que les souris non traitées, ce qui suggère une activité anti parasitaire partielle mais significative des anticorps IgG protecteurs. Cela nous a conduit à suggérer que le transfert de cellules $CD11b^+$ ayant acquit une fonction phagocytaire plus accrue partir de souris protégées par *Pb*NK65-*hrfΔ* serait nécessaire et suffisant pour protéger les souris naïves d'une infection par le parasite sauvage. En conséquence, nous avons réalisé des expériences de transfert adoptif de cellules $CD11b^+$, obtenues à partir de souris sauvages ou de souris protégées par *Pb*NK65-*hrfΔ,* suivi d'une infection avec des parasites WT.

Le transfert de cellules $CD11b^+$ à partir de souris protégées par *Pb*NK65-*hrfΔ*, mais pas de souris naïves, protège les souris receveuses contre des infections avec le parasite sauvage. Les résultats obtenus suggèrent que les anticorps sécrétés sont importants pour l'immunité protectrice à *P. berghei*, mais l'absence apparente de protection complète suggère que ces anticorps doivent agir avec des cellules effectrices $CD11b^+$ $FcγR^+$ "amorcées" telles que les monocytes, les macrophages, les cellules NK et d'autres qui existent chez les souris infectées par des parasites *hrfΔ*, mais pas chez les souris naïves. A l'appui des résultats obtenus, plusieurs études *in vitro* ont indiqué la pertinence biologique des sous-classes de FcγRII et IgG dans la résistance au parasite en montrant l'importance du mécanisme "antibody-dependent cellular inhibition (ADCI)" lors de la protection contre *P. falciparum* en phase sanguine (Bouharoun-Tayoun et al., 1990; Bouharoun-Tayoun et al., 1995; Druilhe and Perignon, 1994; Shi et al., 1999; Zhou et al., 2015). En outre, des études humaines ont également montré l'implication de FcγR à engendrer une puissante immunité antipaludique (McIntosh et al., 2007).

Dans ce travail nous avons démontré que le ciblage précis d'un gène du parasite responsable de la régulation des processus inflammatoires, dans ce cas précis en modulant la voie de signalisation de l'histamine au niveau de l'hôte vertébré, se révèle être une stratégie clé pour le déverrouillage du système immunitaire protecteur de l'hôte induisant une diminution de la charge parasitaire et de la pathogénicité. Ces travaux ont contribué à décrypter les liens entre l'inflammation en général, et de type allergique en particulier, et le pouvoir pathogène du parasite. Cela nous permet d'émettre l'hypothèse que les individus atopiques/allergiques

sont à risque vis-à-vis du paludisme. Cela revient à aborder la prise en charge du paludisme en prenant en compte la susceptibilité des individus aux pathologies allergiques. Ainsi une prise en charge thérapeutique originale pourrait consister à proposer des combinaisons médicamenteuses, incluant des antipaludiques et des anti-histaminiques. D'autre part les mécanismes immunologiques révélés pas l'action de notre mutant HRF conduisent à proposer cet outil comme un nouveau GAP (Genetically Attenuated Parasite) à portée vaccinale visant le développement d'une protection durable.

General Introduction

1.1 MALARIA

Malaria, sometimes called the "King of Diseases", is a terrible disease, one of humankind's oldest and deadliest parasitic diseases in the world. It is caused by protozoan parasites (a group of single-celled microorganisms) belonging to the genus *Plasmodium* that infects humans, birds, reptiles, and other mammals through the intermediary of an infected female mosquito vector of the genus *Anopheles*.

Today half of the world's population is at risk. It hits the young, the poor and the vulnerable the hardest. A farmer sick for just some weeks has a huge consequence when his crops go to waste and his family goes hungry. This is happening to families around the world, causing untold misery and hampering development. In the last two decades of the 20th century the number of malaria cases worldwide seems to be increasing due to appearance of resistance phenomena associated with synthetic antimalarial drugs in use. Under this condition, identification and functional characterisation of key parasite and host proteins involved in this multi-system disorder with many similarities with the sepsis syndromes are a major challenge of the post-genomic era of *Plasmodium* research.

1.2 HISTORY OF MALARIA

1.2.1 The origin

Phylogenetic analyses of Apicomplexa genome sequences suggest that the genus *Plasmodium* evolved around 130 million years ago (Escalante and Ayala, 1995), before the dinosaurs extension. These ancestral malaria parasites were characterized by the two-host life cycle involving blood-feeding Dipterans and land vertebrates (Carter and Mendis, 2002). Mitochondrial genome sequences studies suggest that malaria may have affected human beings ever since the emergence of Homo sapiens 200,000 years ago (Jongwutiwes et al., 2005).

The climatic and ecological changes associated with an early and stable development of agriculture in sub-Saharan Africa with the loss of the forest cover beginning around 8,000 to 10,000 years ago contributed to the transmission of malaria in humans (Coluzzi, 1961; Joy et al., 2003). The forest agriculture development with culture of crops, including breaking up

the soil and clearing the forest cover created new breeding conditions that favoured the reproduction of *Anopheles* mosquitoes that could have accelerated the transmission of *Plasmodium* species among forest-dwelling agriculturalists was pivotal to the subsequent evolution and history of human malaria (Carter and Mendis, 2002; Coluzzi, 1961).

1.2.2 Malaria in the ancient world

The illness is probably from Africa and followed human migrations toward the Mediterranean coasts, to India and Southeast Asia. In the past, malaria was frequent in the Pontinses swamps, around Rome and its name has derived from a medieval italian term “mal’aria” (born in Venezia, in 1571) or bad air.

The first traces of malaria seem back to ancient Egypt, as evidenced by cases of splenomegaly (Ruffer, 1913) and presence of specific *Plasmodium falciparum* (*P. falciparum*) genes such as apical membrane antigen (*ama*) 1 and merozoite surface protein (*msp*) 1 found in 2010 by DNA amplification and sequencing performed on bone samples from the Egyptian Pharaoh Tutankhamon mummified in 1324 B.C at the age of 19 years (Hawass et al., 2010).

Historical records of ancient civilizations (Sumerians, Assyrians, Babylonians, Chinese, Egyptians and Indians) report testimonials, in medical and religious texts, of the presence of seasonal or intermittent fevers that attest the antiquity of the malaria. But at this time the malarial fevers were attributed to demons or to different gods (Crotti, 2005). The Chinese classical medical book Huang Di Nei Jing (the Canon of Medicine), edited in 2007 B.C by the Emperor Huang Ti, describes enlargement of spleen connected with different types of fever. In this document three malaria symptoms (headache, chills, and fever) are represented in the form of three demons: one with a hammer, one with a pail of cold water and one with a stone (Crotti, 2005).

At the end of the fifth century B.C in Greece, Hippocrates (460-377 B.C) attributes the origin of the various diseases to natural causes related to climatic and environmental conditions. In the Hippocrates corpus, he reported the occurrence of seasonal fevers characterized by fever peaks recurring every 48 or 72 hours and associated with rigors, cold and hot spells, profuse perspiration and splenomegaly (Cunha and Cunha, 2008).

In Italy, during the Roman Empire (circa 50 B.C to 400 A.D), malaria was excluded for several centuries, by drainage, husbandry, and building development. New episodes of malaria appeared with the decline of Empire. The presence and the disappearance of malaria have been associated with corresponding rising and falling agricultural and economic prosperity. This behavior, still observable, reflects the dependence of malaria on prevailing human activity and life-style.

All the medical knowledge acquired during the Roman Empire was lost and the malaria fevers returned to be confused with other common fevers. This situation was the cause of the spread of malaria in the cooler countries, Central and Eastern Europe. Around the sixteenth century, with the first colonization, malaria was widespread in the Americas.

1.2.3 Scientific discoveries

The scientific studies on malaria made their first significant advance the 6 November 1880 when a French military doctor in Algeria, Alphonse Laveran (Nobel prize 1907), discovered the malarial parasites by studying, under a microscope, the blood of patients not treated with quinine (natural alkaloid from the bark of a Peruvian tree, the tree of china). Due to the movement of the observed parasite, Laveran named the parasite *Oscillaria malariae.*

But the confirmation of an association between malaria and parasites occurred only in the late nineteenth century with the Italians Angelo Celli, Ettore Marchiafava and Camillo Golgi (Nobel Prize 1906). The first two described how malaria parasites, which they called *Plasmodium*, grow and multiply within the red blood cells before lysing the host cell to infect fresh erythrocytes and Golgi linked the onset of intermittent fever with the destruction of red blood cells and the spread of parasites into the blood (Ferroni et al., 2012). At the same time, morphological differences of the various agents responsible for fever were identified and *P. falciparum* was individualized as the species responsible for fatal cases of malaria (Ferroni et al., 2012). These findings allowed the description of malaria parasite life cycle stages in human blood.

While Golgi described the intra-erythrocyte parasite multiplication, Sir Ronald Ross (Nobel prize 1902), a medical officer in the British Colonial Medical Service, identified in the gut of infected mosquitoes *Plasmodium sp.* oocysts that broke and release sporozoites that

migrated to the mosquitoes salivary glands, highlighting the role of mosquitoes in malaria transmission. This discovery allowed Giambattista Grassi in collaboration with Amico Bignami and Giuseppe Bastianelli to identify the specific species of mosquito responsible for malaria transmission to humans (Ferroni et al., 2012). They showed that the mosquitoes belonging to the genus

Anopheles were the specific carriers of malaria in Italy, and described the development of the *Plasmodium* in the mosquito (Ferroni et al., 2012). To confirm these results Sir Patrick Manson, a Scottish physician working in London (where there was no *P. falciparum*), exposed his son and one of his colleagues to the infected mosquitoes coming from Italy confirming previous observations.

In 1948, Shortt and Garnham showed that the cyst-like bodies (schizonte), filled with thousands of merozoites, are formed in the liver of a rhesus monkey that had been bitten by infected mosquitoes (Cox, 2010). Henceforth, the life cycle of the parasite was fully known.

With the development of the scientific techniques it was possible to initiate the genome sequencing of the *P. falciparum* and *Anopheles gambiae* in 1996 and 1998, respectively. The published results (Gardner et al., 2002a; Winzerling and Pham, 2006) made possible different studies on parasite biology and entomology in order to promote new strategies destined to control the malaria such as diagnoses, medicines and vaccines.

1.3 DISTRIBUTION AND IMPACT

Despite the rise in the knowledge gained on *Plasmodium* parasites and the tremendous efforts to control this disease, malaria remains one of the most deadly infectious diseases in the world with a particularly devastating effect in Africa, where over 90% of deaths due to malaria occur (Fig.1). The endemic areas are mostly found in tropical and subtropical regions at an altitude below 1800 meters. Malaria infects not only in Sub-Saharan Africa, but also in Asia, Latin America, Middle East and even some areas of Europe are at risk (Fig.1).

According to the World Health Organisation, estimated 214 million cases leading to 438,000 deaths where reported in 2015, among the poorest populations in the world (WHO, 2015). The same year, 65% of deaths occur in children under 5 years of age in Africa.

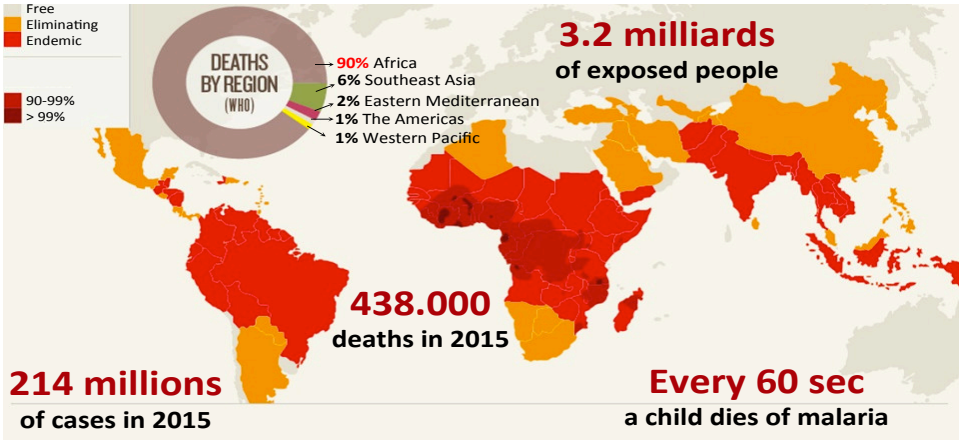

Figure 1: Global distribution of Malaria. (Shetty, 2012)

In highly endemic areas, in addition to an enormous amount of human pain, malaria plays a critical role in the socio-economic development (working and education days lost, costs of health care, and loss of investment and tourism), especially in the widespread regions. The morbidity and mortality constitute a serious obstacle to socio-economic development with a decrease of nearly 1.3% in the gross domestic product of countries (Sachs and Malaney, 2002).

Moreover, in recent years there was a return of malaria in countries where it had been eradicated in the past. This return may be the result of factors such as increasing insecticide-resistance in the vector, increasing multiple drug resistance in the parasite, increasing of large population migrations, increasing of the world proportion climatically suitable for transmission (global warming) (Nabi and Qader, 2009) and increasing of tourism between non-endemic and endemic countries (Moorthy and Hill, 2002).

1.4 PARASITES AND DISEASES

1.4.1 Malaria parasites

Malaria parasites are members of the genus *Plasmodium,* belonging to the Apicomplexa family a monophyletic group composed of at least 6000 species (Levine, 1988; Morrison, 2009), the majority of which have an obligatory intracellular lifestyle. The phylum contains other parasites that are important causative agents of human and animal diseases, such as

Toxoplasma and *Cryptosporidium* (Beck et al., 2009). In nature there are over 250 species of *Plasmodium* (Fig. 2), but only five species naturally infect humans: *P. falciparum, P. vivax*, *P. ovale*, *P. malariae*, and sometimes *P. knowlesi*. The five species differ by clinical criteria, their geographic distribution and their ability to develop resistance to antimalarial drugs. *P. vivax* is the most prevalent world-wide in most countries outside of sub-Saharan Africa, and *P. falciparum* is the most dangerous and virulent species, which is associated with severe complications such as cerebral malaria, renal failure and pulmonary affection leading to death. The other human malaria species can cause acute, severe illness but mortality rates are low. The last species, *P. knowlesi*, has been recently added to the list, originally a malaria parasite of long-tailed macaque monkeys, which has jumped the species barrier and can naturally infect humans (Daneshvar et al., 2009; Singh et al., 2004).

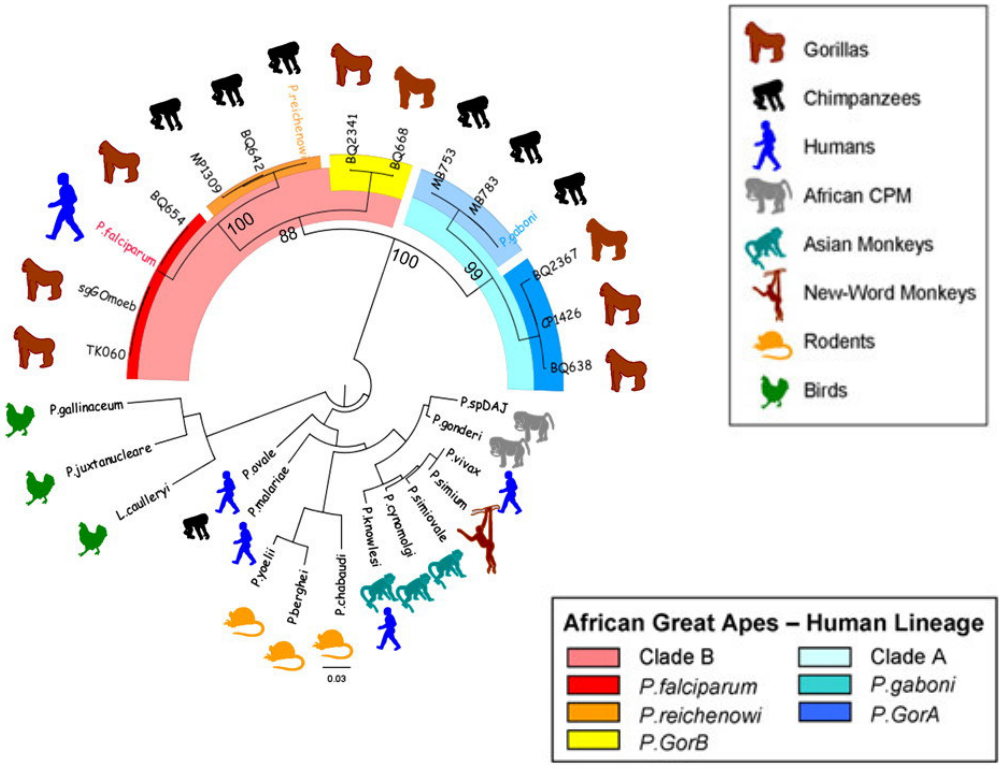

Figure 2: Phylogenetic relationships among *Plasmodium* species (Prugnolle et al., 2010).

1.4.2 Rodent malaria parasites (animal models in experimental malaria)

Rodent malaria parasites are mostly used to study experimentally the development of the disease to understand how parasites adjust traits during infections in response to changes in the host environment to maximise their fitness and escape the host immune response.

Rodent malaria parasites were discovered in Central Africa (Cameroon, Central African Republic, Congo, Democratic Republic of the Congo and Nigeria) between 1948 and 1974. During this period, four species – *P. berghei* (*Pb*), *P. vinckei (Pv)*, *P. chabaudi (Pc)* and *P. Yoelii (Py)* – were described, encompassing 13 sub-species of various strains (e.g. *Pb* ANKA and *Pb* NK65) (Ramiro et al., 2012). Like all mammalian malaria parasites, they undergo several rounds of asexual replication in the vertebrate host and sexual reproduction in *Anopheline* mosquitoes. All four rodent malaria parasites are known to be infective to mice by blood inoculation and by mosquito transmission with some differences in the susceptibility to laboratory animals. Infections caused by parasites such as *P. berghei*, and some strains of *P. vinckei (Pv vinckei*)*, P. yoelii* (*Py* YM*, Py* XL) and *P. chabaudi* (*Pc* CB) cause lethal infections in mice, whereas infections with some strains of *P. yoelii* (*Py* 17XNL), *P. chabaudi* (*Pc chabaudi* AS, *Pc adami*) and *P. vinckei* (*Pv petterei*) are cleared after the initial acute parasitaemia (Wykes and Good, 2009). Moreover, *P. berghei* and *P. yoelli* have a marked predilection to invade reticulocytes with a relatively asynchronous development in laboratory rodents whereas *P. vinckei* and *P. chabaudi* invade both immature and mature RBCs populations showing a synchronous development during the blood stage (Wykes and Good, 2009). These differences are due to some changes in morphology, developmental time and size of different stages and iso-enzymes that influence host-parasite interactions.

Consequently, there is no single model that reflects exactly infections in humans, but taken together, different systems could provide valuable informations on the mechanisms of immunity and immunopathogenesis. Lethal infections are better suited for testing putative vaccine candidates and also examining the efficacy and safety of antimalarial drugs. Non-lethal infections have been used as best models to investigate the mechanism of acquired immunity. For example, *Pb* ANKA and *Py* 17X*,* in C57BL/6J mice are used as experimental models to study the development of cerebral malaria (CM) (Sanni et al., 2002; Wykes and

Good, 2009), and *Pc chabaudi* or *Py* 17XNL are usually used to explore immune response mechanisms. Moreover, *P. yoelii* is used to compare two strains (non-lethal 17XNL and lethal YM) with vastly different pathogenicity to deduce which factors may contribute to more serious malaria infections.

1.4.3 Malaria Genome

The recently available sequence of the *P. falciparum* (clone 3D7) genome provides powerful information for understanding resistance mechanisms and opens exciting new avenues for drug and vaccine development (Arav-Boger and Shapiro, 2005).

In addition to *P. falciparum,* other genome sequences of *Plasmodium* parasites have been published. The rodent malaria *Py* 17XNL clone (Carlton et al., 2002), *Pb* ANKA clone and *Pc* AS clone (Hall et al., 2005), the human malaria *P. vivax* Salvador 1 (Sal 1) strain (Carlton et al., 2008) and the human/simian malaria *P. knowlesi* H strain (Pain et al., 2008).

Comparative analysis of the available *Plasmodium* genomes revealed that they are all haploid and composed of 22.8 megabases distributed among 14 chromosomes that vary from approximately 0.643 to 3.29 megabases. A remarkable feature is its base composition that varies among the different species, with the *P. falciparum* and rodent genomes extremely A + T rich (80.6% on average and close to 90% in introns and intergenic regions in *P. falciparum* (Gardner et al., 2002a; Winzeler, 2008) in contrast with the more G + C rich *P. knowlesi* and *P. vivax* genomes (37.5% and 42.3%, respectively) (Carlton et al., 2008; Pain et al., 2008).

Gene research identified around 5000-6000 protein-encoding genes and, about 60% (approximately two-thirds) of the predicted proteins appear to be unique to this organism and another 5% presents significant similarities to proteins of other organisms (Plouffe et al., 2008; Ward et al., 2004). The two-thirds of the proteins have poor similarities to proteins of other organisms indicating a great evolutionary distance between *Plasmodium* and other eukaryotes (Fig.3) (Gardner et al., 2002a).

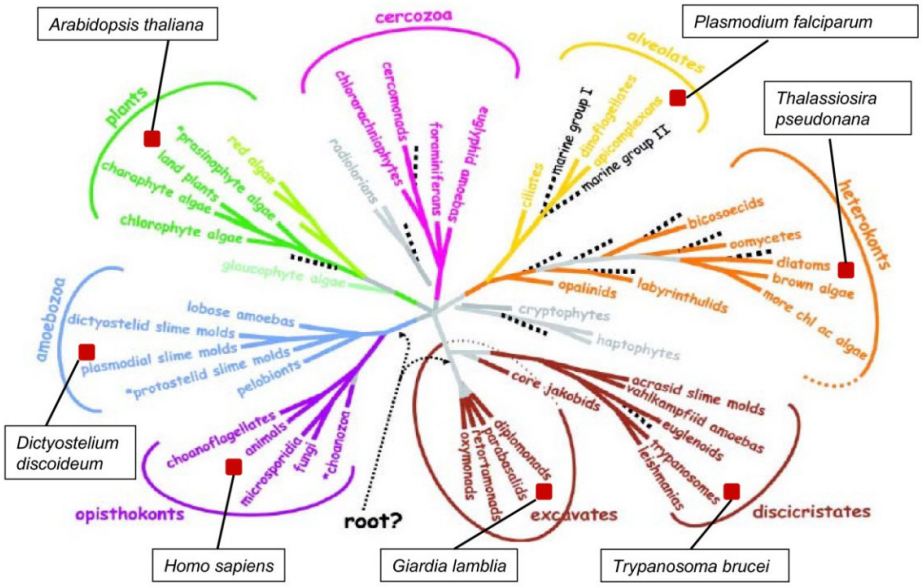

Figure 3: Phylogenetic tree demonstrating the putative relationships between the major types of eukaryotic organisms (Ward et al., 2004).

The evolutionary distance between the host (human) and the parasite proteins makes the use of inhibitors or genetic modifications, targeting the parasite, more interesting since they do not disturb the human protein and allow the development of new anti-malarial drugs or vaccines that target only the parasite vital functions without any influence on the host.

1.4.4 Life cycle

Malaria parasites have a complex life cycle which involves more than one host, an invertebrate primary host and a vertebrate secondary host (Fig. 4). The cycle can be divided into three main steps: (i) the **mosquito phase** characterized by sexual and asexual multiplications allowing the formation of sporozoites (12-14 days) (ii) the **pre-erythrocytic phase** (2-14 days) characterized by an asymptomatic asexual multiplication of the parasite in the liver of the vertebrate host and the (iii) the **erythrocytic phase** (48-72h) where the parasite undergoes an asexual multiplication within red blood cells leading to the symptoms of the disease.

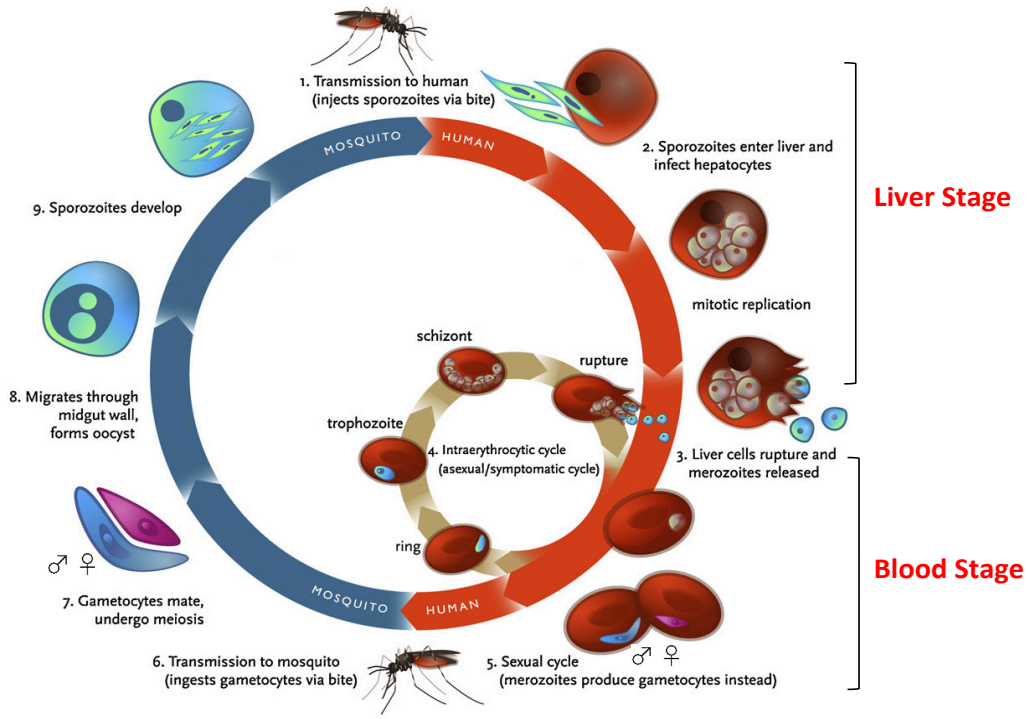

Figure 4: Schematic representation of the *Plasmodium* life cycle. The cycle consists of asexual development phase in the vertebrate host (liver and erythrocytic stages) and one sexual development phase inside the mosquito hosts (Klein, 2013).

1.4.4.1 Mosquito host

During a blood meal when a female *Anopheles* mosquito bites an infected host, it ingests mature male and female gametocytes (Kappe et al., 2010). As soon as the gametocytes are in the tract of the insect, triggered by the temperature drop and by chemicals (Billker et al., 1998), they escape from their surrounding erythrocyte membrane. In the midgut the gametocytes undergo gametogenesis, resulting in the development of female gametocyte into macrogametes while the male gametocyte undergoes exflagellation whereby it develops into eight flagella called microgametes. The male and female gametes fuse together (sexual reproduction) to form a diploid zygote that undergoes meiotic nuclear division and develops into a motile form, called ookinete, which penetrates the midgut epithelium and forms oocysts on the outer surface. Within the oocyst the parasite undergoes several rounds of asexual replication (sporogony) producing thousands sporozoites, elongate slightly curved cells approximately 10 μm long with a central nucleus and apical organelles (Schrevel et al., 2008). Rupture of the mature oocyst releases the sporozoites into the hemocoel (body cavity) of the mosquito. The sporozoites migrate to and

invade the salivary glands, thus completing the life cycle (Aly et al., 2009) for onward transmission, ready to be delivered to the skin of the host during mosquito bite (Zhang et al., 2010).

1.4.4.2 Pre-erythrocytic phase

This obligatory hepatic phase is the only classically described life cycle of the parasite where the parasite development requires invasion of nucleated host cells. Upon mosquito injection of motile salivary gland sporozoites into the human dermis, most of them start migrating in the cutaneous tissues and enter into the blood stream or the lymphatic system (Amino et al., 2006). In mice, around 50% of the inoculated parasites remain in the skin where ~10% can initiate development and lead to the production of new infectious parasites inside skin cells (Gueirard et al., 2010). Another 15% enter lymphatic vessels and reach the proximal draining lymph node where the parasites are eventually degraded. Finally, the remaining 35% invade bloodstream by entering capillaries in a process that usually lasts less than a minute (Amino et al., 2006) after the sporozoite deposition in the skin, but it can extend to hours. Once in the circulation, sporozoites migrate passively to the liver sinusoids where they migrate along the endothelial cell layer and cross the sinusoidal barrier. The parasite traverses different cells, including Kuppfer cells and several hepatocytes, before settling and establishing a parasitophorous vacuole (PV) derived from the host-cell membrane (Frevert et al., 2005; Mota et al., 2001) which is rapidly remodelled by parasite proteins (Bano et al., 2007). Once the PV vacuole is established the parasite start to develop (Frevert et al., 2005) via a process of asexuate multiplication, called hepatic merogony, which leads to the generation of hundreds or thousands of uninucleated merozoites contained vesicles called merosomes. After the lysis of the hepatocytes the merosomes are carried away in the circulation and finally reach the pulmonary vasculature where they break releasing hepatic merozoites in the blood.

Infections of *P. vivax* and *P. ovale* differ from the other types of malaria in that some sporozoites may develop into a dormant forms termed hypnozoites. Under this form the parasite can remain in the liver and relapses months or even years after the primary infection.

1.4.4.3 Erythrocytic phase

Red blood cells (RBCs) are the 'central stage' for the asexual development of the malaria parasite. Within RBCs, repeated cycles of parasitic development occur with precise periodicity. Once release in the blood, hepatic merozoites invade healthy erythrocytes by an active and rapid process, accomplished within 30 seconds of initial attachment to the erythrocyte surface minimizing the parasite surface antigens exposure, thereby protecting these parasite forms from the host immune response (Cowman and Crabb, 2006; Greenwood et al., 2008) (Fig. 5A). This invasion is a process which depends on successive major events: (i) initial weak attachment of merozoite to the erythrocyte surface by random collision mediated by MSP-1 and MSP-9 co-ligand complex that binds erythrocyte membrane transport glycoprotein (Gaur et al., 2004), (ii) reorientation of the merozoite mediated by AMA-1 (Mitchell et al., 2004) and creation of a distinctive tight junction between the apical end of the parasite and the erythrocyte followed by protein and lipid material secretion, and (iii) starting of the invagination process that involves direct interactions of parasite ligands at the apical end with erythrocyte receptors (Gaur et al., 2004). Formation of an invasive pit with the tight junction moving along the surface of merozoite as it penetrates into the erythrocyte, closing of the invasion pit on the erythrocyte membrane and isolation of the parasite within PV in the erythrocyte cytoplasm. At this point the parasite develops (asexual replication) through a change of its morphology (stages) inside the PV (Fig. 5C):

- Ring stage (0–24 hours),
- Trophozoite stage (24–36 hours)
- Schizont stage (40–48 hours)

At the end of the cycle the infected RBCs (iRBCs) lyse and release around 16-32 new merozoites that in turn will infect new RBCs. After several rounds of erythrocytic schizogony, some merozoites differentiate into the extracellular sexual stage gametocytes which develop into male and female gametocytes and are taken by mosquitoes during the blood meal.

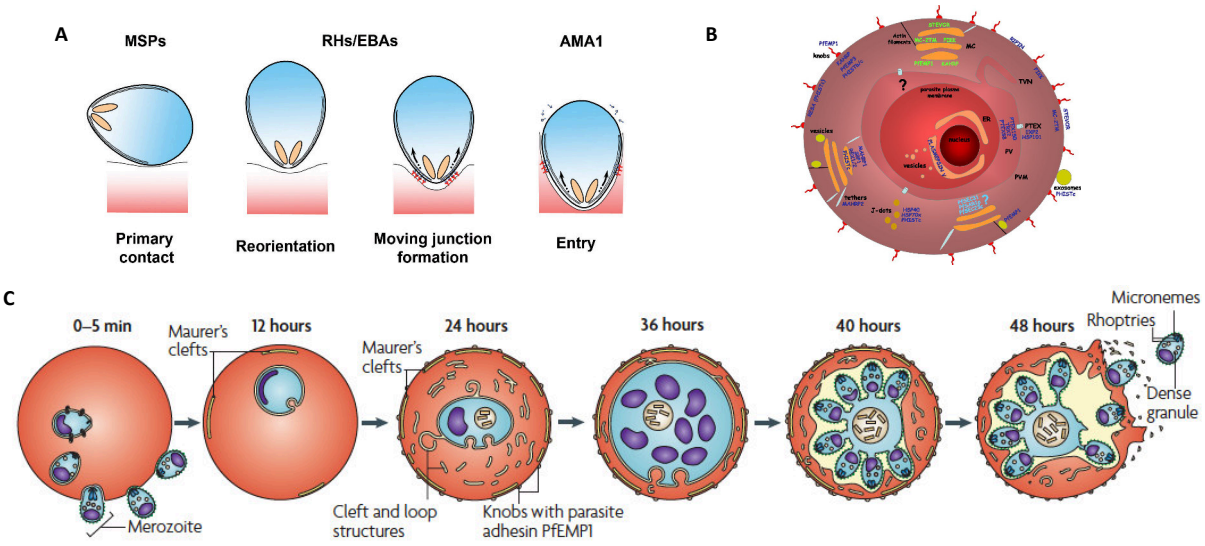

Figure 5: Schematic representation of the different stages of *P. falciparum* development in erythrocyte. (A) Major morphological changes occur during the merozoite invasion of erythrocytes. Initial weak attachment followed by reorientation, formation of tight junction and secretion of apical organelle contents and invagination (Wright and Rayner, 2014). (B) Cellular modifications occurring during parasite growth in the host erythrocyte and confirmed locations of exported proteins within the export. Protein names in dark blue indicate resident proteins of that organelle, names in green indicate transient location, and names in light blue indicate proteins, the of which location has not been confirmed. Abbreviations: ER, endoplasmatic reticulum; MC, Maurer's clefts; PVM, parasitophorous vacuolar membrane; TVN, tubular-vesicular network (Mundwiler-Pachlatko and Beck, 2013). (C) Parasite development process. Merozoite attach and RBCs invasion (0-5min). The parasite develops in a parasitophorous vacuole (PV) through the ring (0–24 hours), trophozoite (24–36 hours) and schizont stages (40–48 hours). In mature-stage (>24 h) news membrane-bound structures appear in RBC (Knobs, Maurer's clefts, etc.). After approximately 48 hours there are the infected RBC lyse and 16–32 daughter merozoites are released (Maier et al., 2009).

One of the *Plasmodium* unique features is the ability to remodelling the host erythrocyte in order to overcome the lack of standard biosynthetic pathways and intracellular organelles in the RBCs that create obstacles for the parasites fast-growing. During the ring stage the major nutrient is the hemoglobin that is ingested into a food vacuole and degraded to obtain amino acids utilized for protein biosynthesis. The remaining toxic heme is converted into harmless hemozoin (malaria pigment) by heme polymerase and sequestred (Coronado et al., 2014). As the parasite grows and multiplies within the cell there is a dramatic expansion of the surface area through the formation of a tubulovesicular network and the parasite exports a variety of proteins to the erythrocyte cytoplasm and cell surface remodeling the RBCs structure (Fig. 5B). These exported proteins have functions in cytoadherence (such as *falciparum* erythrocyte membrane protein 1 (PfEMP-1)), nutrient acquisition and evasion of the host immune response (Charpian and Przyborski, 2008; Winzeler, 2008).

1.5 MALARIA PATHOGENESIS

Malaria pathogenesis is caused by the asexual multiplication of parasites in erythrocytes. The severity and the incubation period of the symptoms vary depending on the species of *Plasmodium* that causes the parasitosis. The red blood cells destruction leads to fever and flu-like symptoms, such as chills, headache, muscle aches, nausea, vomiting and diarrhea. These symptoms usually appear 10-15 days after being bitten. The disease can progress extremely rapidly and if it is not treated quickly with effective drugs, the parasite burden continues to increase and severe malaria may occur. However, symptoms can sometimes appear after weeks or months in patients who have not been appropriately treated. In epidemic region in people who have recently survived an infection, re-infection typically causes milder or no symptoms (uncomplicated malaria) but this partial resistance disappears over months to years if there is no on-going exposure to malaria.

1.5.1 Asymptomatic malaria

In areas of high malaria transmission, *P. falciparum* infections are characterized by persistence of low levels of chronic parasitaemia in the blood stream without manifestation of any clinical symptoms in adult patients (Hamad et al., 2000). These persistent or repeated "asymptomatic" infections allow the development of a natural acquired partial immunity, which controls but not completely eliminate the infection (Doolan et al., 2009). Moreover, microscopic diagnosis is often challenged by very low parasite density and individuals who are not treated with antimalarials remain reservoirs of gametocyte for perpetuating the malaria transmission cycle (Bousema et al., 2004).

During pregnancy, malaria infection is usually asymptomatic associated with placental sequestration of parasite and significant reduction of maternal haemoglobin level (Leke et al., 1999; Matangila et al., 2014) particularly in primigravidae (Ndyomugyenyi and Magnussen, 1999). It is only during subsequent pregnancies that they acquire a form of pregnancy-associated immunity (Rowe and Kyes, 2004).

1.5.2 Uncomplicated malaria

Malaria infection usually results in an uncomplicated, mild febrile disease in which intermittent episodes of fever and peaks of parasitemia are controlled by the body's immune defences and eventually eliminated. Nevertheless, clinical symptoms and signs of malaria infection are not specific and shared with other febrile diseases as viral or bacterial diseases and precise analysis, as blood smear or rapid diagnostic test, are necessary to confirm malaria infection. Moreover, the presence of hepatosplenomegaly, thrombocytopenia and anaemia are clearly associated with malaria, particularly in children (Grobusch and Kremsner, 2005; White et al., 2014).

The clinical features have been attributed to the release of numerous know and unknown substances, such as red cell membrane products, hemozoin pigment (Sherry et al., 1995), and other toxin factors including parasite-specific glycosylphosphatidylinositol (GPI) (Schofield et al., 1996; Schumann, 2007) into the blood. These products, in particularly the GPI, are potent stimulators of cytokines and inflammatory mediators synthesis by macrophages and endothelial cells (Gazzinelli et al., 2014), such as tumor necrosis factor (TNF), interferon (IFN)-γ, interlukin (IL)-1β, macrophage inflammatory proteins (MIP)-1α and MIP-1β (Sherry et al., 1995), and nitric oxide (NO) (Gazzinelli et al., 2014). Additionally, the plasmodial DNA is highly proinflammatory and able to induce cytokinemia and fever. Hemozoin internalizes the DNA that is recognized by the toll-like receptor (TLR)-9 and activate the synthesis of pro-inflammatory cytokines. Cyclooxygenase (COX)-2 release upregulates the production of prostaglandins and leads to fever (Schumann, 2007) (Fig. 6).

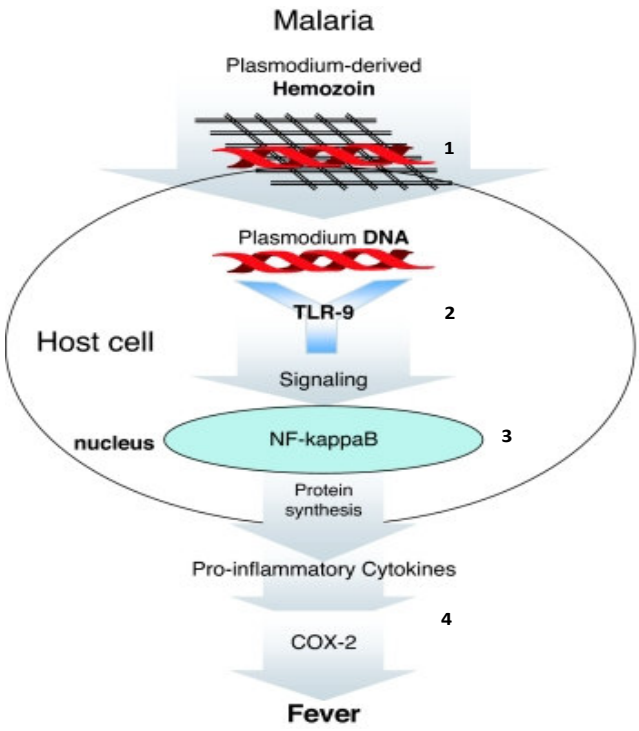

Figure 6: Potential mechanism of malaria-induced fever. (1) Hemozoin presents or internalizes plasmodial DNA. (2) TLR9 recognizes plasmodial DNA and initiates the innate immune response. (3) NF-kappaB activated pro-inflammatory cytokines induce COX-2. (4) COX-2 upregulates prostaglandins leading to a change in the set-point of the thermoregulatory center (fever). (Schumann, 2007)

1.5.3 Severe malaria

The progression of the infection results in progressive and dramatic modification of RBCs that can worsen into severe malaria responsible for a broad spectrum of clinical features accompanying *P. falciparum* infection. Nevertheless in the recent years, several cases of severe infection and even deaths have been reported in non-*falciparum* infections such as *P. vivax* (Naing et al., 2014) and *P. knowlesi* (Cox-Singh et al., 2010; Rajahram et al., 2016) infections.

The manifestations of severe *falciparum* malaria depend on age (Dondorp et al., 2008). In infants and young children the development of metabolic acidosis (respiratory distress), cerebral malaria (CM) and severe malarial anaemia (SMA) are the major complications causing high mortality (Gazzinelli et al., 2014; Schofield and Grau, 2005). In non-immune adults only a minority of infections become severe with the involvement of single-organ, multiple-organ or systemic impairment. Multiple complications could occur in an individual

at the same time and can develop rapidly and progress to death within hours or days if appropriate treatment is not provided in time.

1.5.3.1 Severe Malaria Anaemia (SMA)

Anemia is a common manifestation of all types of malaria, especially in children and during pregnancy, and is globally the leading cause of malaria related to mortality and morbidity. The pathophysiological processes in SMA can include a number of distinct, as well as overlapping features, such as the rupture of infected and uninfected RBCs (Dondorp et al., 1999), splenic sequestration of RBCs (Buffet et al., 2009), impaired erythopoiesis and dyserythropoiesis (Abdalla et al., 1980; Dormer et al., 1983; Kurtzhals et al., 1997; Perkins et al., 2011), co-infections with bacteria (Were et al., 2011), virus (ex. HIV-1) (Davenport et al., 2010) or hookworm (Price et al., 2001; Stoltzfus et al., 2000).

The spleen uptake of infected, altered or uninfected RBCs (uRBCs) is responsible for the clearance of those cells by the phagocytic cells such as monocytes, macrophages and neutrophils due to adherence of malarial IgG or complement binding to their surface (Haldar and Mohandas, 2009). The elimination of uRBCs may also occur by antibody-dependent cell-mediated cytotoxicity (ADCC). In confirmation of this hypothesis, recent studies have detected on the surface of ring stage iRBCs membrane as well as in uRBCs *P. falciparum* rhoptry protein 2 (Sterkers et al., 2007). Moreover, the impaired erythropoiesis due to the involvement of cytokines and other mediators of inflammation (such as hemozoin) causes SMA. The dysregulation of the innate immune response due to the phagocytosis of the malarial pigment hemozoin by effector cells such as neutrophils, macrophages and monocytes down-regulate IL-12 levels, associated with a decreased production of IFN-γ and IFN-α, through an IL-10-dependent mechanism allowing the development of the parasite and SMA (Haldar and Mohandas, 2009; Keller et al., 2006). Increased circulating levels of TNF, IL-6, IL-1b, IL-1RA, MIP-1α, MIP-1β are observed in sick children as well as prostaglandin E, NO, macrophage migration inhibitory factor (MIF), chemokine (C-C motif) ligand 5 (CCL5 or RANTES) and stem cells growth factor suppression. Further, in children with SMA, reduced NO production promotes ineffective parasite killing and may suppress, in the bone marrow, the erythropoiesis (Haldar and Mohandas, 2009). Recent studies in mice using

P. chabaudi AS have highlighted the important role of the Th2 cytokine IL-4 in erythropoiesis suppression (Thawani et al., 2009).

1.5.3.2 Cerebral Malaria (CM)

Cerebral malaria (CM) due to *P. falciparum* parasites sequestration in the brain is the most striking severe malaria syndrome characterized by gradual neurological disorders (Idro et al., 2005) starting from prostration, loss of consciousness, convulsions that occur in one third of the cases and progressive paralysis leading to coma even with appropriate medical intervention (Lovegrove et al., 2008). Disorders develop in 1% of patients infected with *P. falciparum* which cause a mortality rate of 20-30% among infected people, while more than 10% of survivors present neurological sequelae such as spasticity, ataxia, hemiplegia and blindness (Armah et al., 2007). Furthermore, numerous differences exist between paediatric and adult CM. Epidemiological data show that in endemic areas severe malaria is a complication almost exclusively associated with infancy. However, CM does not occur in children upon first infection, but after one or several infections (Artavanis-Tsakonas et al., 2003).

The immunopathogenesis of cerebral pathology in both human CM and experimental cerebral malaria (ECM) is a complicated series of inter-related events involving multiple organs, parasite and host immune factors over a period of some 5-10 days. Several mechanisms, which fall under three major hypotheses (Polimeni and Prato, 2014) are supported today to explain the CM pathogenesis: 1) the mechanical theory, 2) the permeability theory, and 3) the immunological theory.

The mechanical theory proposes that CM pathology is as a result of mechanical obstruction of cerebral microvasculature by iRBCs due to their impaired deformability (Coltel et al., 2004) or cytoadherence to receptors expressed on endothelial cell surfaces (Fig. 7A) (Miller et al., 2002), causing flow perturbations and hypoxia of the surrounding brain parenchyma (Turner, 1997) and increased lactate production due to anaerobic glycolysis (Planche et al., 2005). The cytoadherent properties increased in mature parasites (trophozoïtes, older schizonts) is associated with the expression on iRBCs surface of parasite proteins, such as *Pf*EMP-1, that interact with specific host receptors in the microvascular endothelium,

including intracellular adhesion molecule-1 (ICAM-1), vascular cellular adhesion molecule-1 (VCAM-1), thrombospondin, CD36, and E-elastin (Polimeni and Prato, 2014). Other phenomena such as rosetting (Kaul et al., 1991), characterized by iRBCs forming a flower-like cluster around a uRBCs, clumping a cluster of iRBCs and platelets (Cox and McConkey, 2010; Pain et al., 2001), and decreased deformability of the iRBCs further increase the clogging of the microcirculation (Fig. 7A).

The permeability theory highlights the importance of the blood-brain barrier (BBB) alterations, caused by secondary signalling events activated by sequestered iRBCs, that allow toxic compounds to enter the brain and cause neurological dysfunction (Gitau and Newton, 2005; Hunt et al., 2006). Vascular permeability is found to be mildly increased and associated with cerebral edema in several animal models, however, in human no definite evidence of cerebral edema has been found on imaging studies (Medana and Turner, 2006). A correlation between malaria disease severity and the role of histamine, a vaso-active amine, has also been demonstrated in *P. falciparum* infections; with a markedly increased brain uptake of histidine in infected children that would favour an elevated brain level of histamine (Enwonwu et al., 1999). Accordingly, increased levels in plasma and tissue histamine have been associated with disease severity in human *P. falciparum* infections (Enwonwu et al., 2000). Moreover, our laboratory has highlighted the crucial role of histamine in the pathogenesis of the disease in mice. Mice deficient in histamine due to disruption of the histidine decarboxylase gene ($HDC^{-/-}$) or treated with antihistamines or deficient for the H1 and H2 receptors of histamine are more resistant a to ECM, suggesting a harmful role of histamine pathway signalling through these receptors (Beghdadi et al., 2008). The involvement of histamine in the severity of the disease was confirmed by the absence of neuropathology observed in histamine-deficient mice. This resistance is characterized by the preservation of the integrity of the BBB with a decrease in the expression of ICAM-1 by endothelial cells and the absence of sequestration of iRBCs or adhesion of leukocytes to the cerebral blood vessels (Beghdadi et al., 2008).

The immunological theory, based on animal testing and poorly established, suggests that host factors such as cytokines and chemokines can enter the brain parenchyma after increased BBB permeability and aggravate pathological consequences. Curiously, in contrast

to other encephalitis-causing pathogens, such as *Trypanosoma* spp. or *Toxoplasma gondii* (Elsheikha and Khan, 2010), *P. falciparum* appears to remain in the vascular space without entering the brain parenchyma. Murine and human studies, during the development of CM, have demonstrated the recruitment of leukocytes and platelets in the cerebral microvasculature, caused by the production of local toxins from the sequestered iRBCs, followed by an exaggerated immune response through excessive release of pro-inflammatory cytokines (IL-1, IL-6, IL-12, TNF-α, IFN-γ, lympotoxine, and NO) (Hunt and Grau, 2003; Jain et al., 2013; Schofield and Grau, 2005) and microparticles (Jain et al., 2013). The cellular recruitment is a series of steps, in a first time neutrophils and monocytes via chemotaxis migrate to the brain where they bind to the brain endothelium and stimulate more chemokine and cytokine production (Renia et al., 2006). Furthermore, in mice studies, depletion of neutrophils that contribute to the brain lesions and are important source of cytokines and chemokines, prevents the development of CM by downregulation of the expression of T helper (Th)1 cytokines and monocyte sequestration (Schofield and Grau, 2005). In contrast to mice, neutrophil contribution to CM is unknown in humans. Several days post-infection (p.i.), after neutrophils and monocytes recruitment in the brain, naïve T cells fully primed by spleen $CD11c^{high}CD8^{+}$DCs (Piva et al., 2012) upregulates chemokinereceptor CXCR3 on $CD8^{+}$ T cells (deWalick et al., 2007; Miyakoda et al., 2008), influenced by IFN-γ derived from NK cells, and migrate to the brain in a full assault of the BBB representing the central component of ECM immunopathogenesis also responsible for severe symptoms and mortality. $CD8^{+}$ T cells accumulate within the cerebral vessels, recognise and kill endothelial cells presenting parasite antigen by MHC I on their surface (Monso-Hinard et al., 1997) and initiate BBB tight junction disruption by promoting the central nervous system vascular permeability under neuroinflammatory conditions (Fig. 7B). Additionally mice studies shown that depletion of $CD8^{+}$ T cell at both early and late infection prevents ECM pathology (Belnoue et al., 2002; Hermsen et al., 1997; Yanez et al., 1996).

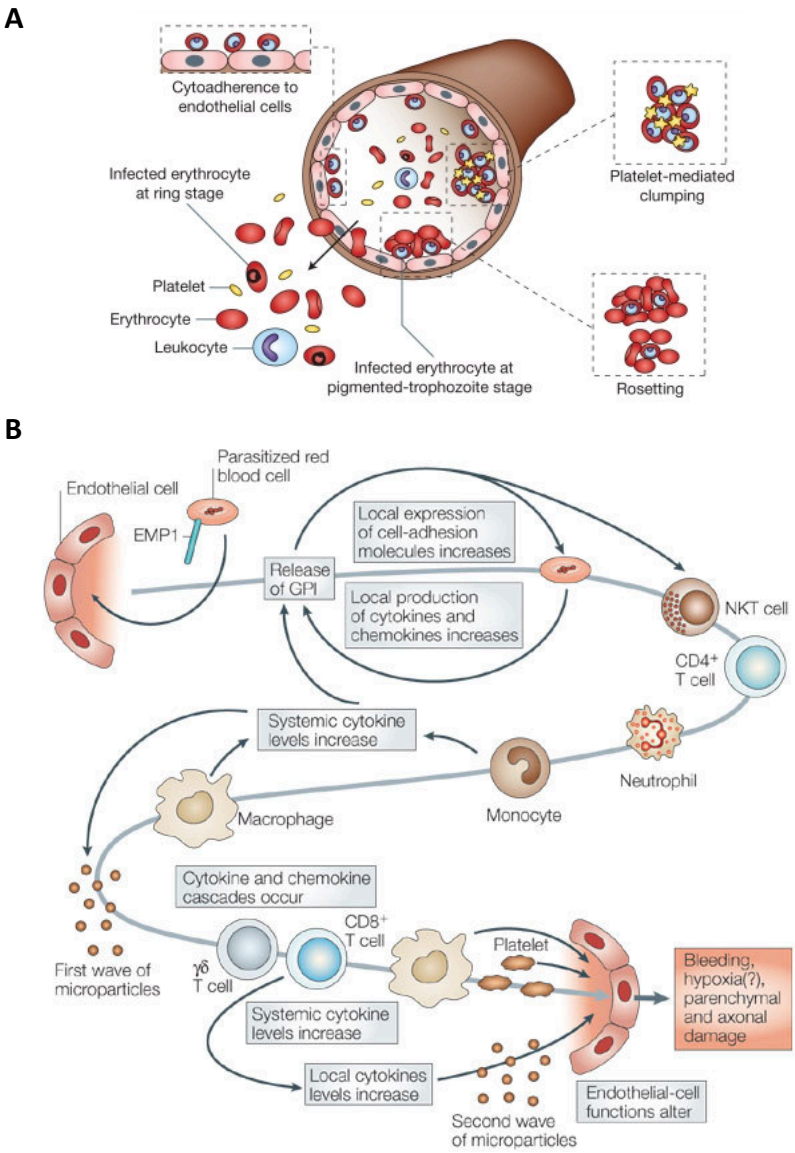

Figure 7: Schematic representation of mechanisms involved in the development of CM. (A) Schematic representation of the adhesion properties of *P. falciparum* iRBCs to different host cells. The iRBCs mature forms have the ability to bind to a range of host cells, such as endothelium, uRBCs (rosetting) and platelets (platelet-mediated clumping)(Rowe et al., 2009). (B) Schematic representation of severe malarial disease events. iRBCs adherence to receptors expressed by brain microvascular endothelial cells is followed by merozoites releasing. The release of parasite GPI induces an inflammatory response. A local acute-phase response then occurs, which involves activation of the endothelium and local production of cytokines and chemokines resulting in upregulation of expression of cell-adhesion molecules by endothelial cells. GPI can also function as a ligand for CD1d-restricted natural killer T (NKT) cells, leading to their activation. Activated NKT cells can regulate the differentiation and activation of $CD4^+$ T cells into Th1 or Th2 cells. In addition, chemokines recruit monocytes and activate neutrophils. Recruited monocytes can then differentiate into macrophages and become arrested in brain microvessels. Macrophages can also be activated by GPI, a process that is amplified by IFN-γ. Local activated macrophages produce more chemokines amplifying the infiltration of cells, sequestration of iRBCs and release of microparticles. After several more cycles, γδ T cells and $CD8^+$ T cells might become involved, releasing more chemokines and cytokines both systemically and locally and possibly inducing perforin-mediated lesions in the endothelium. Together with locally arrested macrophages, platelets are sequestered and participate in altering endothelial-cell functions. More microparticles of platelet, endothelial-cell and monocyte origin are released, which leads to the dissemination of pro-inflammatory and pro-coagulant effects. Finally, damage to the endothelium, with possible perivascular haemorrhage, axonal injury, and neurotransmitter and metabolic changes, can ensue. (Schofield and Grau, 2005).

1.6 MALARIA PROTECTIVE IMMUNITY

In 1980, Bruce-Chwatt wrote, “malaria immunity may be defined as the state of resistance to the infection brought about by all those processes involved in parasite destroying or by limiting their multiplication” (Bruce-Chwatt, 1980). Immunity to malaria is complex, and is essentially both species and stage specific. The multi-stage nature of the *Plasmodium* life cycle, the differential and specific expression of genes at each stage, and the predominantly intracellular lifestyle of the parasite pose challenges to the ability of the vertebrate host to mount an effective defense (Ferreira et al., 2004). Other factors, including the age and genetic background of the host, pregnancy, nutritional status and co-infection, also influence the development of antimalarial immunity (Baird, 1995; Doolan et al., 2009). In humans, various types of acquired or adaptive immunity against plasmodia have been defined (Fig. 8)(Doolan et al., 2009):

- Antidisease immunity, rapidly acquired and results in reduced mortality or severe clinical disease.
- Antiparasite immunity, slowly acquired confers protection against parasitemia by affecting the parasites density and the attendant risk of severe disease (Marsh and Snow, 1997).
- Premunition, phenomenon that describes the asymptomatic status observed in adults with high degree of immune responsiveness together with the nearly permanent presence of relatively low densities of parasite (Sergent, 1935).

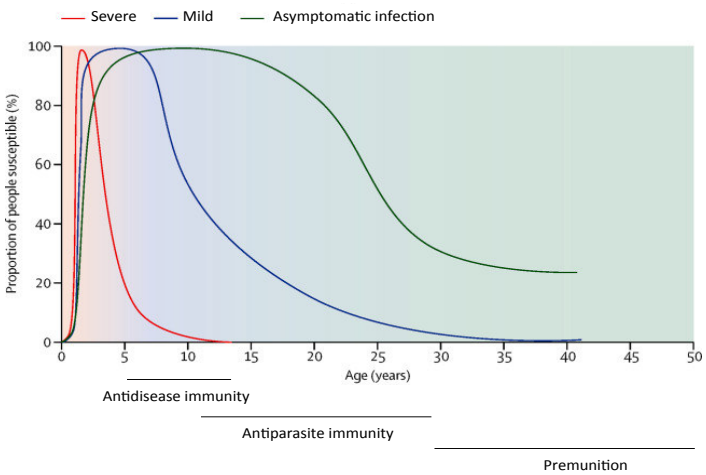

Figure 8: Pattern of *P. falciparum* infection outcome with age in a malaria endemic population. Asymptomatic infection (green), mild disease (febrile episodes caused by malaria; blue) and severe or life-threatening disease (red) (White et al., 2014).

In malaria endemic areas the inhabitants are usually infected repeatedly with malaria parasites and acquired immunity gradually. Sterilizing immunity against infections is never fully achieved. A general trend of increased serological response is seen with age, this is probably due to cumulative exposure over time to various parasite antigens. Acquisition of immunity appears to be sequential, with the ability to limit parasite growth and multiplication, followed by essentially complete protection against severe and then mild clinical disease, and culminating with partial protection against infection. Maintenance of this malaria immunity is dependent on persistent sub-clinical infection and is lost when people leave malaria-endemic areas.

1.6.1 Innate immunity

Animal and human studies have highlighted the importance of innate immune mechanisms to limit the initial phase of parasite replication, controlling the first wave of parasitemia and allowing the host to develop specific adaptive responses that will be enable to clear the infection (Molineaux et al., 2002; Stevenson and Riley, 2004). The initial trigger of the innate immune response is the activation of different cell types including dendritic cells (DCs), monocytes/macrophages, natural killer (NK) cells, natural killer T (NKT) cells, and γδ T cells resulting in the production of different factors among which several inflammatory mediators in particular cytokines and chemokines (Stanisic et al., 2013). This early inflammatory response is involved in various effector mechanisms such as parasite phagocytic clearance through the production of reactive oxygen and nitrogen species, and secretion of anti-microbial peptides, that all aim at destroying the parasites.

The production of inflammatory cytokines is controlled by the type of interactions between host cells and parasite. The innate immune system recognizes *Plasmodium* components (such as hemozoin, CpG-containing DNA motifs bound to hemozoin or AT-rich DNA motif), known as pathogen-associated molecular patterns (PAMPs), via a limited number of pattern-recognition receptors (PRRs). These receptors include the TLRs family that recognize parasite molecules (Coban et al., 2007) or hemozoin (Coban et al., 2005) and cell surface receptors such as CD36 (Stevenson and Riley, 2004), a co-receptor for TLR2 (Langhorne et al., 2008). The strongest evidence of the importance of these interactions comes from mouse models,

specifically *P. berghei, P. yoelii* and *P. chabaudi*. *In vitro* studies show the capacity of *Plasmodium* GPI- anchored molecules to activate and stimulate the production of pro-inflammatory cytokines (TNF-α and IL-1) and NO from mice and human macrophages trough TLR2 and TLR4 (Krishnegowda et al., 2005; Nebl et al., 2005; Schofield and Hackett, 1993). In early infection activation of DCs via TLRs appears to cause inflammatory cytokines, IL-12, release from T cells in mice (Perry et al., 2004) and IL-12 production by NK cells in appropriate dose has been shown to be useful in induction of protective immunity during *P. chabaudi* infection (Stevenson et al., 1995). However, TLR-MyD88-mediated IL-12 production, during blood stage infection with *P. berghei* in mice, was shown to be associated with perforin-dependent liver injury (Adachi et al., 2001). Recently, the role of the CD36 receptor was shown to be involved in opsnonin-independent phagocytosis of *P. falciparum* iRBCs by monocytes from non-immune individuals (Serghides et al., 2003). Moreover, the *in vitro* binding of *P. falciparum* iRBCs to CD36 on the surface of host LPS-activated DCs induces the secretion of IL-10 rather than IL-12 and reduces their ability to activate T cells in an allogeneic mixed lymphocyte reaction or to activate memory $CD4^+$ T cells (Urban et al., 1999).

Another important cytokine implicated in the early inflammatory response and essential for controlling the initial wave of parasitaemia in mice is IFN-γ release by NK cells and $γδ^+$ T cell that strongly expanded during the early phases of malaria infection (Fig. 9) (Hensmann and Kwiatkowski, 2001; Inoue et al., 2013; Stevenson and Riley, 2004), and enhances phagocyte activity, resulting in the elimination of extracellular parasites. Indeed, IFN-γ deletion during *P. chabaudi* infection led to impaired cytokine responses, uncontrolled parasitemia and increased mortality (Favre et al., 1997; Su and Stevenson, 2000). Additionally, IL-12p40 subunit deficiency in resistant mice reduced IFN-γ induction and increased mortality (Su and Stevenson, 2002), while administration of recombinant IL-12 to susceptible mice enhanced IFN-γ production, parasite control, and survival (Stevenson et al., 1995).

A study of *P. falciparum* infection showed that IFN-γ is produced in major quantities by γδ T cells in contrast to NK cells in response to iRBCs before and after *P. falciparum* infection (Teirlinck et al., 2011). Moreover, IFN-γ-producing responses of malaria antigen-specific γδ T cells were shown to be important for the development of a protective immunity against *P.*

falciparum infection in Papua New Guinea children (D'Ombrain et al., 2008). In contrast, several *in vitro* culture studies demostrated the rapid production of IFN-γ by NK cells in response to malaria infection *via* IL-12 and IL-18 signalling from antigen-presenting cells (APCs) (Micallef et al., 1996; Okamoto et al., 1999). Additionally, different studies showed that IFN-γ is also produced by DCs (Suzue et al., 2003) and is important for priming lymphocytes, nevertheless it not clear if IFN-γ production from DCs enhances protective immunity against *Plasmodium* infection.

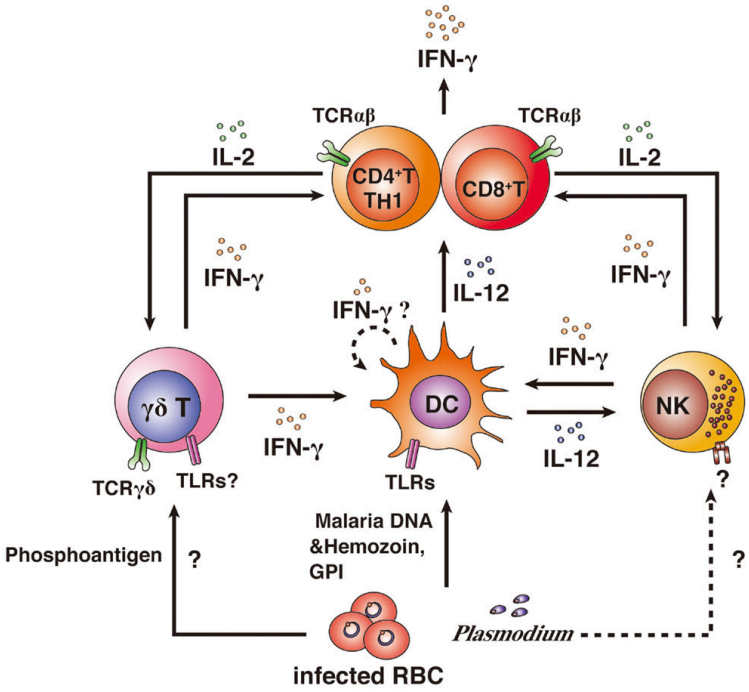

Figure 9: IFN-γ producers and their activation. *Plasmodium* antigens are recognized by innate immune cells (γβ T cells, DCs and NK cells) via PPRs (TLRs and TCR) expressed on cells surface and activate the cells to produce IFN-γ. Moreover, IL-12 production and the antigen presentation from activated DCs strong activate the ab T cells leading to high IFN-γ production. Furthermore, activated ab T cells produce IL-2, resulting in the proliferation of γβ T cells and NK cells and ab T cells themselves. IL-12 can also activate NK cells. (Inoue et al., 2013)

Liver stage production of IFN-γ, capable to inhibit parasite growth *in vito* hepatocyte cultures, seems to be mediated by NKT cells (Pied et al., 2000). Furthermore, during *P. yoelii* low-doses blood infection in nude mice liver NKT cells are required for partial protection, and adoptive transfer of these cells was able to passively transfer resistance to naïve mice (Mannoor et al., 2002; Schmieg et al., 2003). In addition, it has been reported that NKT cells are capable to regulate IgG antibody responses against *P. falciparum* GPI protein of pre-

erythrocytic parasites (Hansen et al., 2003). Nevertheless, additional investigations are required because other reports have shown the opposite (Schmieg et al., 2003).

The role of neutrophil activation in the host response to *P. falciparum* needs further clarification. Host resistance to malarial infection is not classically attributed to a neutrophil response (Stevenson and Riley, 2004). However, examining gene-expression profiles in whole blood from Kenyan children, a cluster associated with neutrophil activity, including absolute neutrophil count and neutrophil-related gene expression was identified (Griffiths et al., 2005). A significant increase in parasite gene-expression intensity corresponded to a significant increase in neutrophil count in subjects who had acute malaria as compared to convalescent subjects. Indeed, it has been observed that neutrophils have been linked with acute malaria (Ladhani et al., 2002).

1.6.2 Adaptive immunity

The parasite-mediated innate inflammatory immune response is the first step of the induction of the subsequent adaptive response responsible for the effector mechanisms against pathogens and ultimately leading to the memory response (Fig. 10). However, during malaria infection long-term memory (central memory) doesn't exist because continued exposure to malarial antigens is needed for: (i) the generation of effector memory cells and (ii) the persistence of memory and effector cells. The underlying mechanisms and antigenic specificity of protective immunity against malaria parasite are not well understood. The acquired anti-malaria immunity has been demonstrated to be strain and stage specific, with some cross reactivity

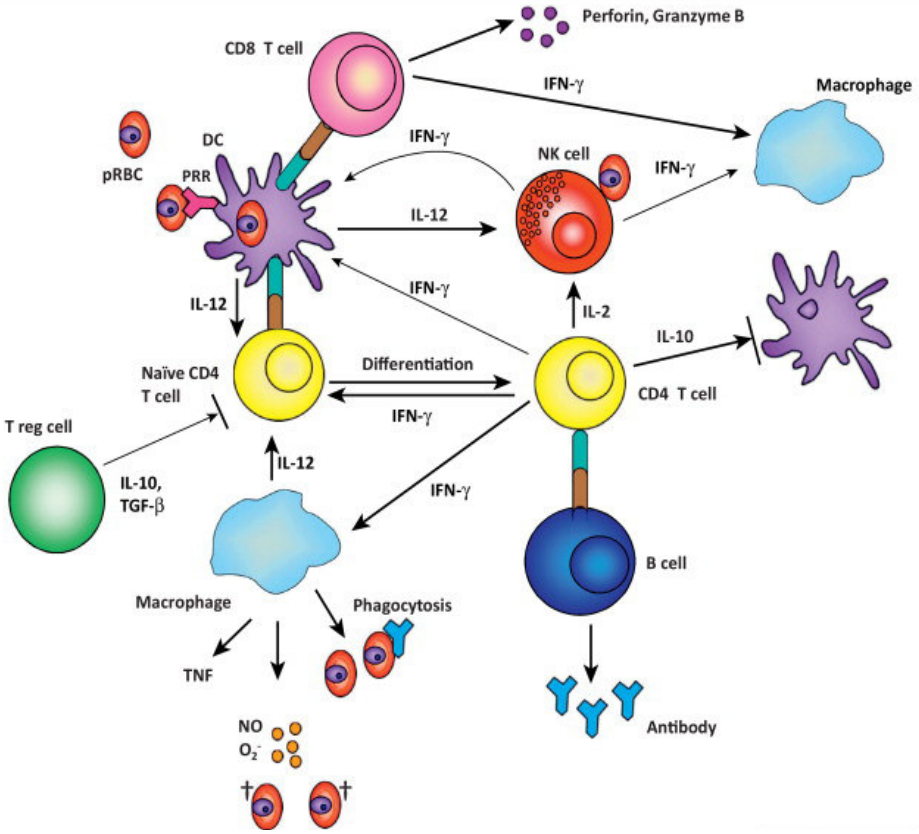

Figure 10: Linking innate and adaptive immunity to blood-stage malaria. In response to parasite ligands recognized by pattern-recognition receptors (PRRs), such as Toll-like receptors (TLRs) and CD36, or inflammatory cytokines, such as interferon-γ (IFN-γ), dendritic cells (DCs) mature and migrate to the spleen. Maturation of DCs is associated with the up regulation of IL-12 secretion, and antigen presentation to $CD4^+$ T cells. DCs can also cross-present antigen to $CD8^+$ T cells. IL-12 activates natural killer (NK) cells to produce IFN-γ and promote adaptive and humoral immune responses via antibody subclass switching. IFN-γ production by NK cells, $CD8^+$ T cells, and differentiated $CD4^+$ T cells primes antigen presenting cells to produce cytokines and chemokines, further assists DC maturation, and also activates macrophages, resulting in phagocytosis and killing of parasites via the production of nitric oxide and reactive oxygen intermediates. $CD4^+$ T cells can also produce IL-10, which can induce apoptosis of DCs. Once activated, macrophages can also participate in antigen

1.6.2.1 Cellular immunity

Control of asexual stages depends on both humoral and cellular immune response mechanisms. Nevertheless, the acquisition and maintenance of protective immunity is largely both $CD4^+$ and $CD8^+$ T cell dependent (Troye-Blomberg et al., 1994). In fact depletion of T cells in animal models does not allow recovery from infections and reconstitution with T cells from immune donors of severe combined immunodeficient mice suppress *P. chabaudi adami* growth suggesting that T cells contribute to immunity (van der Heyde et al., 1994). In contrast, animals unable to make antibodies recover normally from some primary infections

with *P. chabaudi* and when rescued by chemotherapy from other species of malaria parasite develop lasting, nonsterile immunity (Langhorne et al., 1998; von der Weid et al., 1996).

The most important cells implicated in regulating the human immune responses to asexual blood stages of *P. falciparum* are $CD4^+$ T cells. These cells are polarized to make some defined cytokine response and provide help to the humoral component. Activation of malaria-specific αβ TCR^+ $CD4^+$ T cells can be initiated soon after the antigen presentation by APCs (Bruna-Romero and Rodriguez, 2001; Chemtai et al., 1984). The role of $CD4^+$ T cells was observed in *in vitro* experiments in which $CD4^+$ T cells from malaria exposed individuals respond to a diversity of *P. falciparum* antigens by proliferation or by production of IFN-γ or IL-4 (Troye-Blomberg et al., 1990). The T cell production of IL-4 instead of IFN-γ was correlated to Abs levels in exposed individuals (Troye-Blomberg et al., 1990). While the production of IL-4 is typically observed at the end of the acute phase characterized by a Th2 response, the production of IFN-γ is predominant in the early acute *P. chabaudi* infection highlighting a Th1 response involved in the activation of mononuclear and polymorphonuclear leukocytes which phagocytose or lyse iRBCs (Kharazmi and Jepsen, 1984; Orago and Facer, 1991). This high production of IFN-γ declines as the parasitemia decreases, and is replaced by IL-4 and IL-10 production (Taylor-Robinson, 2002; Taylor-Robinson and Phillips, 1994). Moreover, activation of Th2 cells in the early acute phase of *P. chabaudi* infection in susceptible A/J mice results in a severe and lethal course of malaria (Stevenson and Tam, 1993). In human infection with *P. falciparum,* the shift from a Th2 response to a more pronounced Th1 response is associated with the resolution of infection (Winkler et al., 1998). These results show that both Th1 and Th2 cells are involved in protective immunity against blood stage malaria, at different times of infection and maybe according to localization.

Infective dose and kinetics also appears to be important and influence Th1/Th2 response in malaria. The increase in inoculum size in susceptible A/J mice leads to fulminating parasitaemia associated with elevated Th2 responses (Taylor-Robinson and Phillips, 1998). In murine model infected with *P. chabaudi,* changes over time of the ability of splenic DCs ($CD11c^+CD8^+$ or $CD11c^+CD8^-$) to present antigens to $CD4^+$ T cells was observed. High mortality in $CD8^+$DCs population followed by an increase in the number of $CD8^-$DCs

responsible to induce IL-4 and IL-10 production from MSP-1-specific T cells was observed in coincidence with the Th1 to Th2 switch during the immune response to the pathogen (Sponaas et al., 2006).

Although $CD4^+$ T cells are critical for protection against malaria, at the same time they could play a role in the development of lethal complications. The depletion of $CD4^+$ T cells before the infection with *P. berghei* can prevent the convulsions due to ECM (Hermsen et al., 1998).

While $CD4^+$ T cells are important during the blood stage, available evidence indicates that MHC class 1-restricted $CD8^+$ T cells, with a cytotoxic potential, seem to have important effector functions in pre-erythrocytic immunity (Crompton et al., 2014; Nardin and Nussenzweig, 1993) and contribute to protection against severe malaria (Aidoo and Udhayakumar, 2000; Hill et al., 1991). In animal model, $CD8^+$ T cell were shown to directly lyse the infected hepatocytes and destroy intracellular parasite via an IFN-γ- dependent mechanism (Tse et al., 2011). Nevertheless, this $CD8^+$ T cells response appears to be dependent on $CD4^+$ T cells and the persistence of parasite antigens (Cockburn et al., 2010; Overstreet et al., 2011). Moreover, It has been proposed that $CD8^+$ T cells may regulate immunosuppression in acute malaria and down-modulate inflammatory responses. Nevertheless, no available evidence for a protective role of $CD8^+$ T cells against *P. falciparum* blood stage has been reported. This is supported by the fact that RBC do not express classical MHC class I molecules, hence lacking the antigen processing machinery, suggesting that RBC do not represent a target for $CD8^+$ T cells. However, in contrast to the MHC-restricted $\alpha\beta$ T cells, the MHC-unrestricted $\gamma\delta$ T cells may have a direct cytotoxic potential on the asexual blood stages of malaria parasites, as demonstrated by their inhibitory activity in *P. falciparum* cultures (Troye-Blomberg et al., 1999b).

Despite the important role of T cells in the development of anti-malaria immunity, some mechanisms are opposed to its effectiveness. In rodent models the development of memory $CD4^+$ T cells during malaria infection occurs but this memory decays over time in the absence of re-infection. Indeed, mice infected with a chronic *P. chabaudi* are more protected in absence of anti-malaria drug treatment than treated mice suggesting that the persistence of the parasite was necessary for the establishment of the immunity. These non-treated mice

presented enhanced Th1 response compared to those treated with drug (Stephens and Langhorne, 2010; Stephens et al., 2011). In humans Th1 memory responses gradually decline in the absence of infection, whereas central memory IL-10 regulatory responses were maintained (Wipasa et al., 2011).

Moreover, in chronic diseases it was shown that repeated antigen stimulation through the T cell antigen receptor drives the sustained expression of T cell inhibitory receptors, including programmed death 1 (PD-1; also known as PDCD1) and lymphocyte-activation gene 3 (LAG3), on virus-specific CD8+ T cells (Ishida et al., 1992). Transcriptional changes that negatively regulate proliferation and the expression of pro-inflammatory cytokines by virus-specific $CD8^+$ T cells are induced (Barber et al., 2006; Blackburn et al., 2009). Recently it was reported that in mice, infection with nonlethal *P. yoelii* parasites drives specific T cell dysfunction called exhaustion (Butler et al., 2012). Therapeutic blockade of PD-1 ligand (PD-L1) and the inhibitory receptor LAG3 in mice demonstrated a restoration of $CD4^+$ T cells and B cell responses resulting in enhanced parasite control, even in chronically infected mice, suggesting that regulatory pathways may also promote chronicity of infection (Butler et al., 2012; Horne-Debets et al., 2013; Stanisic et al., 2013). The combined blocking of PD-L1 ad LAG3 was also associated with increased numbers of $CD4^+$ T_{FH} cells and germinal center B cells resulting in higher Abs titres which contribute to a better control of blood stage malaria (Butler et al., 2012). Recently, studies using a PD1-KO mouse model infected chronically with *P. chabaudi* confirmed the importance of PD1 and highlighted the important role of $CD8^+$ T cells in the development of a long-term protection against murine malaria. In fact, depletion of $CD8^+$ T cells during a secondary infection in PD1-KO mice results in the development of the parasite in the blood stage, the opposite is observed with $CD4^+$ T cell depletion (Horne-Debets et al., 2016). Furthermore, *P. falciparum*-infected children present higher expression of the inhibitory receptor PD-1 associated with T cell dysfunction (Butler et al., 2012), and the increased frequency of CD4+ T cells expressing PD-1 alone and in combination with LAG-3 appeared to be driven by persistent *P. falciparum* exposure (Illingworth et al., 2013).

1.6.2.2 Humoral immunity

The role of Abs in malaria protective immunity is not completely understood. However different anti-parasite effector functions have been attributed to the strong Abs responses reported during the infection: (i) the inhibition of the invasion of hepatocytes and erythrocytes, (ii) the inhibition of the cytoadherence, (iii) the complement-mediated opsonization of iRBCs, and (iv) monocyte-dependent antibody-mediated cellular cytotoxicity (ADCC) and monocyte-dependent antibody-mediated cellular inhibition (*ADCI*) (Perlmann and Troye-Blomberg, 2002).

The importance of antibodies in protection against malaria has been clearly shown by clinical studies where passive transfer of hyper-immune serum or purified Igs from asymptomatic adults to sick children can modify the course of infection by reducing parasitaemias and clinical diseases (Bouharoun-Tayoun et al., 1990; Cohen et al., 1961). Moreover, until 6 month of age children remain remarkably resistant to *P. falciparum* and *P. vivax* infection. This protection has been associated with maternally derived immunoglobulin G (IgG) antibodies (Logie et al., 1973), acquired by the fetus *in utero* through the passage of Abs across the placenta by an active receptor-mediated transport system (hFcRn) (Ellinger and Fuchs, 2012; Malek et al., 1996). During breastfeeding, infant protection may be associated with parasite growth-inhibitory factors such as lactoferrin and secretory IgA found in maternal milk. As observed in humans in animal models the passive immunization with mAbs (Quinn and Wyler, 1979; Siddiqui et al., 1987), adoptive transfer of B cells (Langhorne et al., 1998) and increased susceptibility of B-cell-deficient hosts (von der Weid et al., 1996) have highlighted the important role of antibodies in malaria immune response.

However, the protection obtained *in vivo* studies with passive transfer of Igs is lost around 10 months after the Abs treatment (Cohen et al., 1961). *In vitro*, clinically effective Igs obtained from the sera of *P. falciparum* immune adults had no detectable inhibitory effect on either parasite invasion or growth, but was shown to supress the parasite growth in co-operation with human monocytes in an antibody-dependent cellular inhibition (ADCI) assay (Bouharoun-Tayoun et al., 1990). Additional studies in humans and squirrel monkeys have shown that opsonic or cytophilic antibodies (IgG1 and IgG3 in humans) are associated with

the protective effect of the Abs (Bouharoun-Tayoun and Druilhe, 1992; Groux and Gysin, 1990; Shi et al., 1999). At this point cooperation between cells of the immune system and Abs appears to be crucial in parasite elimination and acquired protective immunity.

The elimination of the parasite trough an Abs-cell complex is mediated by the capture of Abs on the surface of cells via FcγRs receptors that bind the Fc part of the Ab, while the Fab part of the Ab is bound to antigens on the surface of either merozoites (Bouharoun-Tayoun et al., 1990) or late infected erythrocytes (Gysin et al., 1993) enhancing their phagocytosis by immune cells (neutrophils, monocytes and macrophages) and making parasites available for clearance by the spleen (David et al., 1983; Del Portillo et al., 2012), thus protecting against CM (Carlson et al., 1990). Different Abs with a protective function are produced (IgG, IgM and IgE) but in clinically immune individuals the predominance of the cytophilic IgG1 and IgG3 subclasses has been associated with either lower parasitaemia or a lower risk of malaria attack (Bouharoun-Tayoun et al., 1995). The non-cytophilic IgG4, present in low doses in immune individuals, may inhibit effector mechanisms by competing with cytophilic Ab and are considered nonprotective (Aucan et al., 2000; Garraud et al., 2003; Leoratti et al., 2008). In contrast, recent data suggest that, under certain circumstances, the non-cytophilic IgG2 could be correlated with protection in individuals. This protection is due to a rare mutation on the allele encoding an FcγRIIa that can bind IgG2 (Aucan et al., 2000). Moreover, highest levels of IgG1, IgG2 and IgG3 Abs were observed in individuals with asymptomatic and uncomplicated malaria infection, while high levels of IgG4, IgM and IgE Abs were predominant among individuals with complicated malaria infection in individuals naturally exposed to malaria living in different regions of Brazil (Leoratti et al., 2008).

Althoug many functions have been attributed to IgM Abs in infectious diseases, no specific function has been ascribed to this class of Abs during malaria infection. However, it was observed that natural non-specific IgM bind to the surface of some *P. falciparum* infected erythrocytes via the Fc, and this was shown to correlate with severe malaria in laboratory strains and field isolates (Ghumra et al., 2008; Rowe et al., 2002). Therefore, the understanding of the role of non-immune IgM and its contribution to the development of a protective immunity needs further investigation.

While the role of IgG subclasses has been characterized in some detail, there is some controversy as to the role of specific IgE Abs and whether they serve to protect against the disease or to make the illness more severe (Desowitz, 1989; Perlmann et al., 1994; Troye-Blomberg et al., 1999a). Elevated levels of IgE and anti-plasmodial IgE Abs were observed in human and experimental malaria infections (Desowitz et al., 1993; Helmby et al., 1996; Perlmann et al., 1994). The induction of IgE Abs reflects a switch in the $CD4^+$ T helper cells, from Th1 to Th2, due to repeated exposures to the parasite (Perlmann et al., 1994). However, the rise in IgE levels is controlled by a variety of environmental and genetic factors (Perlmann et al., 1994). IgE bind to high-affinity receptors (FcεRI) on mast cells and basophils as well as to low-affinity receptors (FcεRII/CD23) expressed on the surface of B cells, activated macrophages and eosinophils amongst other cell types (Stone et al., 2010). Cross-linking of receptor bound IgE by the antigens triggers the release of pharmacologically active substances from mast cells and basophils, including histamine, leukotrienes and peptides attracting neutrophils and eosinophils.

Some studies conducted in endemic areas suggest that IgE could play a role in the pathogenesis of malaria. Increased levels of IgE where found in individuals suffering from severe malaria in comparison to uncomplicated malaria (Perlmann et al., 1994; Perlmann et al., 1997; Perlmann et al., 2000; Seka-Seka et al., 2004). Furthermore, immunohistological studies on brain sections revealed the presence of IgE deposits in brain microvessels and on infected erythrocytes from cerebral malaria patients as well as in placentas infected with *P. falciparum* (Maeno et al., 2000; Maeno et al., 1993). Additionally, the expression of high levels of IgE during cerebral *P. falciparum* infection was related to the deepness of the coma (Maeno et al., 2000). *In vitro* studies using malaria patient's sera showed high levels of TNF-α, known to contribute to pathogenesis of CM (Clark et al., 1991), from activated monocytes. In contrast, *P. falciparum* specific IgE in the form of immune complexes, with either antigen aggregates or with IgG anti-IgE autoantibodies, crosslink the CD23 receptor expressed on monocytes which results in TNF-α and NO production (Elghazali et al., 1997; Perlmann et al., 1999). The CD23/NO pathway is believed to play a protective role during the development of malaria pathogenesis by down regulating the expression of ICAM-1 and decreasing iRBCs cytoadherence (Pino et al., 2004). However, it has been shown in Thai patients that during

severe malaria the release of CD23 from the cell surface giving rise to increased sCD23 levels in plasma was higher compared to uncomplicated and asymptomatic malaria (Kumsiri et al., 2016). Indeed, the amount of sCD23 correlates with disease severity (Rujeni et al., 2013; Vouldoukis et al., 2011) and is recognized as an important indicator of disease progression (Schwarzmeier et al., 2005) in other diseases. In the opposite, several findings have demonstrated that high levels of IgE increase with age in parallel with the acquisition of immunity (Desowitz et al., 1993; Maeno et al., 1993) and in the non-comatose patients compared to comatose patients (Calissano et al., 2003). In support of these results studies on the Fulani group, known to be less susceptible to malaria infections, showed a correlation between the decrease of infected cells with the increase of anti-malaria IgG and IgE levels (Farouk et al., 2005). In Tanzania, elevated anti-malaria IgE were associated with the reduction of the subsequent malaria attacks risk (Bereczky et al., 2004).

The impact of malaria parasite on B cells and subsequently on Abs production and in maintenance of B cell memory in individuals chronically re-infected with *P. falciparum* was highlighted during epidemiological studies in malaria endemic areas where atypical memory B cells were reported to be significantly expanded in *Pf*-exposed Malian adults and children. In chronically infected asymptomatic children the number of these atypical MBCs was higher compared with uninfected children, suggesting that the chronic presence of the parasite may drive expansion of these distinct MBCs (Weiss et al., 2009).

Protective immunity against malaria appears to result in a delicate balance, temporally and spatially regulated, between an appropriate innate immunity able to drive a quick Th1 response for the control of the parasite density followed by a Th2 response avoiding Th1-mediated deleterious effects, and maintaining the long term protection against parasite rise.

1.7 MALARIA TREATMENT

Public health efforts to combat malaria take the three-pronged approach of insecticide-treated bed nets, case management of malaria illness and preventative treatment. Despite the intensive efforts over the years to reduce the malaria burden the situation has gradually worsened in the recent years with the increase in resistance phenomena associated with synthetic antimalarial drugs (Ashley et al., 2014; Packard, 2014) and insecticides in use

(Brown, 1958) in addition to the economic instability of affected countries and the lack of infrastructure required to instigate these public health measures *en masse*. For this reason, to further combat the disease, researchers and politicians need to invest in a combination of both traditional and new public health interventions by revising antimalarial drugs and strategy policies. This may take the form of developing new drugs and insecticides, along with novel approaches such as transgenic mosquitoes resistant to malaria parasites and new vaccine design.

Another important aspect, indispensable to prevent the over-medication and avoid the development of resistance, is the development of a reliable diagnostic test. Unfortunately in the past the diagnostic was based on clinical symptoms and sometimes associated to a microscopic examination of a drop of peripheral blood. In recent years new diagnostic techniques have been developed as the rapid diagnostic test based on the identification of three different types of *Plasmodium* antigens (histidine-rich protein 2, *Plasmodium* lactate dehydrogenase and aldolase) (Moody, 2002).

1.7.1 Antimalarial chemotherapy

Several drugs are available to kill the malaria parasite. They can be differentiated into 5 categories: the quinolines, the hydroxynaphthoquinones, the antifolates, the antibiotics not antifolates, and the artemisinin derivatives, depending on their activity on different stages of the parasite life cycle or by their mechanism of action (Arav-Boger and Shapiro, 2005). Antimalarials have been known for a long time but their mode of action has still not been completely elucidated. The commonly used were chloroquine, which inhibits haemoglobin digestion, and sulfadoxine or pyrimethamine that inhibit enzymes in the parasite folate synthesis pathway. With the development and spread of drug resistance to chloroquine and the antifolate drugs alkylating agents, such as artemisinin, are becoming more widely used.

1.7.2 Malaria Vaccine

Over the years one of the greatest challenges in the malaria eradication program has been the development of an effective and safe vaccine. Studies on malaria vaccine started in the 1960 with the first immunization of mice with irradiated sporozoites, but despite the

persistent efforts, the lifelong sacrifice from dedicated physicians and scientists, and the investment of hundreds of millions of dollars, the malaria vaccine has remained elusive.

Development of an effective vaccine seems to be a daunting task, due to the complexity of *Plasmodium* biology (Gardner et al., 2002a; Scherf et al., 2008) which allows the parasite to escape the host immune system following different mechanisms. Additionally, natural infection with malaria does not induce immune protection in fact only partially effective immunity is acquired after repeated and prolonged exposures to malaria parasite over several years and is short-lived and highly stage- and strain-specific (Langhorne et al., 2008; Stevenson and Riley, 2004).

In spite the evidence that an effective malaria vaccine should be possible, numerous challenges need to be overcome. Essentially two main strategies for malaria vaccine development, targeting the different phases of the parasite's life cycle, are currently being pursued, largely in parallel: (i) the subunit approach, and (ii) the whole organism approach.

1.7.2.1 Subunit approach

The first peptide-based candidate vaccine tested extensively in endemic areas was the SPf66 developed in Colombia and appeared to be effective in South America (Patarroyo et al., 1987). Unfortunately, field efficacy trials in Africa and Asia failed to demonstrate protection.

In 1982 the identification of the circumsporozoite (CS) protein as the major component of the sporozoite coat has promoted the development of a sporozoite vaccine. The RTS,S/AS, a recombinant protein that fuses a part of the *P. falciparum* CS protein with the hepatitis B surface antigen (Proietti and Doolan, 2014), is the most advanced subunit vaccine in development. RTS,S/AS induces antibodies and T cell function that are believed to diminish the capacity of the malaria parasite to infect, survive, and develop in the human liver. However, due to the low vaccine efficacy or cover (around 30%) and the development of only a partial protection that is lost about two years after vaccination, alternative strategies need to be considered (Agnandji et al., 2012; Agnandji et al., 2011; RTS, 2015). Moreover, the RTS,S/AS candidate has been tested only in the Africa continent whit no data obtained from outside the continent while it should be effective worldwide.

Other vaccine candidates have been developed (Fig. 11) such as MSP-1 or 2 and AMA-1, targeting the asexual blood-stage, both tested in clinical trials still the lack of clear efficacy (McCarthy et al., 2011; Ogutu et al., 2009; Sagara et al., 2009). Different approaches have been used to make the immune response and the protection more efficient, such as the conjugation of *Plasmodium* antigens to an immunogenic carrier protein (Que et al., 1988) or the generation of chimeric proteins comprising domains of two or more vaccine candidates (Malkin et al., 2008) (ex. PfCP2.9, comprising domain III of the apical membrane antigen AMA1 and the C terminal epidermal growth factor-like domains of the merozoite surface protein MSP1), without any positive result. Although it has been shown that several *Plasmodium* antigens can induce some degree of protection in laboratory animal models and induce antibody response able to inhibit *P. falciparum* development *in vitro* (Richards and Beeson, 2009), the antigenic complexity of the parasite is an obstacle of greater importance in the development of an efficient subunit vaccine and contributes to some disillusionment with the recombinant protein subunit approach.

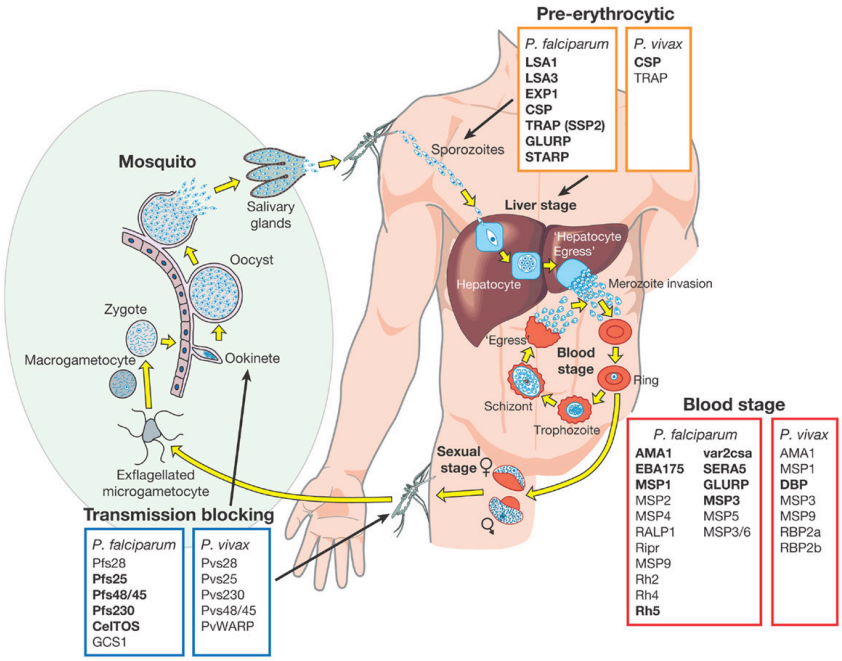

Figure 11: Malaria vaccine candidate antigens. List of *P. falciparum* and *P. vivax* candidate antigens indicated the category of malaria vaccine being developed and the life cycle stage targeted. Antigens evaluated in pre-clinical trials or have entered at least Phase I clinical trails, according to WHO malaria vaccine rainbow tables, are indicated in bold. (Barry and Arnott, 2014)

1.7.2.2 Whole organism approach

In recent years the use of live attenuated parasites (LAPs) as vaccines due to their ability to induce sterile long-lasting immunity has become popular. The advantage of whole-parasite vaccines is to induce immune response to a broad range of parasite antigens, and thus potentially more protective. The necessity of using viable parasite for whole parasite immunization has become evident because protection cannot be induced by heat-killed sporozoites, which are unable to invade and develop in the liver (Alger and Harant, 1976).

The first LAP appeared in 1967 when Ruth Nussenzweig and co-workers showed the necessity to use whole parasite for complete immunization using radiation-attenuated sporozoites (RAS) (Vanderberg et al., 1969). In mice RAS, whose attenuation is based on multiple random DNA breaks, shows an be arrest in the earliest phase of liver-stage, causing no blood infection and conferring sterile, protective immunity against subsequent challenge with wild type sporozoites (Suhrbier et al., 1990; Vanderberg et al., 1969) over at least 10 months (Hoffman et al., 2002). Mice studies carried out to understand the mechanism involved during the immune response highlighted the role of $CD8^+$ T cells (Schofield et al., 1987; Weiss et al., 1988) and sporozoite-specific antibodies against the major surface protein CSP (Doolan and Hoffman, 2000). Interestingly, recent studies have shown the possible implication of $CD4^+$ T cells and IFN-γ in protection (Oliveira et al., 2008). Moreover, it is known that activation of $CD8^+$ T cells is followed by the production of IFN-γ, which in turn provokes the secretion of IL-12, responsible for stimulating NK cells to produce more IFN-γ in a positive feedback loop (Yazdani et al., 2006). This causes the activation of the NO pathway, which induces the killing of the infected hepatocyte (Doolan and Hoffman, 1999; Doolan and Hoffman, 2000). Consistently, humans immunized by the bite of irradiated *P. falciparum* infected mosquitoes were effectively protected from subsequent challenges with homologous and heterologous infectious *P. falciparum* sporozoites (efficacy > 90%) (Hoffman et al., 2002; Vanderberg et al., 1969). Given the high levels of protection achieved by irradiated sporozoites in many malaria models the development of *P. falciparum* RAS vaccine, called PfSPZ, was proposed by Stephen Hoffman (Sanaria) and is actually tested in a

Phase I clinical study with encouraging results (100% protection after challenge) (Richie et al., 2015; Seder et al., 2013).

Although this vaccine presents some safety problem as the efficacy of the irradiated sporozoites that depend on the number of irradiated sporozoites injected (> 100,000 RAS (Luke and Hoffman, 2003)) and on the precise irradiation dose; up to 200 Gray can confer complete protection (Chattopadhyay et al., 2009), less doses allow the parasite to complete the liver stage development and cause blood stage infection, higher doses completely inactivate the sporozoites resulting in no significant protection (Silvie et al., 2002; Suhrbier et al., 1990). In addition to challenges of manufacturing and delivering a viable cryopreserved whole parasite vaccine, it remains to solve the limit of mass-production of vaccines using the sporozoites stage. Besides, animal studies show that several higher doses are needed to protect mice with cryopreserved irradiated sporozoites as compared to fresh ones, demonstrating that the cryopreservation might inactivate the sporozoites (Epstein et al., 2011).

Another approach has been possible with the availability of the whole *Plasmodium* genome sequences (Carlton et al., 2002; Gardner et al., 2002a; Gardner et al., 2002b) and the advancement of gene manipulation technologies (de Koning-Ward et al., 2000; Menard and Nussenzweig, 2000; Thathy and Menard, 2002) which has led to the individualization of genes essential for parasite survival at distinct points during its life cycle. Genetically arrested parasites (GAP) generated by targeted gene deletion(s) designed to arrest at specific points of livers-stage development (Fig. 12) (Butler et al., 2011; Mueller et al., 2005a; Mueller et al., 2005b; van Dijk et al., 2005) were able to successfully invade the liver but arrest prior to liver stage maturity, generating complete protective immunity against wild type sporozoite challenge in a manner comparable to radiation attenuated sporozoites (Butler et al., 2011; Khan et al., 2012; Mueller et al., 2005b). This immune response is mediated by MHC class I-dependent IFN-γ producing $CD8^+$ T cells (Jobe et al., 2007; Mueller et al., 2007), which results in sterile protracted protection (Jobe et al., 2007; Wang et al., 2009) against sporozoite challenge in rodent models. Moreover, a late-blocked liver-stage GAPs have been shown to induce superior protective immunity against sporozoite challenge in outbred mice compared to both early-blocked liver-stage GAP and RAS vaccine (Butler et

al., 2011; Nganou-Makamdop and Sauerwein, 2013). Similar transgenic parasites have been produced in *P. falciparum* and studied in clinical assays. Unfortunately the first clinic study showed considerable but incomplete attenuation (Spring et al., 2013), indicating that complete attenuation of the parasite in humans remains challenging (Bijker et al., 2015).

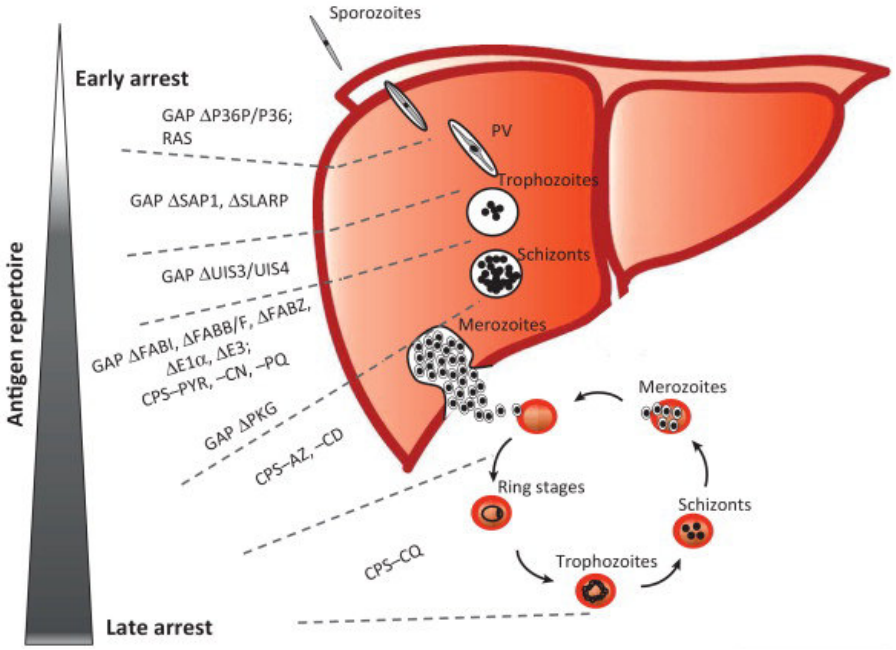

Figure 12: Liver stages of arrest for radiation-attenuated sporozoites (RAS), genetically attenuated parasites (GAP), and wild type sporozoites with chemoprophylaxis (CPS). Shortly after entry, sporozoites form a parasitophorous vacuole (PV) for further development into liver trophozoites, schizonts, and eventually merozoites. Release of merozoites marks the end of the liver stage and the start of blood-stage infection. GAPS are produced by deletion of selected parasite genes (Δ). Both RAS and the GAP ΔP36p/P36, ΔSAP1, ΔSLARP arrest early in the liver before completion or remodeling of the PV. Development into intermediate stages is observed for the GAP (ΔUIS3/UIS4, ΔE1α, ΔE3, ΔFABI, ΔFABB/F, ΔFABZ, and ΔPKG), and the CPS–PYR (pyrimethamine), –CN (centanamycin) and–PQ (primaquine). Later liver-stage arrest is observed for CPS–AZ (azithromycin) and CD (clindamycin). Blood-stage exposure occurs only for CPS–CQ (chloroquine) where the latest parasite arrest is observed (Nganou-Makamdop and Sauerwein, 2013).

More recently, the development of blood-stage GAPs (Table 1) as the *P. yoelii* lacking purine nucleoside phosphorylase (Ting et al., 2008) or nucleoside transporter 1 (Aly et al., 2010) and *P. berghei* ANKA lacking plasmepsin-4 (Spaccapelo et al., 2010) where shown to attenuate the parasite development in the blood stage, resulted in long-lasting protection against sporozoite and iRBCs re-infection respectively.

Alternative strategies have been developed by combining wild type sporozoite immunizations with drugs acting at various stages of parasite life cycle. Several treatments have been tested as primaquine (liver stages elimination) (Putrianti et al., 2009), chloroquine (blood stages elimination) (Belnoue et al., 2004; White, 1997), clindamycin and azithromycin (targeting the apicoplast resulting in non-infectious merozoites) (Friesen et al., 2010). David Pombo and co-workers demonstrated that different challenges of low doses of live blood-stages wild type parasites treated early by appropriate drug treatment could induce a strong cell-mediated immunity to the same strain of malaria parasite in human adults (Belnoue et al., 2004). These results were confirmed in murine models where was highlighted the need of three and more repeated malaria infections followed by drug treatment to obtain semi-immune mice (Bao et al., 2013; Bao et al., 2015; Evans et al., 2006). The fact that the infection and the acquisition of immunity are thereby the result of natural wild type infection, as opposed to the attenuated infection induced by GAPs or RAS, the use of this model seems to be difficult in endemic areas where drug resistance is high and the majority of people have already had malaria episodes with the development of an humoral or cellular response.

The pathogenesis of malaria is complex, and development of pathogenesis depends on the parasite species that cause infection and the immune status of the infected host. While current blood stage sub-unit preparations have not yielded any protection in field trials (Proietti and Doolan, 2014; White et al., 2014), live attenuated parasites appear to confer significantly broader and more lasting protection. However, the development of an adequate and effective vaccine definitely requires well and detailed understanding of the mechanisms underlying natural immunity to malaria associated with all environmental cultural, society and territorial parameters that can be found among the different populations living in endemic areas.

			PROTECTION			IMMUNE RESPONSE				
	Mouse	**Development/ Phenotype**	**Protection (time)**	**Cross stage**	**Cross strain**	**Cytokines**	**T/B cell**	**Ab/ Protection**	**FcγR$^+$ /Protection**	**Ref.**
Py* YM *Δpnp	BALB/c Swiss	Slow/ CLEARENCE **(D15)**	YES (**5 m**)	*Py* 17XNL	*Py* 17XNL	ND	ND/ND	ND	ND	(Ting et al., 2008
Py* 17XNL *Δnt1	BALB/c Swiss C57BL/6	Very slow/ CLEARENCE **(D15)**	YES (3 **m**)	*Py* YM	*Py* YM *Pb* ANKA	ND	YES/YES	ND	ND	(Aly et al., 2010
Py*YM *Δsera2	BALB/c	Normal/ CLEARENCE **(D28-30)**	ND	ND	ND	ND	ND/ND	ND	ND	(Huang et al., 2013
Pb* ANKA *Δnt1	C57BL/6	Normal and no ECM/ CLEARENCE **(D90)**	YES	ND	ND	ND	ND/ND	IgG/ND	ND	(Niikura et al.,
Pb* ANKA *Δpm4	BALB/c	Slow/ CLEARENCE **(D25)**	YES (**1 y**)	ND	*Pb* ANKA *Py* 17XNL	ND	ND/ND	IgG/YES	ND	(Spaccapelo et
Pb* ANKA *Δpm4	C57BL/6	Slow no ECM/ 20% CLEARENCE (**D25**)	ND	ND	ND	ND	ND/ND	ND	ND	(Spaccapelo et
Pb* ANKA *Δmps7	C57BL/6	Slow and no ECM/ Death at D18-22	ND	ND	ND	ND	ND/ND	ND	ND	(Spaccapelo et
Pb* ANKA *Δpm4Δmps7	BALB/c C57BL/6 CD1	Slow and no ECM/ CLEARENCE **(D25, D12,**	YES	ND	*Pb* ANKA *Py* 17XNL	ND	YES/YES	ND	ND	(Spaccapelo et
Pb* ANKA *Δhmgb2	C57BL/6	Slow and no ECM/ Death at D20-25	ND	ND	ND	↓ TNF-α, IL-10 and IL-6	Brain:↓CD4 and CD8 /ND	ND	ND	(Briquet et al.,
Pb* NK65 *Δhmgb2	C57BL/6	Normal/ CLEARENCE **(D15)**	ND	ND	ND	ND	ND/ND	ND	ND	(Briquet et al.,
Pb* ANKA *Δhfr	C57BL/6	Slow and no ECM/ Death at D20-25	ND	ND	ND	↑IL-6	ND/(Liver) ↑ B cell	ND	ND	(Mathieu et al.,
Pb* NK65 *Δhrf	C57BL/6	Normal/ CLEARENCE **(D12-15)**	YES (**1 y**)	*Pb* NK65 *Pb* ANKA	*Pb* ANKA *Py* YM	↑ IL-12, IL-23, IFN-γ and IL-6	YES (↓PD1)/YES	IgG, IgG2a/ Partial	CD11b$^+$/YES	(Demarta-Gatsi et
Pb* ANKA *Δsb1	C57BL/6 BALB/c	Slow and no ECM/ Death at D32-45	ND	ND	ND	ND	ND/ND	ND	ND	(De Niz et al.,
Pb* ANKA *Δmhrp1a	C57BL/6 BALB/c	Slow and no ECM/ Death at D25-38	ND	ND	ND	ND	ND/ND	ND	ND	(De Niz et al.,
***Pb* ANKA Δptex88 (iKD)**	C57BL/6	Slow and no ECM/ Death at D?	ND	ND	ND	↑IL-6 (serum)	ND/ND	ND	ND	(Chisholm et al.,
***Pb* ANKA Δptex88**	C57BL/6	Slow and no ECM/ Death at D?	ND	ND	ND	ND	ND/ND	ND	ND	(Matz et al., 2015

Table 1 – Blood stage GAPs and their characteristics.

Histamine Releasing Factor

2.1 HISTAMINE RELEASING FACTOR (HRF)

Discovered in the 1980s the histamine releasing factor (HRF) protein, also known as translationally controlled tumour protein (TCTP), P23/21, Q23 or fortilin is a housekeeping protein, highly conserved in many organisms, ranging from unicellular, yeast, plants to vertebrates including all *Plasmodium* parasite species with both intracellular and extracellular functions (Fig. 13).

At first, the synthesis of HRF was shown to be regulated at the translational level, based on the fact that the cDNA cloned from a mouse tumour cell line, and derived from an abundant mRNA found to mainly exist as messenger ribonucleoprotein (mRNP) particles was unable to interact with translational apparatus within the cells (Chitpatima et al., 1988). Later, it was shown that the protein could also be regulated at the level of transcription (Bohm et al., 1991; Chung et al., 2000).

Recently HRF has attracted the attention of an increasing number of researchers due to its strong regulation in response to a wide range of extracellular stimuli effecting calcium concentrations both intracellular and within endoplasmic reticulum (Xu et al., 1999). A series of recent reports highlighted the importance of HRF in different intracellular functions including the cell cycle progression (Gachet et al., 1999; Yarm, 2002), apoptosis inhibition (Amson et al., 2012; Li et al., 2001; Liu et al., 2005; Rho et al., 2011) and malignant transformation of a variety of cell types (Li et al., 2001; Tuynder et al., 2002).

Accordingly, multiple studies have implicated the protein in the regulation of growth and proliferation as well as in the control of organ size. Complete gene knockout of HRF in drosophila (Hsu et al., 2007) and mice (Chen et al., 2007; Koide et al., 2009) provided genetic evidence that it plays a critical role in the normal proliferation and survival of embryonic fibroblasts. Moreover, different cellular proteins involved in cell growth have been reported to interact with HRF, including tubulin (Gachet et al., 1999), elongation factor eEF1A and its guanine nucleotide exchange factor eEF1Bβ (Cans et al., 2003; Langdon et al., 2004), Mcl-1 (Li et al., 2001; Liu et al., 2005), TSAP6 (Amzallag et al., 2004) and Na,K-ATPase (Jung et al., 2004).

A series of publications have highlighted the role of HRF in anti-apoptotic activities since a decrease at the protein levels leads to apoptosis or reversion of malignant phenotypes (Amson et al., 2012; Li et al., 2001; Liu et al., 2005; Rho et al., 2011). In tumor cells the expression of HRF is upregulated while during reversion of cells from the malignant phenotype the HRF levels are considerably reduced. Consistently, the inhibition of HRF expression, by anti-sense cDNA or small interfering RNA molecules, results in the suppression of the malignant phenotype (Tuynder et al., 2002).

The extracellular function of HRF presents a cytokine-like activity leading to the release of histamine from mast cells and basophils via IgE antibody-mediated mechanisms during late-phase acute allergy. Moreover, investigations of the actions of HRF on the immune response first indicated that the human recombinant HRF (HrHRF) induces the secretion of histamine, IL-4 and IL-13 from basophils (Schroeder et al., 1997) and IL-8 from eosinophils (Bheekha-Escura et al., 2000) from a subset of allergic donors in a IgE-dependent and IgE-independent manner. Recombinant murine HRF was found to stimulate B cell proliferation in a dose-dependent manner (*in vitro* and *in vivo*) (Kang et al., 2001) and the HrHRF was found to modulate cytokine release from peripheral blood T cells (PBTs) by inhibition of IL-2 and IL-13 secretion, but not IFN-γ (Vonakis et al., 2003).

Unfortunately so far no connection has been made between the extracellular functions of HRF with the variety of intracellular processes attributed to the protein family and many intracellular and extracellular functions that have been ascribed to HRF seem to be contradictory. Therefore overall functions of HRF still remained to be elucidated.

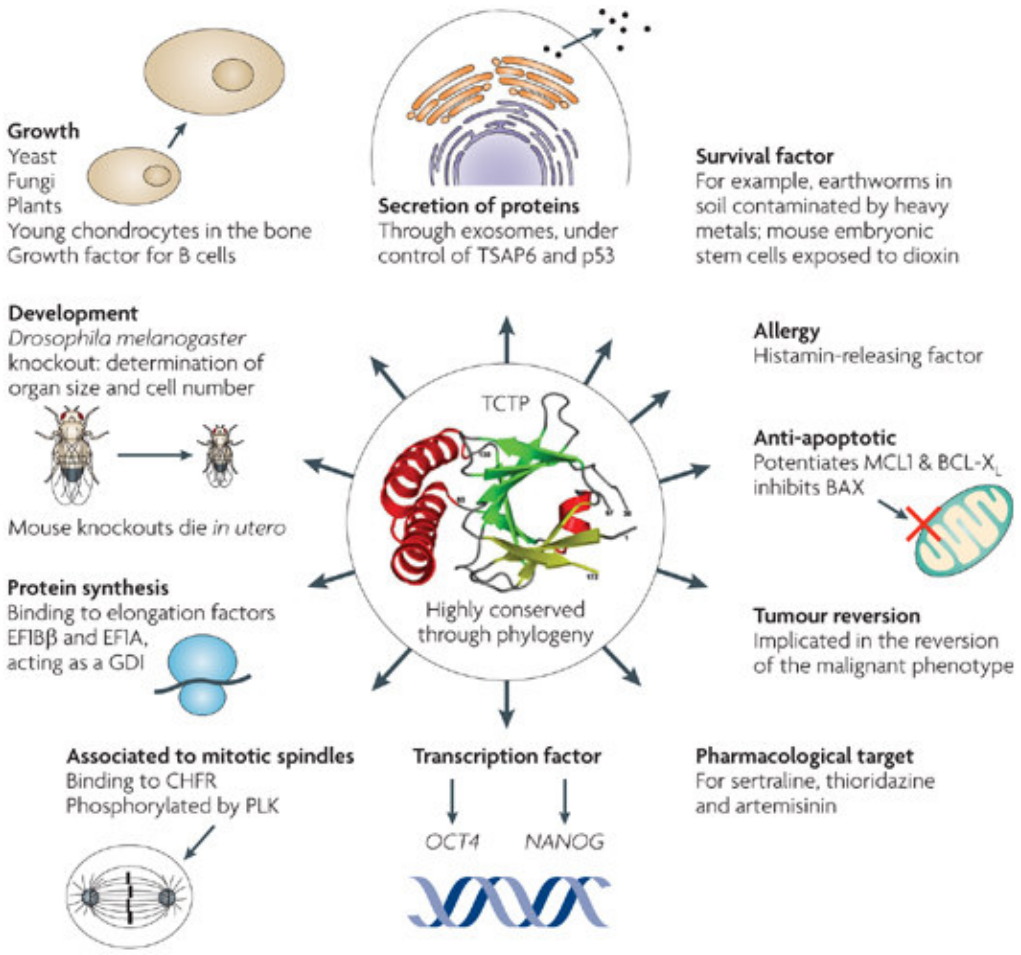

Figure 13: Histamine Releasing Factor (HRF), is secreted through exosomes that are regulated by TSAP6 and p53. *hrf* is a key gene in the process of tumour reversion and a pharmacological target for sertraline, thioridazine and artemisinin. Well conserved through phylogeny as a pro-survival, growth-stimulating and anti-apoptotic factor, it is also a transcription factor for *OCT4*. HRF binds to CHFR and to elongation factor eEF1A and eEF1Bβ, leading to roles during mitosis and in protein synthesis, respectively. GDI, GDP dissociation inhibitor. (Telerman and Amson, 2009)

2.1.1 HRF structure

The translationally controlled tumor protein consists of 170 amino acids in average and has a mean weight of 18-23 kDa.

Bioinformatic analysis was performed to investigate the sequence and predicted structure of the HRFs from many species (Hinojosa-Moya et al., 2008). Extensive sequence analysis revealed that most species, including malaria parasites, have only a single *hrf* gene. Mammals appear to harbour more *hrf* genes (eg. in mouse genome seven *hrf* genes were identified) (Fiucci et al., 2003). Additionally, no similarity to other proteins or functional domains was discovered, so the protein was listed as a 'family' on its own in the databases.

Elucidation of the HRF structure, resolved by NMR spectroscopy and X-ray crystallography, demonstrated that the HRF molecule consists of three distinct domains, the core β–sheet domain, a α-helical domain and a flexible loop structure (Fig. 14) (Thaw et al., 2001). Additional analysis confirmed that this principal structure is highly conserved through phylogeny over a long period of evolution supporting the hypothesis that HRF plays and important role in the cellular physiological functions (Bini et al., 1997; Bommer and Thiele, 2004b; Thaw et al., 2001). Moreover, the sequence alignment demonstrates that 9% of HRF amino acids are absolutely conserved (Bommer and Thiele, 2004b). The invariant residues are largely clustered on one side of the β-strain domain indicating that this side is important for interaction. In fact, three conserved amino acids (Glu12, Leu74 and Glu134) which form a binding surface for G protein interaction are localized in this domain suggests a possible role for HRF in proliferation and growth (Hinojosa-Moya et al., 2008). The other major domains, the flexible loop and the helical domain, are specific for HRF. The flexible loop is predominantly hydrophilic containing a highly conserved area named HRF1. The α-helical domain is characterized by a tubulin-binging region (Gachet et al., 1999) and a Ca^{2+}-binding area (Kim et al., 2000). However, the calcium-binding domain position is controversial. Additionally detailed studies localized the domain in the residues N131, Q132 and D150, located in the β-stranded core domain close to the connection with the α-helical domain (Feng et al., 2007) or in the residues E58 and E60 located in the floppy loop (Graidist et al., 2007).

Interesting, 3D structure of *P. knowelsi* an extra α-helix segment, which in other HRFs correspond to a β-sheet, is present and could potentially interfere with G protein interactions (Fig. 14) (Hinojosa-Moya et al., 2008).

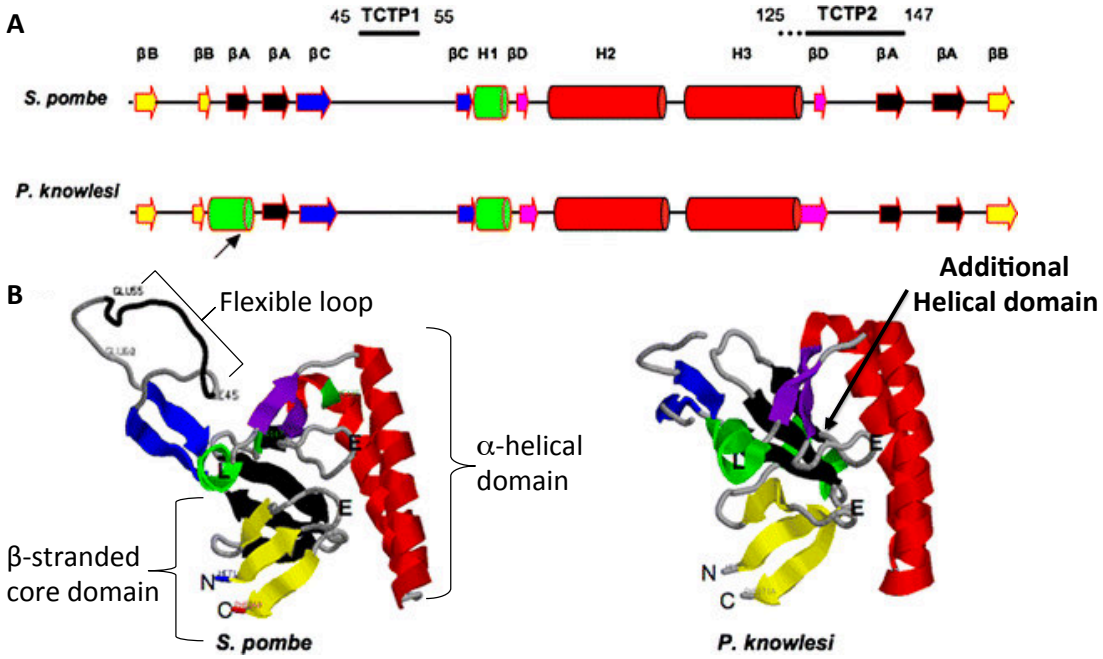

Figure 14: HRF structure of *S. Pombe* and *P. knowlesi*. (A) Two- and (B) three dimensional structure. The arrow indicated the additional a-helix from residue 22 to 30 in *P. knowlesi, which replaces a b-sheet in the oerthologous S. Pombe*. Note that in the 3D structure the torsion induced by the α-helix instigates the resulting protrusion exhibited by the two β-sheets. (Hinojosa-Moya et al., 2008)

2.1.2 HRF secretion

It has been reported that HRF protein lacks the NH_2-terminus signal and no precursor protein has been described suggesting that HRF secretion proceeded via an endoplasmic reticulum/Golgi-independent or nonclassical pathway (Muesch et al., 1990). Interestingly, immune fluorescence assay (IFA) analysis shows a partially co-localisation of HRF with tumour suppressor activated pathway-6 (TSAP6, a p53-regulated gene) in vesicular-like structures at the plasma and nuclear membranes (Amzallag et al., 2004). Furthermore, the overexpression of TSAP6 considerably enhanced the secretion of both endogenous and exogenous HRF in epithelial cells as well as in hematopoietic cell lines (Amzallag et al., 2004). More importantly, these studies show that the TSAP6 gene product could facilitate the secretion of HRF in exosomes (Fig. 15) (Amzallag et al., 2004). Exosomes are nanovesicles (50-150nm in diameter) of cup shape appearance originating from large multivesicular endosomes containing nucleic acids, proteins, lipid rafts and exposed phosphatidylserine (Thery et al., 2009) that induce phenotypic changes in the recipient cells (Raposo and Stoorvogel, 2013). Due to their stability, exosomes protect their cargo against degradation

and denaturation in the extracellular environment. Moreover, they play an important role in many biological processes including pathogen spread: various pathogens utilize the exosomal pathway to facilitate their survival and propagation, including transfer of virulence factors to host target cells (Feng et al., 2013; Ramakrishnaiah et al., 2013).

Recently, another pathway that proposes the involvement of H, K-ATPase was used to describe HRF secretion (Fig. 15) (Kim et al., 2013). A screening of possible HRF secretion inhibitors show that proton pumps inhibitors modulated the protein export. Indeed, the use of omeprazole and pantoprazole, two peptic ulcer drugs, block HRF secretion from HEK293 and U937 cells in a concentration-dependant fashion, corroborated by the observation that H, K-ATPase inhibitor ameliorated the allergic symptoms.

2.1.3 HRF dimerization

The comparison of basophil histamine releasing abilities of mouse recombinant HRF (MrHRF), human recombinant (HrHRF), HRF from nasal lavages, and HRF from supernatants of peripheral blood mononuclear cells (PBMC) cultures have shown that recombinant HRF were less active than the native HRF purified from the biological fluids. Dimerization of HRF seems to be essential for its cytokine-like activity, as the cytokine-releasing activity of extracellular HRF is generated only when the NH_2-terminal is truncated allowing the dimerization via the formation of an intermolecular disulfide bond (Kim et al., 2009). Moreover, dimerized form of the protein was found in sera in allergic patients and bronchoalveolar lavage fluids from airway-inflammed mice (Kim et al., 2013; Kim et al., 2009). Subsequent studies suggested that allergic status might affect the normal biochemistry composition and physiology of the cellular environment, leading to posttranslational modification of the protein, and the activation of inflammatory cells that promote the late phase reaction. Three possible contributors were described: (i) proteolytic enzymes which catalysed the truncation of the NH_2-terminus, (ii) oxidants responsible for the formation of intermolecular disulfure bonds, and (iii) HRF-reactive IgE that possibly confer autoantigen activity (Kim et al., 2013)(Fig. 15).

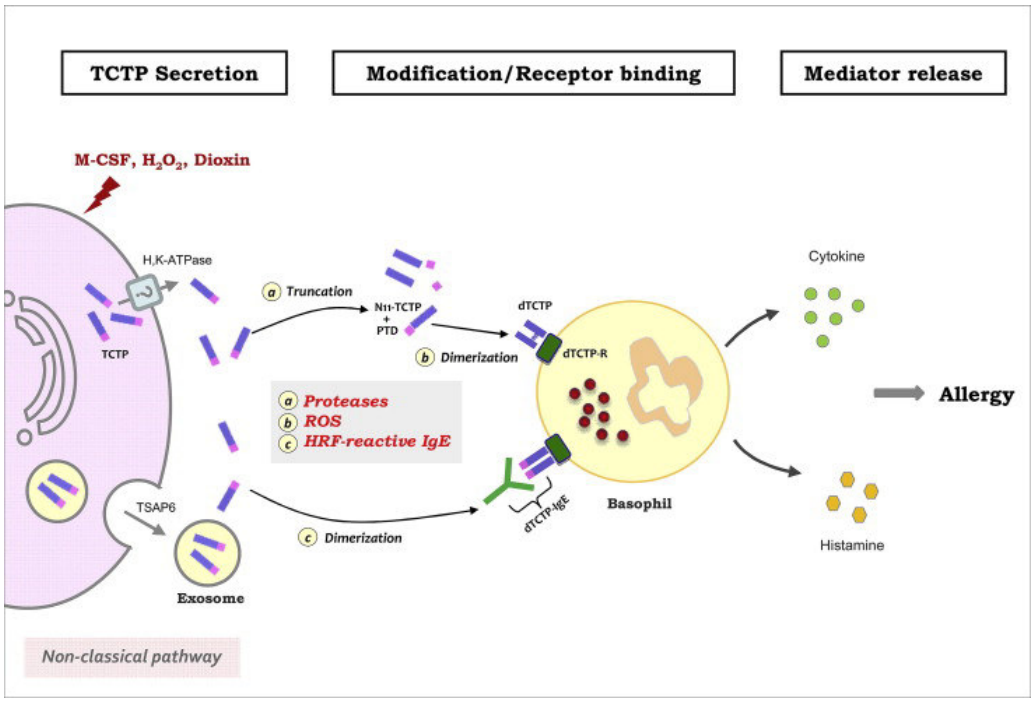

Figure 15: Mechanism of secretion and hypothetical modification of HRF. Secretion of HRF is enhanced by hydrogen peroxide, M-CSF, dioxin, and so forth during late phase allergic inflammation. HRF is secreted outside of cells through H,K-ATPase- or TSAP6-mediated non-classical pathway and undergoes posttranslational modification. (a) Processing of secreted full-length HRF by proteolytic enzyme releases truncated HRF (N11TCTP) and short-length N-terminal fragments (PTD). (b) Oxidants can form an intermolecular disulfide linkage between two of HRF that produces the dimerized HRF (dTCTP). (c) Alternatively, HRF can cross-react with IgE which dimerize the intact HRF (dHRF-IgE) by connecting a HRF monomer with an other HRF monomer. Active structure of HRF now can bind to its specific receptor (dHRF-R) to stimulate the mediator release from basophils. In addition to dHRF-R, subset of IgE can serve as a receptor for HRF dimer in FcεRI-expressing cells. Since these modifications might be possible at inflamed site, extracellular HRF will affect around cells only during the late phase or chronic allergic inflammation. (Kim et al., 2013)

2.2 ROLE OF *PLASMODIUM* HRF DURING MALARIA INFECTION

Evidence of *P. falciparum* HRF (*Pf*HRF) secretion was observed in *in vitro* cultures and in malaria infected individuals. Trophozoite stage culture supernatants of *P. falciparum* contained 87±21μg of *Pf*HRF per 10^9 iRBCs, while schizont stage culture supernatants contained 44±6μg of *Pf*HRF per 10^9 iRBCs. Therefore around half of the total parasite HRF was released during schizogony (MacDonald et al., 2001). *Pf*HRF has been identified in the serum of mildly (0.6μg/mL) and severely (1.4μg/mL) *P. falciparum* infected Malawian children (Janse et al., 2006; MacDonald et al., 2001). There was no correlation between percentage parasitemia of infected individuals and serum *Pf*HRF levels, suggesting that the parasite protein might have local rather than systemic effects.

At the amino acid level, *Pf*HRF is highly homologous to mammalian protein by sharing 33% identity and 54% similarity with human HRF. Molecular mass of recombinant *Plasmodium* HRF molecules is between 20 and 23 kDa for the monomeric form and around 45 kDa for the dimeric form (Bhisutthibhan and Meshnick, 2001). Sequence homology between HRF molecules is very high between different species of *Plasmodium* parasites. It was fond that *P. falciparum* share 88% identity and 97% similarity in HRF amino acids sequence with *P. yoelli* (Walker et al., 2000) and 90% identity with *P. chabaudi* HRF. Aditionally, comparisons of HRF amino acid sequence of *P. chabaudi* with that of *P. yoelli* and *P. berghei* orthologues show amino acid identities of 97 and 98%, respectively (Afonso et al., 2006). IFA and immunolelectron microscopy with immunogold labelling have allowed the localization of intracellular *Pf*TCTP within the cytoplasm, the haem-rich food vacuole and the host or parasite-derived membranes surrounding the parasite (Bhisutthibhan and Meshnick, 2001; Bhisutthibhan et al., 1999).

2.2.1 Consequences of histamine release

Generation of histamine-releasing activity was observed in cultured peripheral blood mononuclear cells and their supernatants which were able to induce the release of histamine from basophils (Thueson et al., 1979). Several studies have attempted to chemically characterize the new cytokine that induce the release of histamine from basophils. In 1995, HRF was found and purified from the nasal, skin blister, and bronchoalveolar lavage fluids during late-stage allergic reaction, implicating HRF in the late-phase allergic reactions (MacDonald et al., 1987; MacDonald et al., 1995). Other studies reported the implication of HRF in promoting the allergic inflammation in the skin and lung from mouse models of asthma and allergy. Furthermore, Ig-interacting HRF peptides, shown to block HRF-Ig interactions *in vitro*, inhibited IgE-HRF-induced mast cell-dependent inflammation *in vivo* (Kashiwakura et al., 2012). Collectively, these studies indicate that HRF has pro-inflammatory features during allergic reactions. At first this release was thought to be the consequence of physical interaction between HRF and IgE on the surface of the responding basophils (MacDonald et al., 1987). However, some evidences suggested that the interaction with IgE may not be required for cell activation mediated by human recombinant HRF and that the protein exerts its activity independently of IgE through a human

recombinant HRF-specific activation pathway other than through FcεRI (Schroeder et al., 1997).

Recent studies suggest a strong relationship between clinical susceptibility to malaria and severe allergic-type responses (Demeure et al., 2005; Griffiths et al., 2005; Sakuntabhai et al., 2008). As previously demonstrated in the laboratory, this allergic-type response starts immediately after the mosquito bite by the intermediary of mosquito saliva which exerts immunomodulatory effects through induction of mast cell degranulation, in the absence of IgE Abs, affecting the maturation of adjacent DCs, which fail to ultimately elicit fully activated effector T cells (Demeure et al., 2005). Additionally, *Plasmodium* infection in a murine model of malaria, upon mosquito bite and saliva inoculation, potentiates the pathogenesis via dysregulation of immune signalling and reduction in the recruitment of key inflammatory cells into the inoculation site perturbing the early antiparasite immune response and effecting downstream disease development (Fig. 16) (Schneider et al., 2011).

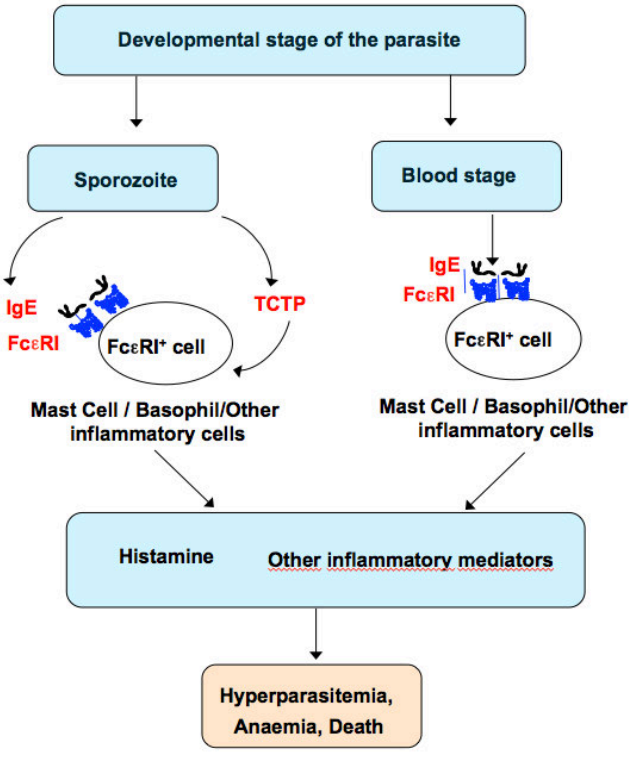

Figure 16: IgE/FcεRI complex and parasite HRF represent two mechanisms by which histamine release is triggered from its cellular stores during malaria infection. Histamine is continuously released into host body starting from the beginning of the infection which allows the development of ECM.

High levels of histamine, a major product of allergic responses derived from basophils and mast cells in plasma and tissue, have been associated with the severity of the disease in humans infected with *P. falciparum* and in many animal models (Enwonwu et al., 2000; MacDonald et al., 1995; Maegraith and Fletcher, 1972; Srichaikul et al., 1976). In addition, during parasitic infection in human and mouse model, higher levels of IgE, which bind to basophils and mast cells and trigger histamine release, are associated with the severity of the disease (MacDonald et al., 2001; Porcherie et al., 2011; Schroeder et al., 1997).

Elevated histamine synthesis (*in situ*) within the brain mediated by the histidine decarboxylase enzyme was five-fold more elevated in plasma of infected children with *P. falciparum* compared with uninfected controls (Enwonwu et al., 2000). Moreover, the proinflammatory activities via histamine signalling pathway was previously demonstrated in the laboratory to be crucial for the development of ECM pathologies and symptoms exhibited in rodent malaria models. Mice treated with antihistamines or deficient for the H1 and H2 receptors were found more resistant to ECM after infection with *P. berghei* ANKA sporozoites (Beghdadi et al., 2008) than untreated or wild type mice respectively. Similar results were obtained in $HDC^{-/-}$ mice, not able to synthesize histamine, infected either with *P. berghei* ANKA iRBCs or sporozoites. This indicated that histamine is produced during all stages of infection and is involved in late stage of pathogenesis (Fig. 16). During the later phase of infection, particularly during the blood stage of parasite development, histamine can be elicited by *Plasmodium* HRF. Parasite HRF has been found in plasma of patients infected with *P. falciparum* and was shown to trigger histamine release from basophils and IL-8 secretion from eosinophils (MacDonald et al., 2001). Significantly elevated plasma histamine concentrations by *Plasmodium* HRF could assist the parasite by inducing vasodilation and increased expression of endothelial adhesion factors such as thrombomodulin, to which *P. falciparum*-infected erythrocytes adhere (MacDonald et al., 2001). These results show that the existence of a parasite protein able to stimulate host histamine secretion can amplify the host inflammatory response, via histamine signalling and highlight a possible strategy developed by the parasite to create conditions advantageous for its own survival and persistence.

2.2.2 Artemisinin chemo-resistance

The artemisinin removes quickly the parasites and eliminates the symptoms associated with malaria by reducing the carriage of the transmission stages. All the artemisinin drugs have activity throughout the asexual intra-erythrocytic stage, also having effect on young gametocytes. However, their mode of action is not completely clear (Klonis et al., 2011). Some researchers agree that artemisinin might interact with intraparasitic haem as well as other parasite proteins (Meshnick, 2002; Robert et al., 2002). Consistently, artemisinin and its derivatives are inactive against the RC strain of *P. berghei*, which doesn't produce hemozoin (Meshnick, 2002). This hypothesis supposes that artemisinin is activated by intraparasitic heme into a carbon-centred free radicals mediated by the decomposition of the artemisinin endoperoxide bridge. The carbon-centred free radicals then act as alkylating agents for specific parasite proteins (Meshnick, 2002). The hypothesis that *Plasmodium* HRF might be a possible drug target for artemisinin comes from the reaction of *Plasmodium* HRF with radio labelled dihydroartemisinin ([^{3}H]DHA) both *in vitro* in the presence of hemin, and in intact infected erythrocytes. Scatchard analysis showed that two hemin-binding sites of modest affinity existed on parasite HRF (Bhisutthibhan et al., 1998). Aditionally, [^{3}H]DHA adducts could be immunoprecipitated using anti-HRF antibodies. Overexpression of *Plasmodium* HRF at the protein level in artemisinin-resistant parasites of *P. yoelii* is consistent with this hypothesis (Walker et al., 2000).

The three-dimensional structure of *Pf*HRF created using the nuclear magnetic resonance structure of the *S. pombe* HRF as a template was used to study the interactions between artemisinin, heme and *Pf*HPF. The center of the peroxide bond of artemisinin was docked within a short distance of 2.6 Å from the iron of the heme, in the same way the center of heme-activated artemisinin (C4-radical) was docked on the *Pf*TCTP sulfur of Cys14 within a distance of 2.48 Å suggesting the possibility of alkylation having occurred (Chae et al., 2006). To confirm this modelling previous study had shown that blocking the single cysteine of *Pf*TCTP reduced artemisinin binding by 67% (Bhisutthibhan et al., 1998).

2.2.3 Calcium binding and self-interaction with malaria HRF

High-affinity binding of HRF to calcium was previously demonstrated from both human and trypanosome (Haghighat and Ruben, 1992; Xu et al., 1999). Depletion of endoplasmic reticulum levels of calcium up-regulates HRF transcription, while an increase in cytosolic calcium up-regulates HRF translation showing that HRF expression is regulated by calcium at both transcription and post-transcriptional level (Xu et al., 1999).

The primary sequence of *Pf*TCTP lacks a calcium-binding motif; but calcium-binding properties have been demonstrated by *Pf*HRF migration and PVDF membrane transfer followed by calcium (^{45}Ca) incubation or by SDS-PAGE gel migration of recombinant *Pf*HRF in presence of calcium (Bhisutthibhan et al., 1999).

2.2.4 B cell inhibition by malaria HRF

Structural analysis of *P. knowelsi* HRF and human HRF shows some variations, particularly for the presence in *P. knowelsi* of an extra α-helix near the GTPase binding pocket while in human HRF is a β-strand. This difference causes a more general structural distortion in the putative G-protein binding pocket suggesting that *Plasmodium* HRF may act as a dominant negative mutant in the host, as it could potentially block the action of B cells due to its proposed inability to bind G proteins (Hinojosa-Moya et al., 2008). Recently it was shown that recombinant *Pf*HRF has a lower B cell stimulatory effect compared to recombinant human HRF. In addition, splenic B cells were shown to incorporate more efficiently and faster the parasite protein than the human one (Calderon-Perez et al., 2014). This result indicated that *Plasmodium* HRF could interfere with the action of the host HRF by inhibiting B cell responses allowing the parasite to escape the immune response. However, additional work is required to confirm these results.

2.2.5 Basophils activation by malaria HRF

Field studies in Senegal had shown an association between high levels of *Pf*HRF in the peripheral blood circulation and basophils activation. At the same time it was observed that a decrease of basophils in the peripheral blood circulation is associated with the severity of symptoms (Pelleau et al., 2012). A significant accumulation of basophils in the spleen during

malaria was similarly observed in the mouse model during *P. chabaudi* AS infection (Poorafshar et al., 2000). Moreover, activation of basophils leads to IL-4 production that might play a crucial role in the initiation of Th2 responses, that when activated too early during the infection, increases host susceptibility to malaria disease. However, in this case also additional studies are required to understand whether basophils have a protective or deleterious effect during the development of pathogenesis.

2.3 Comparative characteristics of mammal HRF and Plasmodium HRF

Many intracellular and extracellular functions have been ascribed to mammal HRF (Table 2) (Bommer and Thiele, 2004b). Many of the functions that have been determined for HRF are unrelated, and some seem to be contradictory. An overall function of HRF has yet to be determined, and much is left to be learned.

Moreover, despite this broad knowledge on mammal HFR, little is know about Plasmodium HRF (Table 2) and the structural differences observed suggest that the parasite protein may have different if not opposite biological functions than the human HRF, especially in influencing the biological responses of immune cells. For this reason, during this work we want to study and identify the possible effects of *Plasmodium* HRF on the host immune response and their implication in the development of disease pathogenesis.

	Mammal HRF	*Plasmodium* HRF	Ref.
Biological function			
Calcium binding	YES	YES	(Xu et al., 1999), (Bhisutthibhan et al., 1999)
Growth and development	YES ($hrf^{-/-}$ mouse lethal)	NO	(Chen et al., 2007; Hsu et al., 2007; Koide et al., 2009; Tuynder et al., 2002), (Mathieu et al., 2015)
Tubulin binding activity	YES	ND	(Gachet et al., 1999)
Chaperon activity	YES	ND	(Cans et al., 2003)
Anti-apoptotic	YES	ND	(Amson et al., 2012; Li et al., 2001; Liu et al., 2005; Rho et al., 2011)
Tumour reversion	YES	ND	(Li et al., 2001; Tuynder et al., 2002)
Pharmacological target	YES	YES	(Tuynder et al., 2004), (Bhisutthibhan et al., 1998)
Histamine release	YES (from basophils)	YES (from basophils and mast cells?)	(MacDonald et al., 1987; MacDonald et al., 1995; Thueson et al., 1979), (Briquet et al., 2015; MacDonald et al., 2001)
Cytokines release	YES (IL-4, IL-13 from basophils and IL-8 from eosinophils)	YES (IL-8 from eosinophils)	(Bheekha-Escura et al., 2000; Schroeder et al., 1997), (MacDonald et al., 2001)
B cell	YES (Proliferation-Stimulation)	YES (Inhibition of proliferation)	(Mathieu et al., 2015), (Calderon-Perez et al., 2014)
T cell	YES (Inhibition of IL2 and IL13 secretion)	ND	(Vonakis et al., 2003).
Other Characteristics			
Copy nb of *hrf* genes	Several copies	Only one copy	(Fiucci et al., 2003)
Structure	YES	YES	(Hinojosa-Moya et al., 2008; Thaw et al., 2001), (Hinojosa-Moya et al., 2008)
Secretion	Exosomes or H,K-ATPase channel	ND	(Amzallag et al., 2004; Kim et al., 2013)
Active form	Dimer	ND	(Kim et al., 2013; Kim et al., 2009)

Table2 – HRF characteristics.

Objective

3. SCOPE OF THE THESIS

The purpose of this thesis was to assess the importance of *Plasmodium* HRF in the modulation of immune mechanisms during malaria infection and evaluate whether its expression is associated with the severity of malaria disease.

Specific Aims

Our specific objectives were as follows

- Design and generation of HRF-deficient parasites (*hrfΔ*) in a *P. berghei*-GFP ANKA and NK65-GFP strains by homologous recombination
- The proof of concept that the HRF protein plays a role in malaria pathogenesis
- To assess whether mutant parasites show an altered phenotype that may ultimately cause a sterile immunity
- Characterize the cellular and molecular alterations induced by the presence or the absence of HRF and the immune mechanisms by which the *hrf* gene modulates the immune response

  In the present thesis, the methodological part has been omitted since it is described in detail in the enclosed articles.

Results

4.1 ARTICLE I

Plasmodium berghei histamine-releasing factor favours liver-stage development via inhibition of IL-6 production and associates with a severe outcome of disease.

In this work we were interested in the role of the *P. berghei* HRF protein during malaria infection since it could affect the immune responses of the host *in vivo*, at least through its potential to induce secretion of histamine and to contribute to the pathogenesis of cerebral malaria. To characterize the role of *Plasmodium* HRF in parasite transmission, development and disease pathogenesis, HRF-deficient *P. berghei* ANKA (*Pb*ANKA-*hrfΔ*) parasite were generated. The parasite ability to induce ECM was studied comparing mice infected with either WT or mutant *Pb*ANKA-*hrfΔ* parasites. Parasitemia, survival rates, and immunological monitoring were all used to establish the mutant phenotype.

Highlights

- Identification of *hrf* as a gene playing a role during parasite development in pre-erythrocytic stages. The development of *Pb*ANKA-*hrfΔ* parasite was specifically inhibited during liver stage development *in vivo*, resulting in reduction of merozoites numbers initially released in the blood, and an extension of the time between sporozoite injection and blood stage development.
- The prolonged liver-stage development influenced the clinical outcome of infection by decreasing the frequency of ECM and by increasing the survival rate.
- Impairment in the development of *Pb*ANKA-*hrfΔ* parasite in liver stages was associated with an early rise in liver and systemic IL-6 that in case of WT parasite infection was directly inhibited by *Plasmodium* HRF production.
- Increase in B-cell population in the liver of mice infected with mutant parasites at 40 h p.i. in association with early rise of IL-6 reflects the possibility that the HRF molecule could interfere with B-cell memory.

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Plasmodium berghei histamine-releasing factor favours liver-stage development via inhibition of IL-6 production and associates with a severe outcome of disease

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Summary

***Plasmodium* spp., which causes malaria, produces a histamine-releasing factor (HRF), an orthologue of mammalian HRF. Histamine-releasing factor produced by erythrocytic stages of the parasite is thought to play a role in the pathogenesis of severe malaria. Here, we show in a rodent model that HRF is not important during the erythrocytic but pre-erythrocytic phase of infection, which mainly consists in the transformation in the liver of the mosquito-injected parasite form into the erythrocyte-infecting form. Development of *P. berghei* ANKA cl15cy1 liver stages lacking HRF is impaired and associated with an early rise in systemic IL-6, a cytokine that strongly suppresses development of *Plasmodium* liver stages. The defect is rescued by injection of anti-IL-6 antibodies or infection in IL-6-deficient mice and parasite HRF is sufficient to decrease IL-6 synthesis, indicating a direct role of parasite HRF in reducing host IL-6. The target cells modulated by HRF for IL-6 production at early time points during liver infection are neutrophils. Parasite HRF is thus used to down-regulate a cytokine with anti-parasite activity. Our data also highlight the link between a prolonged transition from liver to blood-stage infection and reduced incidence of experimental cerebral malaria.**

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Introduction

Histamine-releasing factor (HRF), originally classified as a tumour protein (translationally controlled tumour protein, TCTP) in mouse erythroleukemia, is found in a wide range of eukaryotes including yeast, plants and animals. The name TCTP was coined as a consequence of cDNA cloning from a human mammary carcinoma and based on the fact that TCTP is regulated at the translational level. Histamine-releasing factor/TCTP was first described as P21, Q23 and P23 by different groups and the mouse and human cDNA were cloned in the late eighties (Chitpatima *et al.*, 1988; Gross *et al.*, 1989). HRF plays many different functions and is involved in many physiological processes such as cell proliferation, stress and heat shock responses, and cell death. As an intracellular product, HRF has a calcium- and tubulin-binding properties and has been shown to transiently associate with microtubules during cell cycle (Gachet *et al.*, 1999). As a calcium-binding protein, HRF was found to be up-regulated in response to a loss of calcium homeostasis, which could be part of a role of HRF in general stress response (Xu *et al.*, 1999). HRF was shown to be associated with prostate, breast and colon cancers (Chung *et al.*, 2000; Vercoutter-Edouart *et al.*, 2001; Arcuri *et al.*, 2004), which is consistent with its anti-apoptotic activity (Zhang *et al.*, 2002). This was supported by the capacity of HRF to interact with the B-cell lymphoma-extra large (Zhang *et al.*, 2002; Yang *et al.*, 2005; Fenner *et al.*, 2010; Rid *et al.*, 2010) and to antagonize bax dimerization (Susini *et al.*, 2008) and to control the stability of the tumour suppressor p53 (Rho *et al.*, 2011). As a secreted product, HRF has immuno-modulatory roles. In humans, HRF induces the release of histamine and modulates cytokine secretion from basophils, eosinophils and T-cells. Histamine-releasing factor stimulates eosinophils to produce IL-8 (Bheekha-Escura *et al.*, 2000), induces

secretion of IL-4 and IL-13 from basophils (MacDonald *et al*., 1995; Schroeder *et al*., 1997) and inhibits IL-2, IL-4 and IL-13 production from stimulated primary T-cells (Vonakis *et al*., 2003). Recently, HRF was found to have an inflammatory role in mouse models of asthma and allergy and to exist as a dimer bound to a subset of IgE and IgG antibodies, suggesting the possibility for HRF to cross-link IgE on the surface of basophils and mast cells (Kashiwakura *et al*., 2012).

HRF is also expressed by a number of eukaryotic parasites, including *Plasmodium*, the causative agent of malaria. Malaria is the most deadly parasitic disease worldwide, causing almost a million deaths each year. The symptoms of the disease are due to the multiplication of the parasite inside host erythrocytes. Infection starts by an asymptomatic phase, during which the highly motile parasite form injected by mosquitoes reaches the liver, invades hepatocytes and multiplies into the erythrocyte-infecting parasite form. The latter, once released into the bloodstream, invades erythrocytes and initiates the clinical phase of infection, consisting in repeated cycles of parasite multiplication inside erythrocytes. A severe complication of the erythrocytic phase of malaria infection is cerebral malaria (CM), a syndrome caused by the cytoadherence of infected erythrocytes to endothelial cells in brain capillaries, the release of inflammatory cytokines and chemokines and the accumulation of activated $CD8^+$ T-cells.

Plasmodium HRF, which has a high homology to human HRF (their amino acid sequences are 33% identical and 54% similar) and similar activity (MacDonald *et al*., 2001), has been proposed to play an important role during the erythrocytic phase of malarial infection. *Plasmodium falciparum* HRF was detected in the plasma of malarial patients in Malawi (MacDonald *et al*., 2001) and in higher concentration in patients with severe malaria in Senegal (Pelleau *et al*., 2012). Increased levels of plasma histamine have been associated with disease severity during *P. falciparum* infection as well as in animal models of malaria (Maegraith and Fletcher, 1972; Srichaikul *et al*., 1976; Bhattacharya *et al*., 1988; Enwonwu *et al*., 2000). Accordingly, CM does not develop in histamine-free, histidine decarboxylase-deficient mice (Beghdadi *et al*., 2008). Therefore, parasite HRF is thought to play a role in malaria pathogenesis, at least in part through its action on histamine.

Although the contribution of cytokines and other inflammatory molecules during malarial erythrocytic infection is extensively studied, still little is known about host factors that control *Plasmodium* development in the liver. Work in rodents and cultured hepatocytes showed that several host cytokines strongly inhibit parasite development inside hepatocytes, including IFN-γ, TNF-α, IL-1 and IL-6 (Schofield *et al*., 1987; Maheshwari, 1990; Pied *et al*., 1992; Vreden *et al*., 1992). Both TNF-α-induced (Vreden *et al*., 1992) and IL1-induced (Nussler *et al*., 1991) inhibitions of liver-stage development are mediated by IL-6. In contrast, recombinant IL-6 is sufficient to reduce liver-stage development inside cultured hepatocytes in the absence of non-parenchymal cells (Pied *et al*., 1992) and exerts its action throughout liver-stage maturation (Nussler *et al*., 1991). One of the mechanisms by which IL-6 might control parasite liver infection is by regulating iron homeostasis through hepcidin (Nemeth *et al*., 2004), which limits *Plasmodium* development inside hepatocytes (Portugal *et al*., 2011).

In this work, we investigated the role of HRF in *P. berghei*, a rodent-infecting species of *Plasmodium* that can be cycled *in vivo*. We found that HRF has no contribution during the erythrocytic phase of infection, but plays an important role during the pre-erythrocytic phase of infection in the liver by decreasing IL-6.

Results

HRF is important for P. berghei *pre-erythrocytic infection*

To test the role of HRF in *P. berghei*, we replaced in erythrocytic stages of a GFP-expressing *P. berghei* ANKA clone (Ishino *et al*., 2006) the entire coding sequence of *PbHRF* (PBANKA_111050) by the *hDHFR* selectable marker (Supporting Information Fig. S1). Three clones were selected from independent transfection experiments, called HRFΔ1–3, which were verified to harbour the expected *HRF* null-mutant locus by PCR (Supporting Information Fig. S1A–C), and by Southern blot analysis (Supporting Information Fig. S1D). Blood stages of the wild type (WT) and of the three mutant clones exhibited similar growth rates in the blood of C57BL/6 mice, as measured by Fluorescence-activated cell sorter (FACS) analysis during exponential growth (Fig. 1A). When similar numbers of erythrocytes infected with HRFΔ1 or WT parasites were injected in mice, a similar proportion of mice (> 90%) succumbed to CM in the two groups, with similar kinetics (Fig. 1B). Similar results were obtained with HRFΔ2 and HRFΔ3 parasites (not shown). We conclude that HRF is not essential for *P. berghei* blood stages.

To test pre-erythrocytic stage infectivity, sporozoites were inoculated into mice by mosquito bite (Fig. 1C and D), intradermal (ID, Fig. 1E) or intravenous (IV, Fig. 1F) injection and emergence of blood-stage parasites monitored by FACS. Only 40% to 50% of animals became infected after injection of HRFΔ1 sporozoites when 100% animals were infected after injection of WT parasites (Fig. 6B). Animals became patent with HRFΔ1 blood-stage parasites on average 2 to 3 days after the WT following infection by mosquito bites (Fig. 1C, insert) or inoculation of isolated sporozoites (Fig. 1E and F),

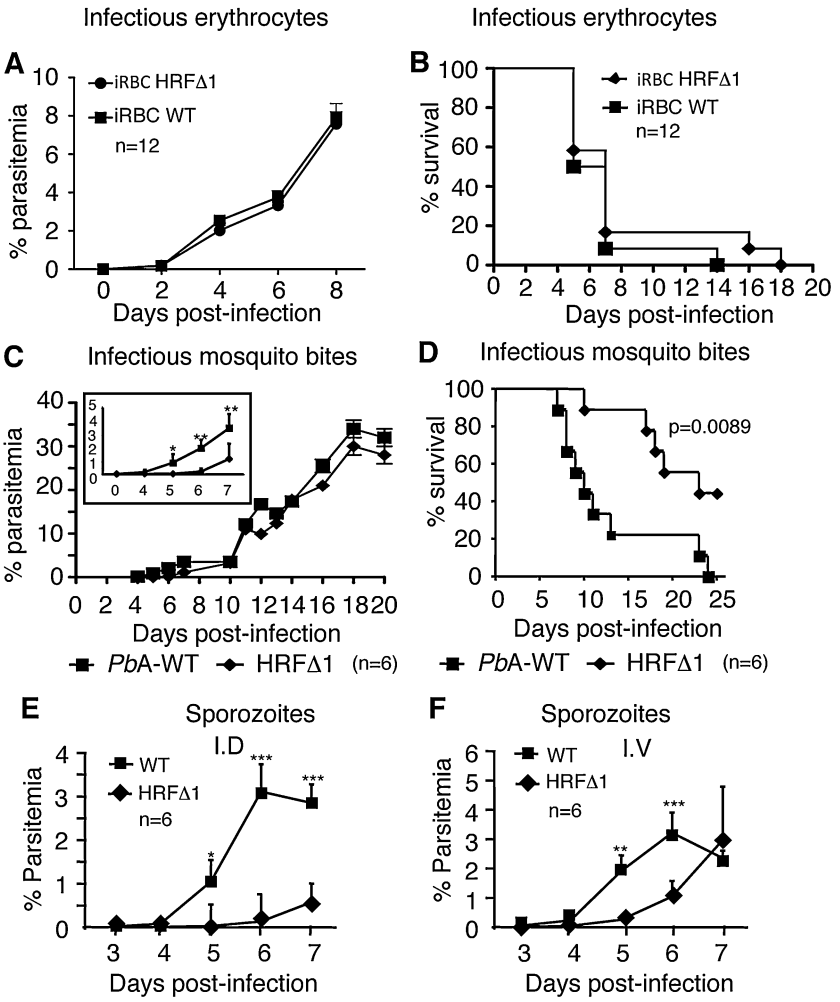

Fig. 1. Marked differences in parasitaemia between WT and HRFΔ1 *Pb* ANKA-infected mice are observed only at the pre-erythrocytic stage. C57BL/6 mice (12 mice per group) were infected with 10^6 WT or HRFΔ1 *Pb* ANKA-infected RBCs (A, B) or via bites of 10 mosquitoes (C, D), or inoculated with 10 000 isolated sporozoites ID (E), or IV (F). In C, D, E, and F panels, as 50% of mice did not show parasitaemia, only six mice per group were shown. Kaplan–Meier survival plots (log-rank test) and parasitaemia (Mann–Whitney test; $*P < 0.028$; $**P < 0.007$; $***P < 0.002$) were recorded over time. Results are from three independent experiments.

corresponding to a 100- to 1000-fold decrease in infectivity of mutant parasites (day of patency 4 and 6 for WT and HRFΔ1-infected mice, respectively, Fig. 1C; day of patency 4 and 6 for WT and HRFΔ1-infected mice, respectively, Fig. 1E; day of patency 3 and 5 for WT and HRFΔ1-infected mice, respectively, Fig. 1F). Similar results were obtained with clones HRFΔ2 and HRFΔ3 (Supporting Information Fig. S2A and B). As expected, the delayed development of the mutant in the liver greatly decreased its ability to induce CM after sporozoite inoculation, as already reported for other mutants with a developmental block in the liver (Butler *et al.*, 2011) and in humans after RTS,S (repeat and T-cell epitope in the circumsporozoite protein (CSP) of Plasmodium falciparum malaria parasite and a viral envelope protein of the hepatitis B virus, HBsAg) immunization (Heppner *et al.*, 2005). Indeed while 100% C57BL/6 mice developed CM on day 7 or 8 after WT sporozoite inoculation by mosquito bite, only 10% of the mice inoculated with HRFΔ1 sporozoites showed signs of neuropathology (Fig. 1D). Similar CM resistant phenotype was observed in C57BL/6 mice infected by intravenous injection of isolated sporozoites (Supporting Information Fig. S2). We conclude that in *P. berghei* HRF is crucial for the pre-erythrocytic, but not erythrocytic phase of infection.

HRF is produced by sporozoites and liver stages

To detect HRF in *P. berghei* pre-erythrocytic stages, specific antibodies were generated by rabbit immunization with recombinant *P. berghei* HRF protein (*Pb*HRF). Expression of HRF by WT sporozoites was assessed by immunofluorescence assay (IFA), which showed a diffuse staining pattern suggesting a cytoplasmic localization (Fig. 2A). In contrast, no detectable HRF could be seen in HRFΔ1 parasites (Fig. 2A). Expression of HRF in parasitized hepatocytes was assessed by incubating cultured HepG2 hepatoma cells with WT or HRFΔ1 sporozoites. After 48 h, HRF was detected specifically in fixed/permeabilized cells infected with WT, not HRFΔ1, parasites (Fig. 2B). Staining of WT and mutant sporozoites, as

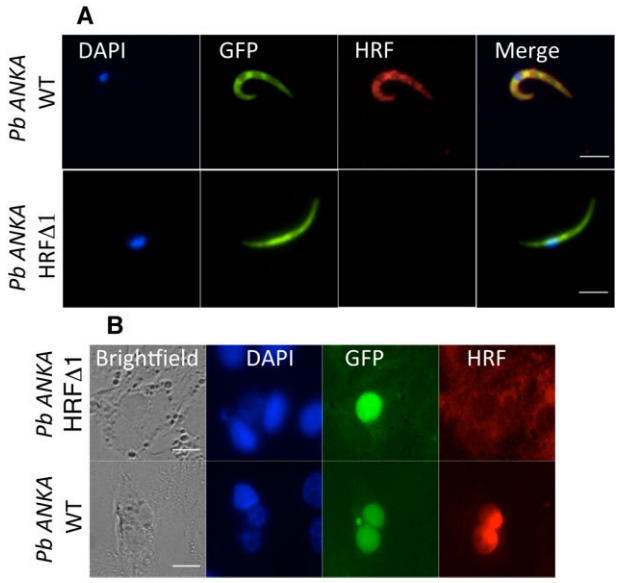

Fig. 2. Detection of HRF protein in pre-erythrocytic stages.
A. Immunofluorescent detection of HRF in fixed WT and HRFΔ1 GFP-tagged sporozoites. Representative images are shown for anti-HRF antibody together with bright-field images and DAPI stained nuclei. Scale bar 10 μm.
B. Intracellular staining for detection of HRF within hepatocytes was performed by incubating HepG2 cells with WT or HRFΔ1 sporozoites at MOI of 1:1 for 48 h. Cells were fixed and stained with anti-HRF rabbit IgG antibodies followed by Alexafluor 568-labelled donkey anti-rabbit IgG antibodies. Experiments were replicated twice with similar results. Scale bar, 10 μm.

well as HepG2 cultures harbouring WT or mutant parasites, with only secondary antibodies were negative.

HRF is important for liver-stage development in vivo

In vitro, HRFΔ1 sporozoites did not display any detectable defect in gliding motility (Supporting Information Fig. S3A–C), host cell traversal (Supporting Information Fig. S3F) or host cell invasion of HepG2 cells (Supporting Information Fig. S3D and E). HRFΔ1 and WT developed indistinguishably inside HepG2 cells into exo-erythrocytic forms (EEF, liver stages; Supporting Information Fig. S3G). Therefore, HRF does not appear to be important for a basic parasite developmental step detectable *in vitro*. After transmission to mosquitoes, WT and HRFΔ1–3 parasites generated similar numbers of sporozoites in the insect salivary glands. Indeed, at day 20 following parasite passage into female mosquitoes, similar prevalence of mosquito infection and similar numbers of HRFΔ1 and WT sporozoites per infected mosquito salivary gland were counted (Supporting Information Fig. S3H), indicating that HRF was dispensable for parasite development inside mosquitoes.

We then tested whether HRF might play a role during sporozoite progression *in vivo* using a putative vasodilatory effect of histamine. A potential role of HRF in facilitating sporozoite passage across the liver sinusoidal endothelium to reach hepatocytes was addressed by intravital imaging. Mice were injected IV with a 1:1 mixture of GFP$^+$ HRFΔ1 and RFP$^+$ control *P. berghei* sporozoites and sporozoites present in the parenchyma were counted after 3 h, when sporozoite motility has ceased. As shown in Fig. 3A and B, sporozoites of each type crossed the sinusoidal barrier with similar efficacy. We next tested whether HRF might help sporozoites penetrate blood capillaries in the skin and thus exit the skin. For this, sporozoites were injected IV or ID and parasite loads in the liver were compared by quantitative real-time polymerase chain reaction (qRT-PCR) at 40 hpi, when individual liver stages have initiated nuclear division (Fig. 3C). The HRFΔ1 parasite load was 3.7- and 3.3-fold smaller than that of the WT after IV and ID injections respectively (Fig. 3C). This indicated that the mutant is not more impaired compared with WT after ID than IV injection, i.e. that the mutant is not impaired in exiting the skin. Clones HRFΔ2 and HRFΔ3 gave similar results (Fig. 3D).

To analyse liver-stage development *in vivo*, mice were injected with sporozoites IV and liver samples collected at 40, 96 or 144 hpi were subjected to qRT-PCR analysis of parasite LISP1 RNA (Fig. 3E). At 40 hpi, the HRFΔ1 parasite load was ~ 2.5 times lower than that of the WT. At 96 hpi, WT parasites were undetectable, as expected from the release of merozoites from liver stages starting ~ 55 hpi, while the amounts of HRFΔ1 parasites had risen to levels similar to those of the WT at 40 hpi. At 144 hpi, both WT and HRFΔ1 parasites were undetectable in the liver. Therefore, RT-PCR analysis in the liver indicated a > 48 h delay in the completion of the pre-erythrocytic phase. In parallel, as expected, blood smears and FACS analysis detected WT parasites 2 days earlier than HRFΔ1 parasites (Fig. 3F).

Lack of HRF increases IL-6 production in the infected liver

Since HRF is important for liver-stage development specifically *in vivo*, we assessed the levels of various molecules of the host immune system during parasite liver infection (Fig. 4). Liver samples from naïve C57BL/6 mice and mice infected with WT or HRFΔ1 parasites were harvested at various hpi and host gene expression measured by RT-qPCR. Strikingly, at 40 hpi, the transcripts for IL-6 were the sole among those tested that were differentially expressed between infections with WT and HRFΔ1 parasites, being increased fivefold in HRFΔ1-infected liver samples. At 96 and 144 hpi, the levels of transcripts for MIP-1β, MIP-2, ICAM-1, TNF-α and IFN-γ were all higher

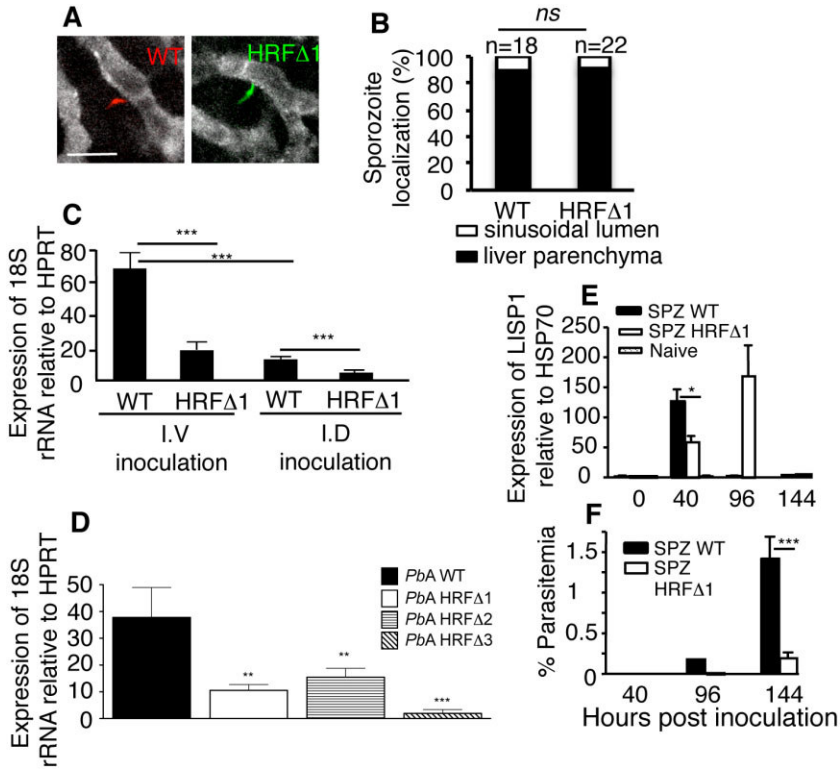

Fig. 3. Comparison of the liver infection by the WT and HRFΔ1 parasites.
A, B. 5×10^5 WT and HRFΔ1 sporozoites were injected together in the tail vein of C57BL/6 mice to compare the parasite ability to cross the sinusoidal barrier. WT and mutant parasites were imaged in the liver of the same infected mice at 3 h post-inoculation.
A. Representative intravital confocal images of WT (red) and HRFΔ1 sporozoites (green) that invaded the liver parenchyma at 3 h post-inoculation. The liver parenchyma (black) is delineated by the liver sinusoids (white), which are labelled by the IV injection of Alexa Fluor 647 BSA. Scale bar, 20 μm.
B. Percentage of sporozoites that invaded the liver parenchyma (black bar) or not (white bar) after 3 h post-inoculation. Similar numbers of parasites were detected in the imaged area ($n = 18$ WT sporozoites, $n = 22$ HRFΔ1 sporozoites).
C. Role of HRF in infectivity of pre-erythrocytic stages of the parasite. Forty hours after inoculation of C57BL/6J Rj mice IV or ID with 10 000 WT or HRFΔ1 sporozoites, livers were isolated and total RNA extracted. Parasite loads were determined using real-time RT-PCR by measuring *P. berghei* 18S rRNA. Gene mRNA expression was normalized to the endogenous control gene HPRT. Data are presented as the means ± SD ($n = 5$) from two independent experiments. ***$0.02 < P < 0.004$ (Kruskal–Wallis multiple comparison test).
D. Comparative development of independent HRF mutant clones. Analysis of the three mutant clones was performed as in E. Data are presented as the means ± SD from two independent experiments. For statistics, $n = 5$, **$P < 0.01$, *** $P < 0.001$ according to the Kruskal–Wallis test.
E, F. Groups of C57BL/6 mice were injected IV with 10 000 GFP-WT or GFP-HRFΔ1 sporozoites and livers and blood were collected at indicated time points. Parasite loads in the liver were assessed by measuring parasite LISP1 mRNA by real-time RT-PCR (E). Gene mRNA expression was normalized to the parasite control gene HSP70. Data are presented as the means ± SD from six individual values. *$P < 0.01$. In parallel, parasitaemia (F) was determined and means ± SD from six individuals values are shown. Significant differences were obtained using The Kruskal–Wallis multiple comparison test (*$P = 0.02$, ***$P < 0.002$). Shown are data from two distinct experiments (E, F).

in response to WT compared with HRFΔ1 parasites (Fig. 4), while the levels of IL-6 transcripts had reverted and reached higher levels in WT-infected livers. Transcripts for IL-4, IL-5 and IL-13 were not detectable both in WT and HRFΔ1 parasites. To verify specificity of the IL-6 increase in HRFΔ1-infected livers, we tested *P. berghei* ANKA parasites lacking the *p36* gene, previously shown to be rapidly blocked in development after hepatocyte invasion (van Dijk *et al.*, 2005; Ishino *et al.*, 2005; Labaied *et al.*, 2007). After IV injection of 10 000 p36Δ sporozoites into C57BL/6 mice, RT-PCR at 40 h post-infection confirmed the arrested development of the mutant in the liver (Fig. 4H). In addition to the abortive intrahepatocytic development, p36Δparasites did not up-regulate IL-6 (Fig. 4I), confirming that the latter was not a mere consequence of parasite developmental arrest in the liver but indeed the consequence of the absence of HRF.

To characterize the IL-6-producing cells regulated by HRF, C57BL/6 mice were inoculated IV with WT or HRFΔ1 sporozoites and, 40 h later, cells were harvested from the liver and characterized for IL-6 production. For this, liver cells gated on $CD45^+$ $IL\text{-}6^+$ cells were subsequently labelled for various leukocyte markers. As shown in Supporting Information Fig. S4A, a higher proportion of liver cells producing IL-6 upon infection with HRFΔ1 parasites compared with WT parasites were confined to $Ly6G^+$,

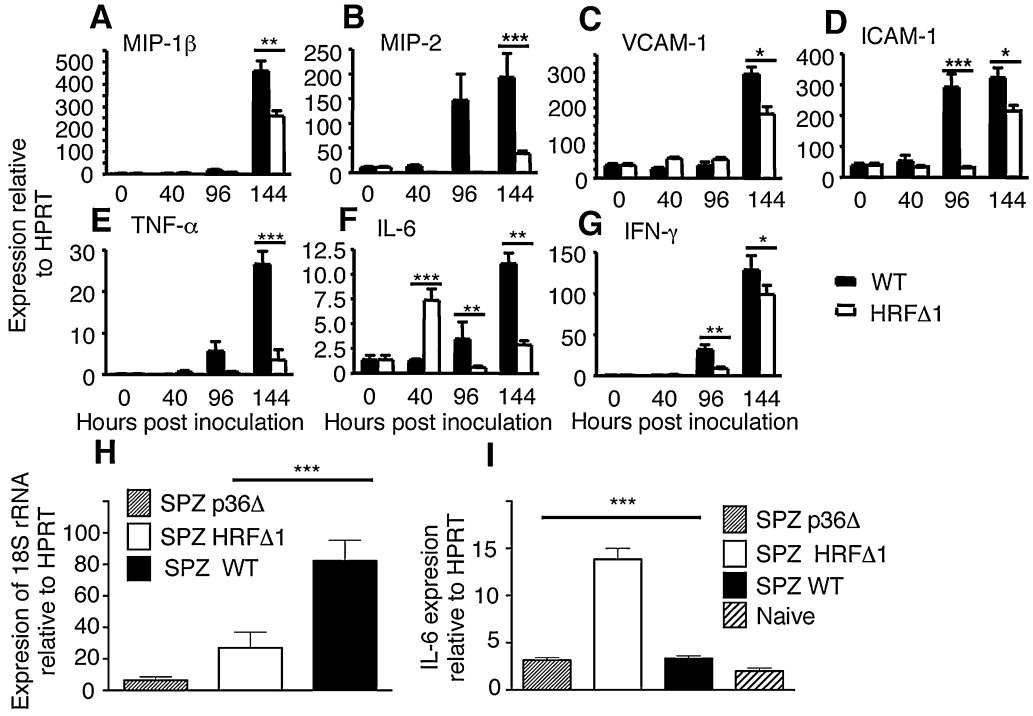

Fig. 4. Immune response genes are differentially regulated by WT and HRFΔ1 parasites. Transcription of the immune response genes including MIP-1β (A), MIP-2 (B), VCAM-1 (C), ICAM-1 (D), TNF-α (E), IL-6 (F) and IFN-γ (G) in the liver ($n = 6$ per group) at different time points post-infection as evaluated by real-time RT-PCR. mRNA expression was normalized relative to HPRT expression for each mouse strain. The asterisk indicates that differences are significant (Mann–Whitney test, $0.01 < P < 0.05$), double asterisk indicates that these values are significantly different (Mann–Whitney test, $0.005 < P < 0.01$) and triple asterisk indicates that differences are significant (Mann–Whitney test, $0.001 < P < 0.007$). (H) C57BL/6 mice (6/group) were inoculated with 10 000 WT, HRFΔ1 or p36Δ sporozoites. Forty hours later, livers were isolated and total RNA extracted. Parasite load was determined by using primers specific for the *P. berghei* 18S rRNA by real-time RT-PCR, which expression was normalized relative to HPRT expression. (I) IL-6 expression was determined in the same liver samples using real-time RT-PCR by measuring IL-6 mRNA. Triple asterisk indicates that differences are significant (Mann–Whitney test, $0.003 < P < 0.01$).

7/4$^+$, CD11b$^+$ and CD11c$^+$ cells, presumably neutrophils and inflammatory monocytes. At early time point of infection (40 h post-infection), gated Ly6G$^+$, 7/4$^+$ double-positive neutrophils (R2) were twice more frequent in mice infected with HRFΔ1 compared with WT parasites (Supporting Information Fig. S4B). Further characterization within this gate of neutrophils expressing IL-6 showed higher mean fluorescence intensity for HRFΔ1 parasites (1200) than those induced by WT parasites (800) (Supporting Information Fig. S4B). Other cells potentially expressing IL-6 including CD4, CD8, B-cells and macrophages were apparently not regulated by HRF (Supporting Information Fig. S4A). These data support the view that HRF modulation of IL-6 at early time points during liver infection may involve neutrophils.

HRF impact on host cell recruitment in the infected liver

We then analysed host cell recruitment in livers infected with HRFΔ1 or WT parasites (Fig. 5). Innate immunity-associated leukocytes such as neutrophils, eosinophils, monocytes, macrophages, NK cells and adaptive immunity-related leukocytes such as CD4$^+$, CD8$^+$ and B-cells were investigated. Leukocyte populations in the livers of C57BL/6 mice were collected from 40 hpi to 4 days post-injection (dpi) after IV injection of HRFΔ1 or WT sporozoites. At 40 hpi, 3.5- to 4-fold more CD19$^+$/B220$^+$ cells, likely B-cells, were found in mice infected with HRFΔ1 parasites and, conversely, twofold fewer NK1.1$^+$ cells than in mice infected with WT parasites. At 4 dpi, WT infection was associated with higher numbers of CD8$^+$, CD11b$^+$, Ly6-G$^+$ (neutrophils) and CD11c$^+$ CD11b$^+$ dendritic cells. Other leukocyte populations including CD4$^+$, eosinophils, basophils and macrophages were similarly affected during WT and HRFΔ1 infections.

Since the 40 h time point appears to be the critical point for events distinctly elicited by the HRFΔ1 parasite, namely increased B-cell number, we examined whether activation markers on leukocytes were modulated as well. To test whether distinct activation phenotypes could serve a cellular marker for HRFΔ1 infection in the liver, leukocytes were analysed for their level of CD62L expression, a key adhesion molecule that is expressed on the surface of most circulating leukocytes, including lymphocytes,

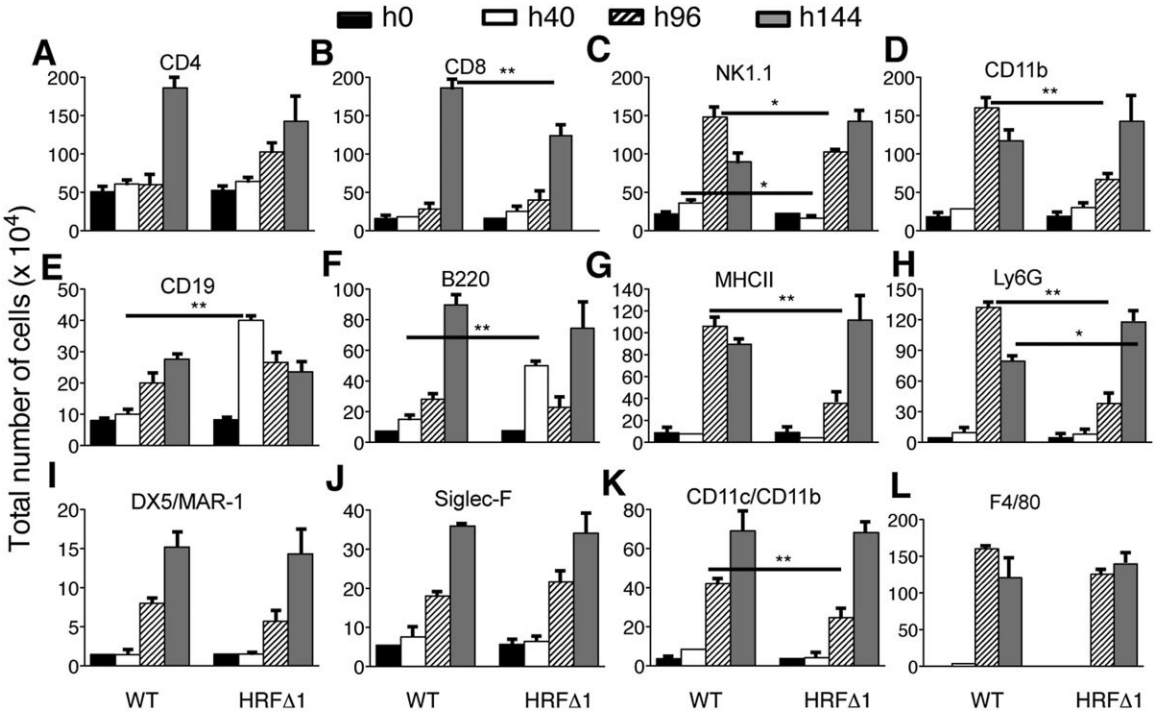

Fig. 5. Leukocyte patterns are differentially regulated by WT and HRFΔ1 parasites. At indicated time points post-infection, livers from C57BL/6 mice infected with 10 000 GFP-WT or GFP-HRFΔ1 sporozoites of *Pb* ANKA strain ($n = 6$ per group) were taken and leukocytes associated with liver tissue were analysed by using the following leukocyte markers: CD4 (A), CD8 (B), NK1.1 (C), CD11b (D), CD19 (E), B220 (F), MHCII (G), Ly6G (H), DX5/MAR-1 (I), Siglec-F (J), CD11c/CD11b (K) and F4/80 (L). Data are expressed as number of cells. The asterisk indicates that differences are significant (Mann–Whitney test, $0.018 < P < 0.05$), double asterisk indicates that these values are significantly different (Mann–Whitney test, $0.008 < P < 0.025$). Values represent the mean ± SD of two experiments.

neutrophils, monocytes, eosinophils and basophils and is down-regulated on activated leukocytes. As shown in Supporting Information Fig. S5, we determined the proportion of various subsets of leukocytes including B-cells ($B220^+$, $CD19^+$), $CD4^+$, $CD8^+$, NK cells and $Ly6G^+$ (neutrophils) that express low levels of CD62L among $CD45^+$ cells taken 40 h after infection from the liver of naïve mice or mice inoculated with WT or with HRFΔ1 parasites. Regardless of the parasite used, only B-cells ($B220^+$ $CD62L^{low}$ and $CD19^+$ $CD62L^{low}$) were found to be slightly activated compared with naïve mice. CD4, CD8, NK cells and neutrophils were not affected by any parasite. MHC class II expression used as an activation marker for dendritic cells ($CD11c^+$ MHC class II^{high}) was also found to be indistinctly up-regulated by both parasites. We infer from this analysis that the slightly increased proportion of activated B-cells and dendritic cells in the liver at early time points is independent from HRF, and that the only HRF-specific effect is the increase in the B-cell number.

To assess whether B-cells contributed to impaired infection by HRFΔ1 parasites, B-cell-deficient mice were inoculated IV with 10 000 WT or HRFΔ1 sporozoites, and the parasite load and IL-6 gene expression were examined at 40 hpi. Data showed, similarly to WT C57BL/6 mice, a ~ 3-fold reduced parasite load for the mutant parasite compared with the WT in B-cell-deficient mice (Supporting Information Fig. S6A). The IL-6 gene was also up-regulated in B-cell-deficient mice infected with HRFΔ1 sporozoites (Supporting Information Fig. S6B). Mortality rates caused by WT and HRFΔ1 sporozoites were similar in B-cell-deficient and WT mice (Supporting Information Fig. S6C), and the absence of CM induced by HRFΔ1 sporozoites was also observed in B-cell-deficient mice. These data suggested that B-cells were not involved in the HRFΔ1 parasite developmental block in the liver.

The mutant phenotype is rescued by decreasing IL-6 levels

To test whether the impaired HRFΔ1 liver-stage growth *in vivo* might be caused by increased IL-6, we attempted to rescue the phenotype of the mutant by decreasing IL-6 amounts. Mice received anti-IL-6 antibodies IV, were injected IV 24 h later with HRFΔ1 sporozoites, and parasite loads in the liver were measured at 40 hpi by qRT-PCR. Anti-IL-6 antibodies increased the HRFΔ1 load in the liver ~ 2.5-fold (Fig. 6A), thus largely rescuing the

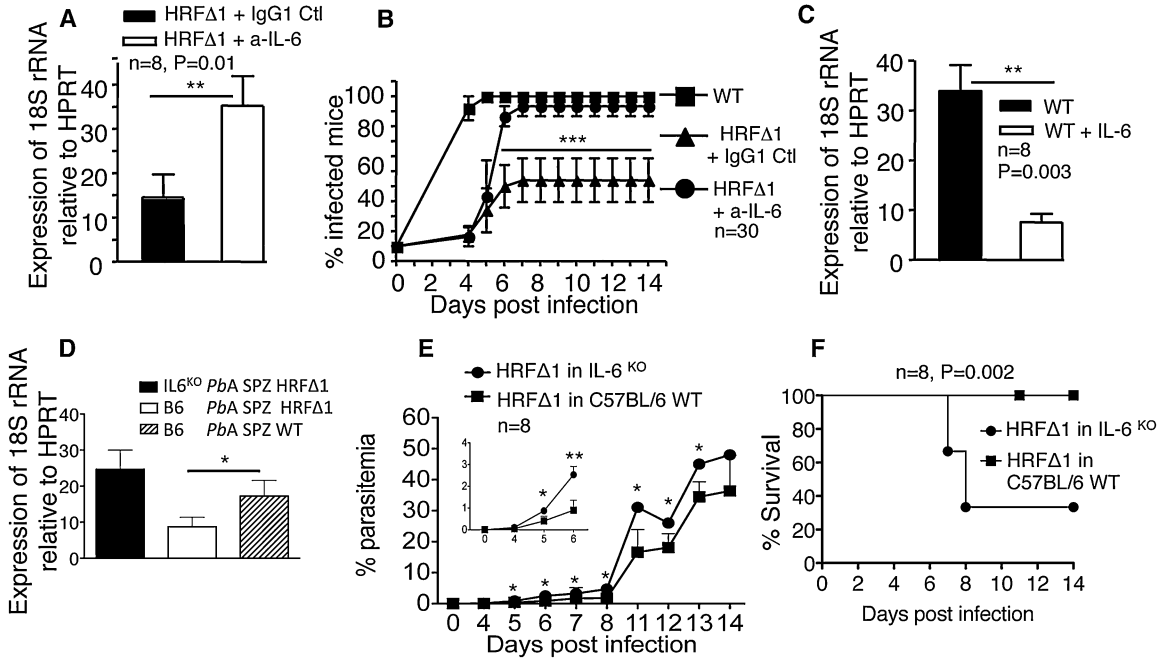

Fig. 6. HRFΔ1-mediated regulation of IL-6 expression in the liver is critical for parasite development. C57BL/6 mice were infected with 10 000 WT GFP-HRFΔ1 sporozoites in the presence of rat anti-mouse IL-6 mAbs or normal rat IgG1 as control (A) or with GFP-WT sporozoites treated or not with 5 μg of rHuIL-6 (C), and 40 h later parasite loads in the liver were assessed by measuring parasite 18S rRNA by real-time RT-PCR, which expression was normalized relative to HPRT expression. Data are presented as the means ± SD from one representative experiment out of two ($n = 8$). Double asterisk indicates that differences are significant (Mann–Whitney test: $0.01 < P < 0.03$). (B) Mice were treated as in A, but were followed for parasitaemia and the proportion of mice developing blood-stage parasites were determined (Mann–Whitney test; $^{***}P = 0.006$). (D) C57BL/6 or IL-6 ^{KO} mice were inoculated IV with 10 000 GFP HRFΔ1 sporozoites. As control, C57BL/6 mice were inoculated with 10 000 WT sporozoites. Forty hours later, livers were isolated and total RNA extracted. Parasite load was determined by using primers specific for the *P. berghei* 18S rRNA. mRNA expression measured by real-time PCR was normalized to the endogenous control gene HPRT. Data are presented as the means ± SD from two independent experiments ($n = 5$, *Mann–Whitney test, $P = 0.028$). (E) C57BL/6 or IL-6 ^{KO} mice were infected IV with 10 000 GFP HRFΔ1 sporozoites and parasitaemia (Mann–Whitney test; $^{*}P = 0.032$, $^{**}P = 0.009$) and (F) survival rates (log-rank test) were followed over time.

defective phenotype. Moreover, while only 40% of the mice infected with 10^4 HRFΔ1 sporozoites became blood-stage patent (Fig. 6B, triangles), all mice infected similarly and pretreated with anti-IL-6 antibodies developed blood-stage parasites (Fig. 6B, closed circles). Conversely, when WT sporozoites were injected IV into mice immediately before human recombinant IL-6 (5 μg per mouse), WT parasite loads in the liver decreased ~ 4-fold at 40 hpi (Fig. 6C). As controls and to see whether IL-6 treatment affects liver parasite load in HRFΔ1-infected mice, mice infected with HRFΔ1 plus/minus IL-6 displayed the same liver parasite load (Supporting Information Fig. S7A). To assess whether treatment with exogenous IL-6 would affect endogenous liver IL-6 mRNA expression, HRFΔ1-infected mice displayed similar IL-6 mRNA expression in IL-6-treated and untreated mice (Supporting Information Fig. S7B).

Next, experiments were conducted in IL-6-knockout (IL-6^{ko}) C57BL/6 mice. First, sporozoites were injected IV into mice and parasite loads in the liver assessed by RT-PCR. While the liver load of HRFΔ1 parasites was lower than that of the WT in WT C57BL/6 mice, the load of HRFΔ1 parasites in IL-6^{ko} mice was higher than in WT mice and similar to that of WT parasites in WT mice (Fig. 6D). Second, pre-patent periods of liver-stage development of HRFΔ1 parasites were assessed in IL-6^{ko} and WT mice. Figure 6E shows that HRFΔ1 parasites developed at a higher rate in IL-6-deficient mice than in WT recipients, reverting to a fully complemented phenotype. The control experiment which consists of inoculation of 10 000 SPZ of WT or HRFΔ1 *Pb*ANKA parasites in IL-6^{KO} mice shows that the phenotype of the mutant parasite in IL-6^{KO} mice is not completely rescued; however, the delay in the patent period is reduced as compared with the WT parasite (1 day instead of 2 to 3 days in WT mice, day of patency 3, and 4 for WT and HRFΔ1-infected IL-6^{KO} mice, respectively; Supporting Information Fig. S7C) but the mortality caused by HRFΔ1 parasites is re-established as 5 mice died out of 7 as compared with no death in WT mice (shown in Fig. 6F). Also the death rate is not significantly different than those IL-6^{KO} mice infected with *Pb*ANKA WT parasites (6 mice died out of 7; Supporting Information Fig. S7C).

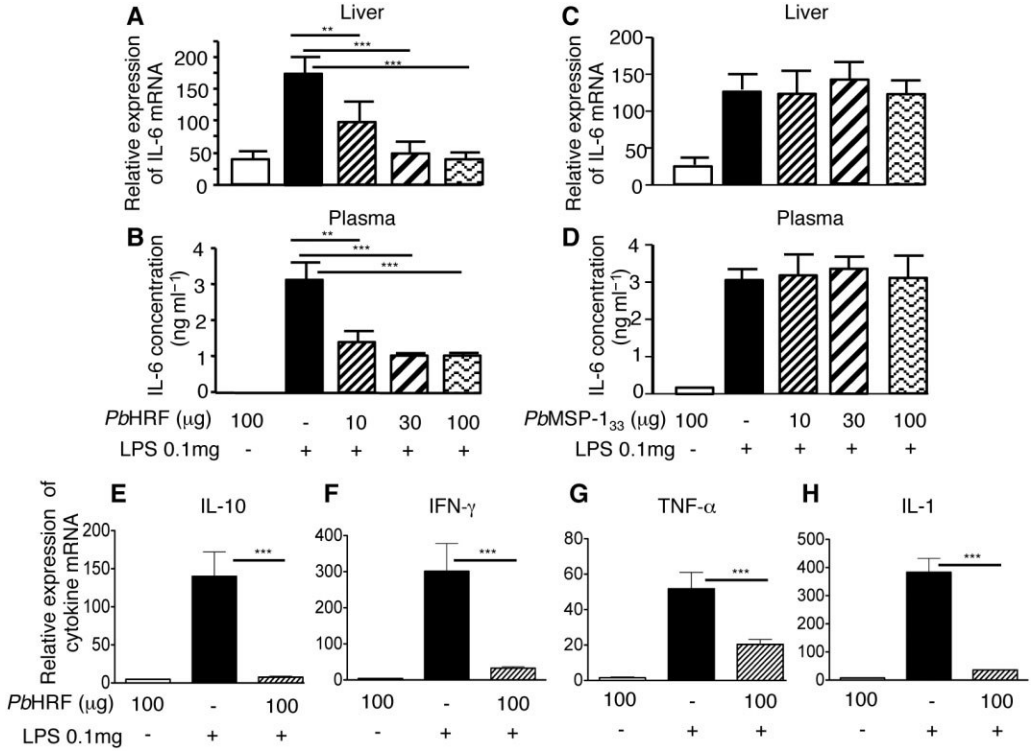

Fig. 7. Capacity of the recombinant HRF to down-modulate LPS-induced IL6- and other cytokine-mRNA expression and protein synthesis. C57BL/6 mice were injected i.v with the indicated doses of recombinant *Pb* ANKA HRF (A and B) or with recombinant *Pb*MSP1$_{33}$ (panels C and D) 15 min prior to the administration of 0.1 mg of LPS. Two hours later, livers were harvested and processed for IL-6 mRNA transcript analysis by RT-PCR (A, C) and in parallel plasma were tested for IL-6 content by ELISA (B, D). As control, HRF was injected alone at 0.1 mg per mouse (far left bar). In the same experimental setting, mRNA transcripts for IL-6, IL-10, IFN-γ, TNF-α and IL-1 (E-H) were measured in mice injected with LPS alone, HRF alone, or in combination. One representative analysis out of 2 is shown and the means ± SD of values obtained in three mice. For statistics in all graphs, $^{**}P < 0.05 < 0.01$, $^{***}P < 0.001$ according to the Kruskal–Wallis test.

Last, we compared the ability of sporozoites to induce CM in WT and IL-6^{ko} mice. HRFΔ1 sporozoites injected IV did not induce CM in WT mice, whereas 70% of IL-6^{ko} mice developed CM (Fig. 6F), similar to the CM frequency caused by WT parasites in WT mice infected via infectious mosquito bites (Fig. 1D). Similar results were obtained with HRFΔ2 and HRFΔ3 parasites. Together, these data indicate that the phenotype caused by lack of HRF in the parasite is mainly, if not only, due to IL-6 overexpression in the host.

HRF is sufficient to decrease IL-6 production

We then tested whether HRF directly affected the production of IL-6 in the liver and systemically in the mouse. For this, recombinant *P. berghei* HRF (*Pb*HRF) was expressed in pRSETA plasmid, with an N-terminal his tag and purified/collected from *Escherichia coli* BL21 strain. Mice were injected IV with various doses of recombinant *Pb*HRF prior to the administration of 0.1 mg of Lipopolysacharaide (LPS), a known IL-6 inducer, and 2 h later IL-6 mRNA expression and plasma levels of IL-6 were determined by RT-qPCR and ELISA respectively. IL-6 expression was down-regulated both at the mRNA (Fig. 7A) and protein levels (Fig. 7B) by HRF, in a dose-dependent manner. As a control, we tested the ability of another parasite protein, merozoite surface protein 1 fragment 33 (*Pb*MSP1$_{33}$), to modulate IL-6 production in the same conditions. MSP1$_{33}$ had no inhibitory effect on the levels of LPS-induced IL-6 mRNA (Fig. 7C) or IL-6 protein (Fig. 7D). Therefore, the differential regulation of IL-6 in the liver in response to infections with WT and HRFΔ parasites can be directly ascribed to HRF itself. The mRNA transcripts for IL-10, IFN-γ, TNF-α and IL-1 (Fig. 7E–H) were also inhibited by the co-injection of HRF, while those for IL-4, IL-5 and IL-13 remained undetectable in mice treated with LPS alone or LPS combined with HRF.

HRF is detected in the extracellular environment in the liver and in the blood

The phenotype of the mutant predicted that HRF would be secreted by the liver stage of the parasite. To test this, the supernatant of 24 h cultures of HepG2 cells harbouring WT parasites was collected and analysed by immunoblot

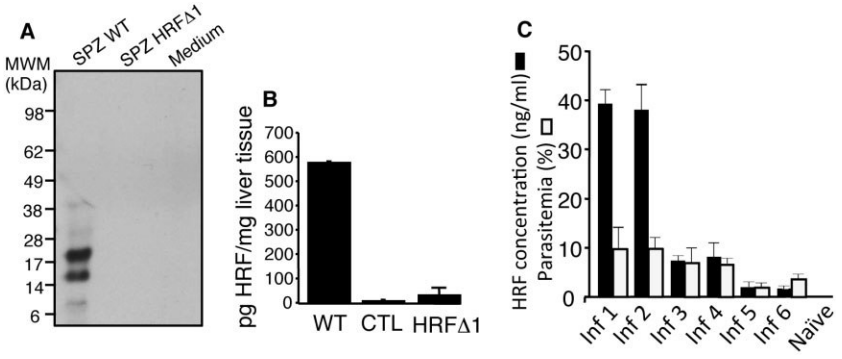

Fig. 8. Detection and quantification of HRF protein in various biological fluids.
A. Supernatants from HepG2 cells cultured in the presence of WT, HRFΔ1 sporozoites, or in the absence of parasite were immunolabelled with anti-HRF antibody.
B. Presence of HRF was measured in the liver extract of mice infected with 10^5 WT sporozoite at 40 h post-infection by using ELISA.
C. The level of HRF in plasma of infected mice with WT and HRFΔ1 (not shown) was measured along with parasitaemia. One representative experiment among three are shown.

using anti-HRF antibody (Fig. 8A). The presence of HRF (22 000 Da molecular weight) was detected, as well as other bands at around 15 000 and 7000 molecular weights, likely representing fragments from the native protein. No signal was detected in supernatants from HepG2 cells containing HRFΔ1 parasites or uninfected HepG2 cells (Fig. 8A). To confirm that HRF was produced by liver-stage parasites *in vivo*, liver extracts obtained 40 h post-infection with WT sporozoites were tested for the presence of HRF. As shown in Fig. 8B, HRF was detected in liver extracts. To assess whether HRF is present in the plasma of infected mice during the blood phase of infection, we measured HRF in the plasma of mice with various levels of parasitaemia. In order to correlate the level of parasitaemia with the amount of circulating HRF, selected sera from two mice with 10% parasitaemia (day 7 post-infection), two mice with 7% parasitaemia (day 5 post-infection) and two mice with 3% parasitaemia (day 3 post-infection) were examined for their content of HRF using a quantitative ELISA assay. As shown in Fig. 8C, HRF was present in the plasma and appeared to correlate with the parasite load in the blood. HRF was not detected in the plasma of mice infected with HRFΔ1 parasites.

Discussion

This work shows that, in rodents, plasmodial HRF is involved in the pre-erythrocytic phase of malarial infection. Given the high (97%) similarity between *P. falciparum* and *P. berghei* HRF, it is likely that the same applies in humans. Histamine-releasing factor was not involved in the erythrocytic phase of infection in rodents, including in the development of CM. This, however, does not exclude a role for HRF in severe *P. falciparum* malaria in humans. It was striking to observe that deletion of *hrf/tctp* gene was not lethal for *Plasmodium* parasites in contrast to the loss of *hrf* expression in mice which results in increased spontaneous apoptosis during embryogenesis (Susini *et al*., 2008). Therefore, in mice, HRF is regarded as both a key intracellular anti-apoptotic regulator beside other functions and as a secreted protein exerting extracellular biological functions. A possible explanation for the lack of lethality of HRFΔ mutants we obtained in our laboratory is that *Plasmodium* parasites have preferentially selected extracellular functions rather than essential intracellular functions of HRF for a better fitness within infected hosts.

The HRF mutant was specifically inhibited during liver-stage development *in vivo*. *In vitro*, mutant development was normal in cultured hepatocytes and, *in vivo*, the mutant was not significantly impaired in leaving the skin and reaching the blood, or in leaving the blood and reaching hepatocytes. In the liver, mutant development was slowed though not blocked, as shown by qRT-PCR analysis of parasite amounts over time. However, the impaired development of HRF mutant liver stages *in vivo* is likely to induce some parasite death, as suggested by intravital imaging studies. This resulted in a 10^2- to 10^3-fold reduction in the numbers of merozoites initially released in the blood, and an extension of the time between sporozoite injection and a threshold of parasitaemia. This prolonged liver-stage development influenced the clinical outcome of a parasite infection by decreasing the frequency of CM. A link between expression of ECM in susceptible C57BL/6 mice and a lengthy development period of parasite in the liver was already suggested by other *P. berghei* parasites deficient in apicoplast protein important for liver merozoite formation (PALM) (*palm*$^-$), which displayed a strong impairment in liver merozoite formation with a severe delay in blood-stage infection and a reduced incidence of ECM (Haussig *et al*., 2011). Our data strengthen the wider concept that the efficiency of pre-erythrocytic infection controls CM occurrence during erythrocytic infection.

Like other pathogens, *Plasmodium* must fine-tune activation/inhibition of host immune effector cells to ensure host survival and parasite dissemination. However, few examples exist in the literature describing particular pathogen genes that manipulate specific host immune response pathways, in particular *Plasmodium*-associated genes regulating specific host cytokine

responses. Three lines of evidence indicate that the mutant defect is mainly caused by increased IL-6 in the infected host. (i) IL-6 increase in the mutant-infected liver was the earliest detectable difference with WT among the tested factors, while the earliest cellular difference was an increase in B-cell counts in the mutant, in line with the B-cell-activating activity of IL-6. (ii) The mutant defect was complemented by decreasing IL-6 levels in the host, using injection of anti-IL6 antibodies in WT mice or infection in IL-6^{ko} mice. (iii) Recombinant HRF was sufficient to inhibit LPS-induced IL-6 production in the liver and release in the circulation. Therefore, although HRF might still have other targets than IL-6, the defective phenotype of the mutant appears to be largely caused by unrepressed IL-6.

How the parasite controls IL-6 expression via HRF remains unclear. During the initial phase of the liver development, the main target of IL-6-producing cells appears to be neutrophils, in which IL-6 production was enhanced by the HRFΔ1 parasite. Consistent with this finding, IL-6 was previously found in murine neutrophils as a pre-stored protein, which makes it readily available upon stimulation (Terebuh *et al*., 1992). Evidence that HRF is able to directly prevent or inhibit IL-6 production was provided by the direct inhibitory effet of HRF on LPS-induced cytokine production. At the same time, B-cells increase in number, perhaps as a result of increased Il-6 production. The interplay between neutrophils and B-cells via IL-6 may represent the mechanism by which the delayed development of the mutant parasite occurs. Such an interplay between neutrophils and B-cells has been recently elegantly demonstrated by the discovery of a B-helper neutrophil population whereby splenic neutrophils can function as professional helper cells for marginal zone B-cells, leading to the generation of affinity-matured antibodies (Puga *et al*., 2012). However, using B-cell-deficient C57BL/6 *mu*MT mice, homozygous for the *Ighm*^{tm1Cgn} targeted mutation, the phenotype of the mutant was similar in B-cell-deficient and WT mice. This suggests that B-cells are not involved in protection during infection with HRFΔ1 parasites.

Both the sporozoite and the intracellular liver stage produce HRF (Fig. 2). The extracellular sporozoite might secrete HRF during gliding motility and/or the liver stage might export HRF through the PVM and out of the infected cell. Here, we showed that HRF from *Plasmodium berghei* was secreted by infected HepG2 cells in the culture supernatant and *in vivo* in the liver of infected mice (Fig. 8). Histamine-releasing factor secretion by liver stages might occur via exosomes. In the human embryonic kidney 293T cells, HRF was found secreted via a non-classical route and exosomes (Amzallag *et al*., 2004). Immunofluorescence and immunoelectron microscopy studies on *P. falciparum* show that some of the malarial HRF is present in the food vacuole membranes, where it is in proximity to the heme-rich food vacuole (Bhisutthibhan *et al*., 1999).

Whether mammalian and malarial HRF, despite their high (54%) similarity, have similar targets and modes of action remains uncertain. Mammalian HRF was reported to act on basophils, eosinophils and B-cells (Macdonald, 2012). Interestingly, HRF was reported to increase IL-6 production from B-cells. Examination of immune cells at early time point, 40 h post-infection, indicated a unique association between the absence of HRF expression and an increased accumulation of B-cells. One of the characteristic features in malaria infection is the induction of short-lived B-cell memory. It has been shown that malaria parasites interfere with the immune response by disabling B-cell development, possibly undergoing apoptosis (Wykes *et al*., 2005). In light of these findings, increased B-cell population in the liver of mice infected with mutant parasites reflects the possibility that the HRF molecule could interfere with B-cell memory. In support of this observation, early rise in IL-6 induction, a B-cell-activating and anti-apoptotic cytokine, at 40 h post-infection was induced by mutant parasites. *Plasmodium falciparum* HRF stimulates histamine release from basophils and IL-8 secretion from eosinophils *in vitro* (Bheekha-Escura *et al*., 2000; MacDonald *et al*., 2001). We showed here that *P. berghei* HRF is sufficient to decrease IL-6 production in the liver. Numerous cell types produce IL-6 in the liver, including T-cells, macrophages, endothelial cells and hepatocytes themselves, where it eventually exerts its anti-parasite effect. Clearly, HRF does not affect the levels of IL-6 only, as co-injection of HRF with LPS down-regulates other pro-inflammatory cytokines like IFN-γ, TNF-α and IL-1. More work is needed to understand the mode of action of malarial HRF.

Few molecules of malaria parasites have been shown to counteract host innate immunity. In early liver stages, the major sporozoite surface protein called circumsporozoite protein translocates into the hepatocyte cytosol and nucleus, where it outcompetes NF-kB nuclear import and suppresses hundreds of genes involved in the host inflammatory response. Another parasite product, the migration inhibitory factor (MIF) orthologue of mammalian MIF, was shown to increase inflammatory cytokine production during the blood phase of infection in rodents and to induce antigen-experienced CD4 T-cells to develop into short-lived effector cells rather than into memory cells, causing decreased CD4 T-cell recall responses to homologous parasites (Sun *et al*., 2012). However, parasites lacking MIF were shown to have no growth defect throughout the parasite life cycle in *P. berghei* (Augustijn *et al*., 2007) or a growth defect during liver-stage development in *P. yoelii* (Miller *et al*., 2012) although in this latter case, the causes of MIF-deficient liver-stage attenuation were not investigated.

In conclusion, *Plasmodium* HRF is the first secreted parasite molecule that is found to down-regulate host cytokines, in particular IL-6, which inhibits the development of the *Plasmodium* liver stage. The importance of the contribution of HRF to *Plasmodium* liver-stage development makes it an attractive target for intervention strategies, given its secreted nature and diffuse action a target of antibody-mediated immunity.

Experimental procedures

Ethics statement

All animal care and experiments described in the present study involving mice were conducted at the Institut Pasteur, approved by the 'Direction Départementale des Services Vétérinaires' de Paris, France (Permit Number N° 75-066 issued on September 14, 2009) and performed in compliance with institutional guidelines and European regulations (http://ec.europa.eu/environment/chemical?s/lab_animals/home_en.htm). A statement of compliance with the French Government's ethical and animal experiment regulations was issued by the Ministère de l'Enseignement Supérieur et de la Recherche under the number 00218.01.

Mice

Seven- to eight-week-old female C57BL/6J Rj mice were purchased from Charles River Laboratories. The IL-6^{KO} mice have been backcrossed 10 times on C57BL/6 mice from the Jackson Laboratory. To make sure the background of IL-6 ^{KO} mice does not alter at least the course of infection and disease in our ECM model, comparable survival rates and parasitaemia are shown between IL-6 ^{KO} mice and C57BL/6J Rj used as WT control in this study. C57BL/6 *mu*MT mice, homozygous for the *Ighm*^{tm1Cgn} targeted mutation and lacking mature B-cells were obtained from The Jackson Laboratory, Bar Harbor, ME, USA.

Parasites

Mice were inoculated with red bloods cells (RBCs) infected with either GFP-transgenic *P. berghei* ANKA (MRA-867) or HRFΔ1 *Pb*ANKA parasites. In a few control experiments, HRFΔ2 and HRFΔ3 *Pb*ANKA parasites were used. The *P. berghei* deficient for the *Pb*s36 gene (van Dijk *et al*., 2005; Ishino *et al*., 2005; Labaied *et al*., 2007), the disruption of which was previously found to nearly abolish sporozoite infectivity in the mammalian liver host was kindly provided by Dr. T. Ishino (Department of Medical Zoology, Mie University School of Medicine, Edobashi, Tsu, Japan).

Murine model of ECM

Six- to eight-week-old C57BL/6 mice were inoculated with 10^5 RBCs infected with *Pb*ANKA or their knockout counterparts. Parasites, before every experiment, were reactivated by previous passage in C57BL/6 mice. Parasitaemia was determined by flow cytometry and the results expressed in percentage of pRBCs. C57BL/6 mice infected with *Pb*ANKA or HRFΔ *Pb*ANKA were monitored for clinical symptoms of ECM: hemi- or paraplegia, deviation of the head, tendency to roll over on stimulation, ataxia and convulsions.

Quantification of parasite loads in the liver

C57BL/6J Rj mice were injected IV or ID with 10 000 WT or HRFΔ1 sporozoites. Forty hours later, livers were isolated and total RNA extracted. Parasite load was determined by using primers specific for the *P. berghei* 18S rRNA (forward: 5′-AAGCATTAAATAAAGCGAATACATCCTTAC-3′ and reverse: 5′-GGAGATTGGTTTTGACGTTTATGTG-3′). Gene mRNA expression measured by real-time RT-PCR is normalized to the endogenous control gene HPRT.

For kinetic studies spanning 6 days after sporozoite inoculation, groups of C57BL/6J Rj mice were injected IV with 10 000 GFP-WT or GFP-HRFΔ1 sporozoites and livers were collected at indicated time points. To avoid possible contamination of liver stages by blood-stage parasites, we decided to use LISP1 as a specific marker for late stage liver merozoites. Parasite loads in the liver were assessed by measuring parasite LISP1 mRNA by real-time RT-PCR. Gene mRNA expression was normalized to the parasite control gene HSP70.

Detection and quantification of HRF

A plasmid containing the DNA encoding for the *Pb*NK65 HRF was obtained from Kim Taylor (RMIT University, Australia). *Pb*HRF plasmid was in pRSET A (Life Technologies Paisley, United Kingdom), with an N-terminal his tag and *E. coli* BL21 was used for expression. Bacteria grew in a 10 ml overnight preculture in LB medium with 100 μg ml^{-1} ampicillin, and we then inoculated 500 μl of LB with 1–2% of the preculture. The culture was kept at 37°C for ~ 3 h until the OD at 600 nm was ~ 0.5. The expression was then induced for 3 h with 1 mM IPTG. We used freeze/thaw cycles and lysozyme to lyse the bacteria, followed by filtration through a 0.2 μm syringe filter. Purification was performed by using the Profinity resin from Bio-Rad (Marnes-la-Coquette, France). Endotoxin was removed by passing HRF solution on Detoxi-Gel Endotoxin Removing Gel column (Thermo Fisher Scientific, Brebières, France). The recombinant protein (100 μg) was injected intradermally into the back skin of a rabbit in the presence of complete Freund's adjuvant followed by two booster injections in the presence of incomplete Freund's adjuvant. Rabbit IgG antibodies were isolated using a protein A column as an immunoabsorbent. For intracellular detection of HRF, isolated sporozoites were washed in phosphate buffered saline (PBS) buffer and resuspended in fixation/permeabilization solution (BD Pharmingen) in the presence of anti-HRF rabbit antibodies for 20 min at 4°C. Fixed/permeabilized sporozoites were resuspended in Perm/Wash buffer and incubated with Allophycocyanin (APC)-anti-rabbit antibodies before flow cytometric analysis. A total of 4×10^5 living cells were analysed using a four-colour FACSCalibur flow cytometer with ProCellQuest software (BD Biosciences, Mountain View, CA, USA). *In vivo* production of HRF by hepatic merozoites was demonstrated by IV inoculation of 100 000 sporozoite to C57BL/6 mice, and 40 h post-infection liver extracts were made. Quantitative determination of HRF in the

liver and in the plasma was performed by using quantitative ELISA sandwich using anti-HRF rabbit antibody as capture and a range of concentrations of recombinant HRF for the titration curve and the same rabbit antibody labelled with horse radish peroxidase for detection. Liver extract from naïve mice was used as negative control.

Western blot

Supernatant samples (14 μl) of HepG2 cells cultured in the presence of sporozoites were loaded onto 4–12% pre-cast gel gradients (Bolt Bis-Tris Plus; Life Technologies, Carlsbad, CA, USA). Gels were placed in the Novex Bolt Mini Gel system (Life Technologies). At the completion of SDS-PAGE, the gel was washed in distilled water and assembled with the iBlot Gel Transfer Stacks (Life Technologies). At the completion of the Western transfer, the membrane was removed from the sandwich and was incubated in blocking solution for 1 h, shaking (5% milk solution in PBS tween 0.01%), then incubated with the rabbit anti-HRF antibody in diluent solution overnight shaking at 4°C.

The membrane was washed three times with PBS tween 0.01% shaking for 10 min, and then incubated in biotinylated-goat anti-rabbit antibody (Life Technologies) for 1 h, shaking at room temperature. The membrane was washed three times in PBS tween for 10 min shaking, and then was incubated with HRPO-streptavidine for 1 h at room temperature. After several washings, the membrane was incubated in a solution of Supersignal West Pico Chemiluminescent substrate (Thermo Scientific, Rockford, IL, USA) in the dark. The blot was recorded either by scanning the image or by photography.

Sporozoite development in HepG2 cells and intracellular detection of HRF

HepG2 cells ($3–4 \times 10^4$ per well) were plated in eight-well chamber slides (Lab-Tek® Chamber Slide™) and cultured overnight in DMEM + GlutaMAX-I media (Gibco) supplemented with 10% heat inactivated FBS (Fetal Bovine Serum, Gibco) at 37°C in the presence of 5% CO_2. Wild type and/or mutant purified *P. berghei* salivary gland sporozoites were used for HepG2 infection at a ratio of 1:1 (parasite/cells) for 48 h, at 37°C, 5% CO_2 in the presence of PSN (penicillin-streptomycin-neomycin solution, Sigma). The *Pb*HRF was detected by immunofluorescence staining as described above.

HepG2 cells fixation and permeabilization was done with 4% paraformaldehyde + 0.1% triton or acetone 1:1 mix, and blocked with 1% gelatin from porcine skin (Sigma) for 30 min at RT. Thereafter, cells were incubated with anti-HRF rabbit antibodies (diluted 1:500) at 4°C overnight, and then incubated with Alexa Fluor® 568 donkey anti-rabbit IgG (Life Technologies; diluted 1:500) and 0.02 mg/ml 4′, 6-diamidino-2-phenylindole (DAPI) for nuclear staining for 30 min at RT. The presence of *Pb*HRF was detected under the fluorescence microscope. Similar IFA was performed to detect HRF in sporozoites.

Flow cytometric analysis of liver leukocytes

Liver cells obtained after various periods of time after infection were stained for FACS analysis according to standard protocols in cold PBS containing 2% Fetal calf serum (FCS) and 0.01% sodium azide (FACS buffer) with the following Abs: APC-labelled CD4, phycoerythrin (PE)-labelled anti-CD8, Fluorescein isothiocyanate (FITC)-labelled anti-F4/80 antibody, and PE-labelled ant-Ly6G antibody, Alexa Fluor 647-labelled anti-B220, APC-anti-NK1.1 antibodies. Red blood cells were eliminated using cell lysis buffer, and cells were washed in FACS buffer. A total of 4×10^5 living cells were analysed using a four-colour FACSCalibur flow cytometer with ProCellQuest software (BD Biosciences, Mountain View, California).

Cytokine and chemokine quantification in the liver

Cytokine and chemokine expression in the liver taken at different periods of time after infection was analysed by the real-time RT-qPCR. RNA utilized for these assays was isolated by means of a two-step extraction process. First, livers were surgically removed from mice as previously described and placed immediately in RNAlater at 4°C overnight. After RNAlater infused the samples, it was removed and samples were maintained at −80°C until processing. Livers were thawed in 1 ml of Trizol and subjected to bead disruption in a polytron three times from 2 min at a setting of 30 cycles per second. Samples were spun at high speed (10 000× g) for 3 min to remove debris and lipids. Half of the sample was transferred to a new tube and mixed with 500 μl of Trizol reagent by vortexing. Following this step, RNA extraction proceeded according to manufacturer's protocol. Precipitated RNA was resuspended in 100 μl of RNase-free water. The second step of this extraction was followed by Qiagen's protocol for RNA clean-up including steps for removal of protein and DNA (Qiagen RNeasy Kit). Samples were eluted with 50 μl of RNase-free water and quality and quantity assured by photospectroscopy. Real-time RT-qPCR utilized various primer-probe sets and standard Taqman protocols (Applied Biosystems).

Assessment of cell traversal

To determine the ability of sporozoites to traverse host cells, a standard cell traversal assay was used. GFP-labelled WT and HRFΔ1 sporozoites, isolated from *Anopheles stephensi* mosquito salivary glands, were incubated with human HepG2 cells, grown in 24-well plates in the presence of rhodamine dextran. After 3 h, the cells were washed with PBS/1% FCS, fixed and analysed by FACS using a BD Bioscience FACSCalibur flow cytometer. Percentage of traversed HepG2 cells was labelled with rhodamine dextran.

Intravital microscopy and development of EEF

Freshly isolated WT or HRFΔ1 sporozoites were resuspended in Ringer's solution containing 1% FBS, and allowed to glide on glass-bottom dishes (MatTek Corp., Ashland, MA, USA) precoated with 2% FCS. Video microscopy was conducted using a Zeiss Axiovert equipped with phase-contrast and epifluorescence microscopy. Time-lapse images and videos were collected under low-light illumination with a Hamamatsu ORCA ER camera (Hamamatsu Photonics KK, Hamamatsu, Japan). Videos were recorded digitally at approximately eight frames per second. Colours represent different frames from a time-lapse movie.

Development of EEF of HRFΔ1 as compared with WT *Pb* ANKA sporozoites was assessed by plating HepG2 cells (10^5 in

1 ml) in 8-well Labtek chamber slides for 24 or 48 h, respectively, in the presence of 2.5×10^4 genetically GFP tagged WT or HRFΔ1 sporozoites. Cells were examined with a fluorescence microscope. Exo-erythrocytic forms in each well were counted and expressed as EEFs $\pm$ SD of duplicate wells. Exo-erythrocytic forms were counted randomly and expressed as number of EEF per field.

In vivo *treatment with IL-6 and anti-IL-6 antibodies*

C57BL/6 mice were injected IV with 5 μg of human recombinant IL-6 (kindly provided by Barbara Willi, Novartis Pharma AG, Basel, Switzerland) in 200 μl of sterile PBS 15 min before sporozoite inoculation. To neutralize endogenous IL-6, recipient mice were injected IV with 1 mg per mouse of anti–IL-6 (rat anti-mIL-6 IgG1 MP5-32C11 kindly provided by Dr Paola Minoprio, Institut Pasteur, Paris), or with 1 mg per mouse of isotype control (rat IgG1), 1 day prior to sporozoite inoculation. The rat anti-mouse IL-6 neutralizing mAb is a very potent and specific antagonist of mouse IL-6 bioactivity.

Determination of IL-6 in the plasma of infected mice was performed using ELISA kit assays according to the manufacturer's instructions (BD Biosciences, San Diego, CA, USA).

Statistical analysis

Significant differences in survival were evaluated by generation of Kaplan–Meier plots and log-rank analysis. $P < 0.05$ was considered statistically significant. For parasitaemia, after verification using a Jarque–Bera test, our data did not follow normal distribution, and thus, when differences between groups of mice were to be compared at a given time point, the Mann–Whitney test was performed with significance set at $P < 0.05$.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Disruption of the *pbHRF* gene. Schematic representation of the strategy utilized to knock out the *Pb*HRF gene via double-crossover homologous recombination. Red lines represent regions of homology for recombination. Integration of the targeting sequence disrupt the HRF-coding sequence and replace it by drug resistance (*h*DHFR) marker. Specific PCR primers indicated genomic integration of the *h*DHFR targeting vector (within the *hrf*/*tctp* locus) within the *Pb*ANKA clone. A, B and C primers were used for PCR analysis. The amplicons are diagnostic for lane 1, wild-type locus; lane 2, integrated plasmid; lane 3, H_2O. (A) Band sizes corresponding to 2681 bp in lane 2 indicated the integration of the plasmid (*h*DHFR cassette (1600 bp) and 5′ (487 bp) and 3′ (594 bp) *Pb*HRF UTRs). A PCR product of 1760 bp in lane 1 indicated wild-type locus. (B) In lane 2 a PCR product of 700 bp indicated the HRFΔ1 mutant. (C) PCR product of 1100 bp indicated the HRFΔ1 mutant. (D) Southern blot analysis of the *Pb*WT and *Pb*HRFΔ1 mutant locus in *Pb* ANKA parasites. Genomic DNA was cleaved with EcoRV, transferred and probed with a HRF probe. The sizes of the bands are indicated in blue in locus representation. Line 1 represents the WT locus (1840 bp), whereas line 2 represents the genomic integration of the *h*DHFR targeting vector (2780 bp).

Fig. S2. Assessment of the phenotype of additional *HRF*-deficient clones. C57BL/6 mice were inoculated with 10 000 isolated WT or HRFΔ2 or HRFΔ3 *Pb* ANKA. (A) Parasitaemia (Mann–Whitney test; $*P < 0.02$; $***P < 0.001$) and (B) Kaplan–Meier survival plots (log-rank test) were recorded over time. Results are from two independent experiments.

Fig. S3. Comparable gliding locomotion capacity, EEF formation, cell traversal ability and *in vivo* cycling of WT and HRFΔ1 *Pb* ANKA sporozoites. (A, B) Circular sporozoite motility of WT and HRFΔ1 *Pb* ANKA parasites expressing GFP fluorescence images of WT and HRFΔ1 sporozoites was tracked on glass cover slides. Individual frames are 2 s apart. The rings indicate the maximum intensity projection over 30 frames showing sporozoite gliding motility (arrows) or passive floating parasites (stars). (C) Percentage of gliding parasites expressed as a ratio between ring forms and total parasites, WT (54% ± 8), HRFΔ1 (48.8% ± 12). (D, E) Comparison of exoerythrocytic forms between cultured GFP-WT and GFP-HRFΔ1 *Pb* ANKA sporozoites on HepG2 cells for 24 h. EEFs in each well were counted and expressed as EEFs plus SD of duplicate wells. EEFs were counted randomly and expressed as number of EEF per field. No significant difference was observed at both 24 and 48 h of culture. (F) Cell traversal was determined by counting wounded HepG2 cells labelled with rhodamine dextran and incubated with WT or HRFΔ1 sporozoites. Percentage of positive cells was determined by FACS analysis. Results shown are from two experiments. (G) Quantification of hepatic merozoites (EEFs) numbered on HepG2 cells cultured in the presence of WT or HRFΔ1 merozoites. Results shown are from two experiments. (H) HRFΔ1 parasites cycle normally between the anopheles and the vertebrate host. Cages of 200 WT *Pb* ANKA- or HRFΔ1 *Pb* ANKA-infected *Anopheles stephensi* female mosquitoes showing GFP-labelled sporozoites in their salivary glands were counted at day 20 post-blood feeding on infected C57BL/6 mice. Prevalence of infected mosquitoes was expressed as a ratio between positive ones and total mosquitoes. Sporozoites were extracted from salivary glands of 10 mosquitoes and counted. The number of sporozoites was expressed as per pair of salivary glands.

Fig. S4. HRFΔ1 sporozoites preferentially modulate the frequency and the magnitude of IL-6-producing neutrophils. C57BL/6 mice were inoculated IV with 10 000 isolated from WT or HRFΔ1 *Pb* ANKA parasites. (A) Liver leukocytes, taken 40 h post-infection, were gated on $CD45^+$ cells and stained with anti-Ly6G-PE, anti-7/4-FITC, anti-CD11b-FITC, anti-CD11c-PE, anti-CD4-FITC, anti-CD8-PE, anti-B220-FITC, anti-F4/80-PECy5 and anti-IL-6-Alexa647. Indicated numbers show the proportion in this gate of various cell subsets expressing IL-6. (B) Proportion and mean fluorescence intensity of gated $Ly6G^+$ $7/4^+$ neutrophils (R2) were determined for IL-6 expression in liver cells from WT and HRFΔ1 *Pb* ANKA-infected mice. Shown data are representative of one mouse out of three.

Fig. S5. Regulation of activation markers on leukocytes is independent of HRF at early time points of infection. C57BL/6 mice were inoculated IV with 10 000 isolated from WT or HRFΔ1 *Pb* ANKA parasites. Liver leukocytes, taken 40 h post-infection, were gated on $CD45^+$ cells and stained with anti-Ly6G-PE, anti-CD19-FITC, anti-CD4-FITC, anti-CD8-PE, anti-B220-FITC combined with anti-CD62L as an activation marker and with anti-CD11c-PE combined with anti-MHC class II as an activation marker for these cells. Results are expressed as the % of respective cell markers and $CD62L^{low}$ expression or MHC class II^{high} expression. Values are expressed as means ± SD obtained in three mice. For statistics in all graphs, $*P < 0.05 < 0.01$, according to the Kruskal–Wallis test.

Fig. S6. B-cells are not involved in protection during infection with HRFΔ1 parasites. (A, B) C57BL/6 and B-cell-deficient muMT mice were inoculated IV with 10 000 sporozoites from WT or HRFΔ1 *Pb* ANKA parasites. Livers were taken 40 h after infection and total RNA extracted. Parasite loads and IL-6 expression were determined using real-time RT-PCR by measuring *P. berghei* 18S rRNA and IL-6 mRNA respectively. Gene expression was normalized to the endogenous control gene HPRT. Data are presented as the means ± SD ($n = 5$) from two independent experiments and whenever indicated, differences with the respective control groups were significant (Mann–Whitney test; $*P = 0.022$, $**P = 0.008$). (C) Kaplan–Meier survival plots (log-rank test) were followed over time ($P < 0.0004$).

Fig. S7. Relationship between IL-6 and the reversibility of the phenotype of HRFΔ1 parasites. (A, B) effect of exogenous delivery of IL-6 on liver parasite load and IL-6 mRNA expression.

C57BL/6 mice were inoculated IV with 10 000 sporozoites from WT or HRFΔ1 *Pb* ANKA parasites. Livers were taken 40 h after infection and total RNA extracted. Parasite loads and IL-6 expression were determined using real-time RT-PCR by measuring *P. berghei* 18S rRNA and IL-6 mRNA respectively. Gene expression was normalized to the endogenous control gene HPRT. Values are expressed as means ± SD obtained in five mice. (C) Rescue of the phenotype of HRFΔ1 *Pb* ANKA parasite in IL-6 ^{KO} mice. IL-6 ^{KO} mice (seven mice per group) were infected IV with 10 000 GFP *Pb* ANKA WT or *Pb* ANKA HRFΔ1 sporozoites and parasitaemia (Mann–Whitney test; $*P = 0.02$, $**P = 0.007$) and survival rates (log-rank test) were followed over time.

Table S1. List of the oligonucleotides used for PCR of wild-type and recombinant parasites.

Appendix S1. Supporting experimental procedures.

Figure S1

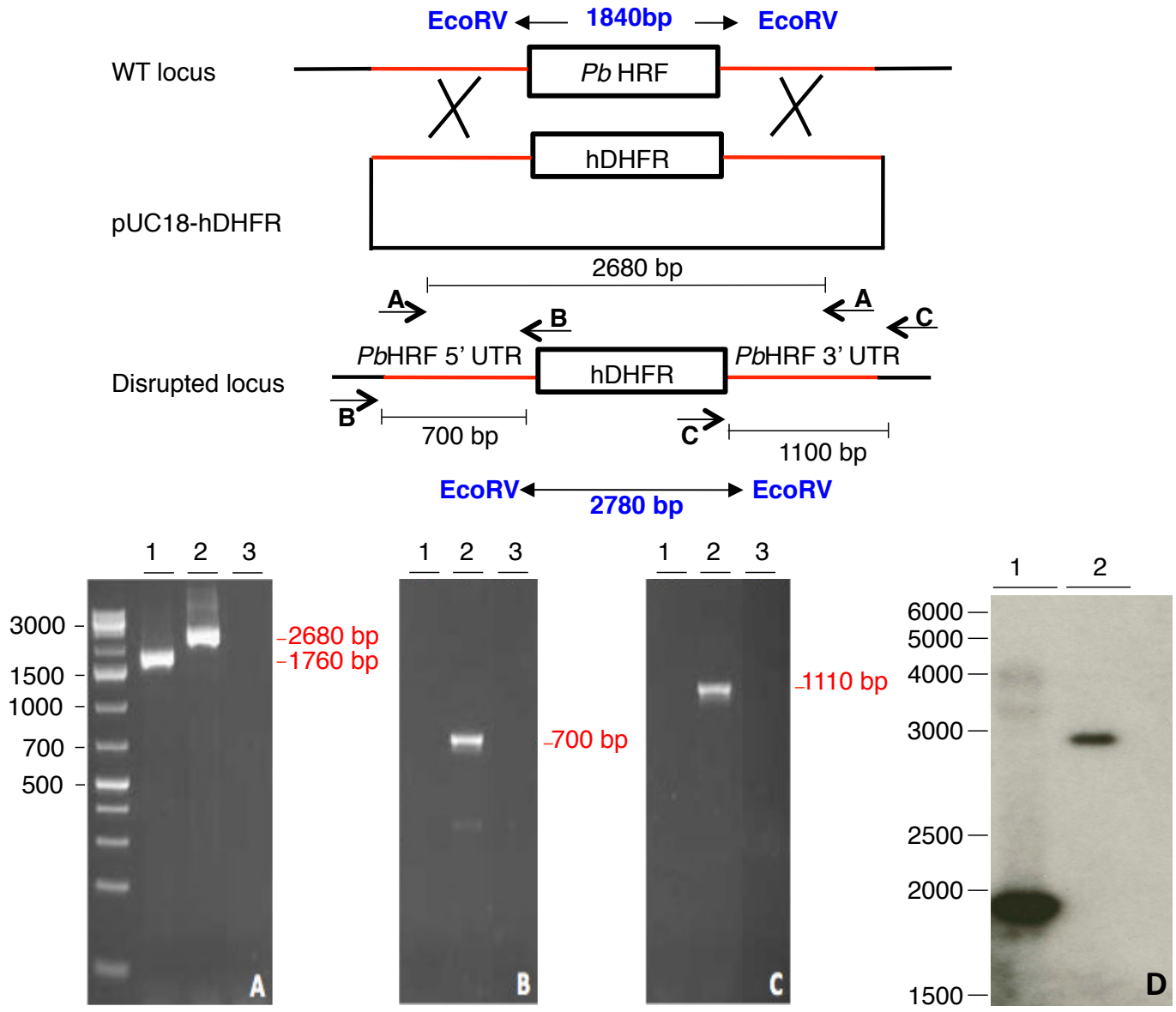
EcoRV
1840bp
EcoRV
WT locus
Pb HRF
hDHFR
pUC18-hDHFR
2680 bp
A
A
B
C
PbHRF 5' UTR
hDHFR
PbHRF 3' UTR
Disrupted locus
B
700 bp
C
1100 bp
EcoRV
2780 bp
EcoRV
1 2 3
3000
1500
1000
700
500
2680 bp
1760 bp
A
1 2 3
700 bp
B
1 2 3
1110 bp
C
1 2
6000
5000
4000
3000
2500
2000
1500
D

Figure S2

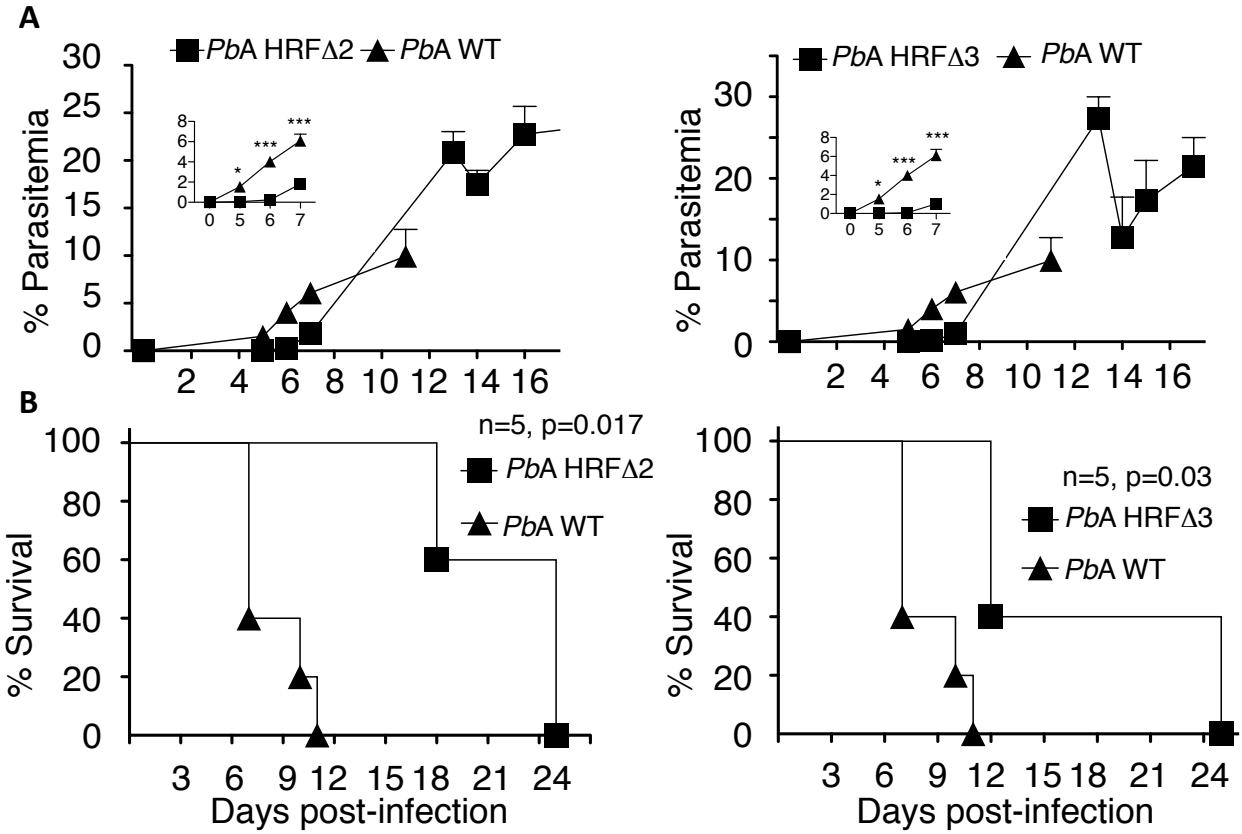
A
PbA HRFΔ2
PbA WT
% Parasitemia
PbA HRFΔ3
PbA WT
% Parasitemia
B
n=5, p=0.017
PbA HRFΔ2
PbA WT
% Survival
Days post-infection
n=5, p=0.03
PbA HRFΔ3
PbA WT
% Survival
Days post-infection

Figure S3

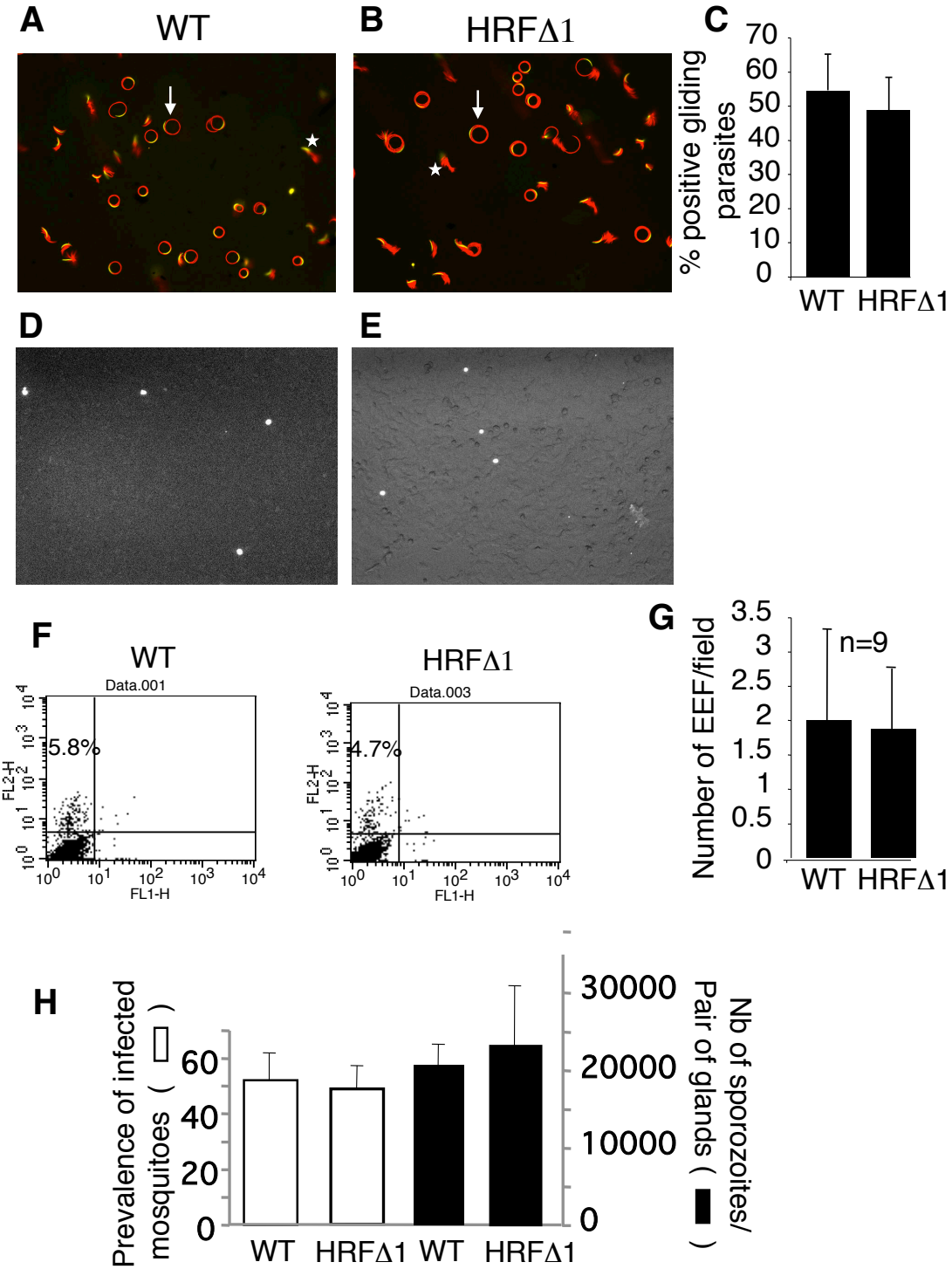
A
WT
B
HRFΔ1
C
% positive gliding parasites
70
60
50
40
30
20
10
0
WT
HRFΔ1
D
E
F
WT
Data.001
5.8%
HRFΔ1
Data.003
4.7%
FL2-H
FL1-H
G
Number of EEF/field
3.5
3
2.5
2
1.5
1
0.5
0
n=9
WT
HRFΔ1
H
Prevalence of infected mosquitoes ( )
60
40
20
0
30000
20000
10000
0
Nb of sporozoites/ Pair of glands ( )
WT
HRFΔ1
WT
HRFΔ1

Figure S4

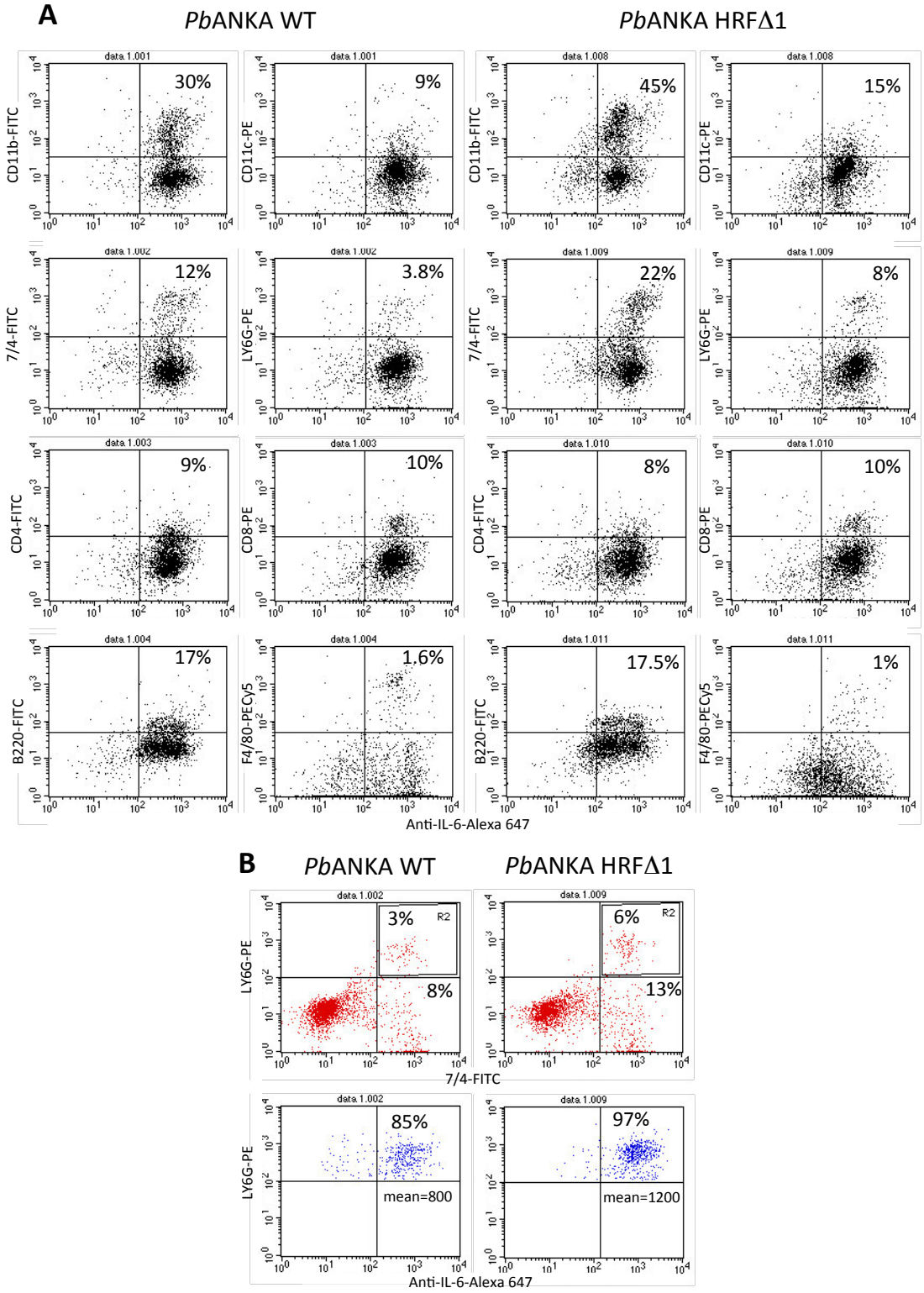
A
PbANKA WT
PbANKA HRFΔ1
CD11b-FITC
CD11c-PE
7/4-FITC
LY6G-PE
CD4-FITC
CD8-PE
B220-FITC
F4/80-PECy5
30%
9%
45%
15%
12%
3.8%
22%
8%
9%
10%
8%
10%
17%
1.6%
17.5%
1%
Anti-IL-6-Alexa 647
B
PbANKA WT
PbANKA HRFΔ1
LY6G-PE
7/4-FITC
R2
3%
8%
6%
13%
85%
97%
mean=800
mean=1200
Anti-IL-6-Alexa 647

Figure S5

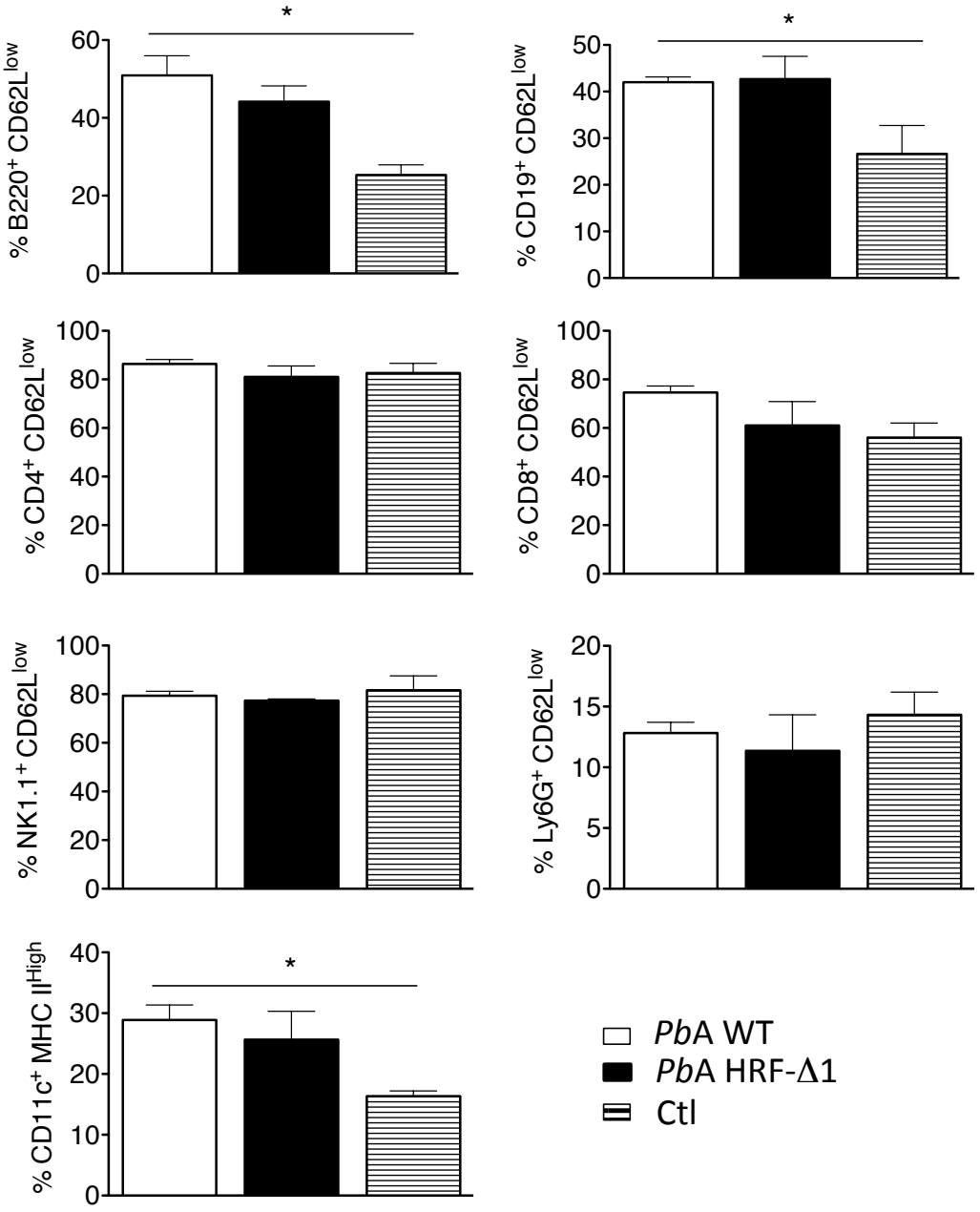
% B220+ CD62Llow
% CD19+ CD62Llow
% CD4+ CD62Llow
% CD8+ CD62Llow
% NK1.1+ CD62Llow
% Ly6G+ CD62Llow
% CD11c+ MHC IIHigh
*
PbA WT
PbA HRF-Δ1
Ctl

Figure S6

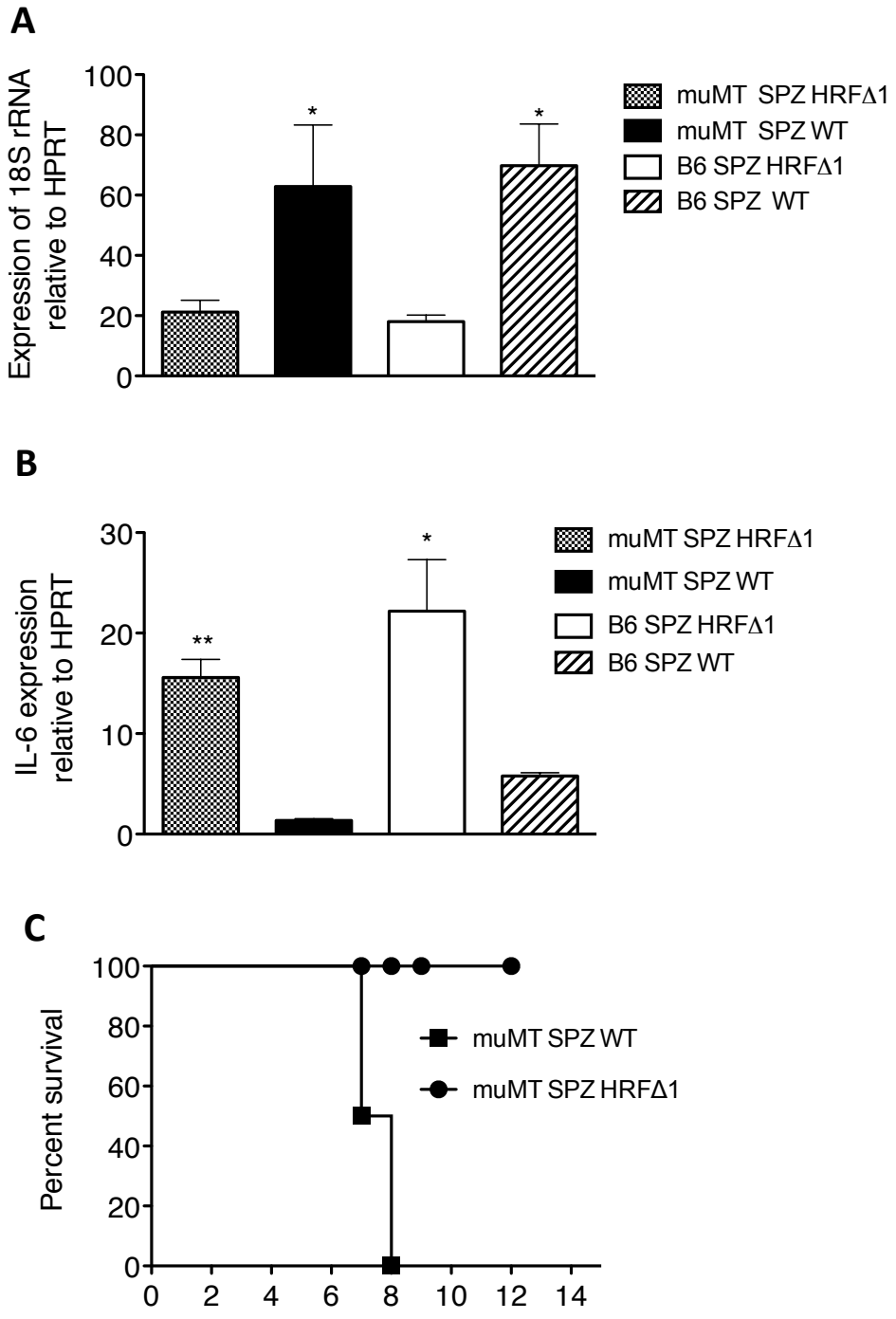
A
Expression of 18S rRNA relative to HPRT
100
80
60
40
20
0
*
*
muMT SPZ HRFΔ1
muMT SPZ WT
B6 SPZ HRFΔ1
B6 SPZ WT
B
IL-6 expression relative to HPRT
30
20
10
0
**
*
muMT SPZ HRFΔ1
muMT SPZ WT
B6 SPZ HRFΔ1
B6 SPZ WT
C
Percent survival
100
80
60
40
20
0
0
2
4
6
8
10
12
14
muMT SPZ WT
muMT SPZ HRFΔ1

Figure S7

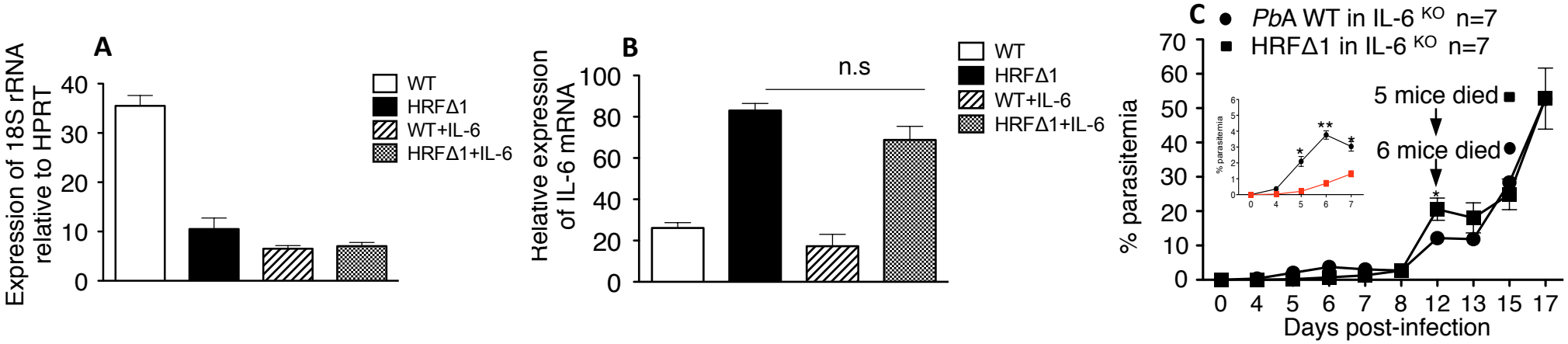
A
Expression of 18S rRNA relative to HPRT
40
30
20
10
0
WT
HRFΔ1
WT+IL-6
HRFΔ1+IL-6
B
Relative expression of IL-6 mRNA
100
80
60
40
20
0
n.s
WT
HRFΔ1
WT+IL-6
HRFΔ1+IL-6
C
PbA WT in IL-6 KO n=7
HRFΔ1 in IL-6 KO n=7
% parasitemia
70
60
50
40
30
20
10
0
5 mice died
6 mice died
0 4 5 6 7 8 12 13 15 17
Days post-infection

Table S1

List of the oligonucleotides used for PCR of wild-type and recombinant parasites

ApaI-5'UTR-PbHRF-F	5'- cgcgggcccgcgcattattaccgttgtca -3'
PstI-5'UTR-PbHRF-R	5'- cgcctgcagggcttatgcaagtatcgaacaa -3'
KpnI-3'UTR-PbHRF-F	5'- cgcggtaccttgctacatgacgcataaacc -3'
EcoRI-3'UTR-PbHRF-R	5'- cgcgaattctgtgaaatcgacaatgttttgg -3'
PbHRF -5'anal-F	5'- gcgatacaaacaaatttattcagc -3'
PbHRF -3'anal-R	5'- cgcaagatatcagagcttttca -3'
hDHFR 3'-F	5'- tgttgtctcttcaatgattcataaatagttgg -3'
hDHFR 5'-R	5'- tgctttgaggggtgagcatttaaagc -3'
PbHRF-5'orf-F	5'- ccatttggaaatgcggaat -3'
PbHRF-3'orf-R	5'- tttttcttcaaataaaccatctga -3'

4.2 ARTICLE II

Protection against malaria in mice is induced by blood stage-arresting histamine-releasing factor (HRF)–deficient parasites.

In this work the attention was focused on the role and the possible immune mechanisms modulated by *Plasmodium* HRF during *P. berghei* NK65 infections using a mutant parasite where *hrf* gene was deleted (*Pb*NK65-*hrfΔ*). Instead of using *Pb*ANKA which causes a rapid death of infected mice around day 7-8 p.i., we introduced the same mutation in a distinct lethal stain of parasite, *Pb*NK65, which causes severe anemia and death around day 20-25 p.i., allowing the tracking of anti-blood stage immune responses over a relatively extended period of time.

Highlights

- Identification of *hrf* as a gene which deletion confers to the lethal parasite strain *Pb*NK65 an attenuated virulence and the ability to induce a long lasting protection and immune memory against homologous and heterologous strains of parasites.
- Elevated IL-6 production in the absence of HRF expression is key in establishing parasite clearance and acquisition of sterile immunity.
- In an effort to identify the cellular source of IL-6 that was negatively regulated by HRF, examination by intracellular cell staining showed that $Ly6G^+$ and $CD11c^+$ cells are the main targets.
- Furthermore, our data showed the immunological role played by B and T cell as mice lacking these lymphocytes were unable to control the infection.
- Absence of the HRF protein increased the survival of T cells by the down-regulation of PD1 expression in both $CD4^+$ and $CD8^+$ T cells and the production of specific anti-parasite antibodies that recognized multiple *Pb* antigens.
- Parasite antigen-specific antibodies and $Fc\gamma R^+$ $CD11b^+$ cells play an important part in mutant-induced protection.

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Protection against malaria in mice is induced by blood stage–arresting *histamine-releasing factor* (*HRF*)–deficient parasites

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Although most vaccines against blood stage malaria in development today use subunit preparations, live attenuated parasites confer significantly broader and more lasting protection. In recent years, *Plasmodium* genetically attenuated parasites (GAPs) have been generated in rodent models that cause self-resolving blood stage infections and induce strong protection. All such GAPs generated so far bear mutations in housekeeping genes important for parasite development in red blood cells. In this study, using a *Plasmodium berghei* model compatible with tracking anti–blood stage immune responses over time, we report a novel blood stage GAP that lacks a secreted factor related to histamine-releasing factor (HRF). Lack of HRF causes an IL-6 increase, which boosts T and B cell responses to resolve infection and leave a cross-stage, cross-species, and lasting immunity. Mutant-induced protection involves a combination of antiparasite IgG2c antibodies and FcγR$^+$ CD11b$^+$ cell phagocytes, especially neutrophils, which are sufficient to confer protection. This immune-boosting GAP highlights an important role of opsonized parasite-mediated phagocytosis, which may be central to protection induced by all self-resolving blood stage GAP infections.

Live attenuated parasites, in particular genetically attenuated parasites (GAPs), are increasingly being considered as vaccines against malaria. Preerythrocytic GAPs fail to develop in the liver, whereas blood stage GAPs cause abortive infections in the blood. In both cases, GAP infection induces solid protection against challenge.

The notion that attenuated blood stage parasites can confer protection originated in early studies using irradiated parasites (Waki et al., 1982; Miyagami et al., 1987). More recently, it was found that infecting individuals with low doses of *Plasmodium falciparum*–infected RBCs (iRBCs) followed by rapid curative treatment induced strong cell-mediated immunity and durable protection against challenge (Pombo et al., 2002). Subsequently, blood stage GAPs were generated in rodent parasites, targeting genes involved in the purine salvage pathway in *Plasmodium yoelii* (Ting et al., 2008; Aly et al., 2010) or genes encoding a protease involved in hemoglobin degradation (Spaccapelo et al., 2010) and a merozoite surface protein involved in adhesion to RBCs (Spaccapelo et al., 2011) in *Plasmodium berghei*. These GAPs multiply suboptimally in the blood and cause infections that eventually self-resolve. Notably, abortive GAP-induced infections confer lasting protection against challenge with blood stages or mosquito transmission stages and depend on both cellular and humoral immunity (Ting et al., 2008; Aly et al., 2010).

Recently, we described the crucial role of histamine-releasing factor (HRF), also known as translationally controlled tumor protein, during development of *P. berghei* ANKA (*Pb*ANKA) in the host liver (Mathieu et al., 2015). In vivo development of *HRF*-deficient *Pb*ANKA parasites is severely impaired in the liver, caused by elevated levels of IL-6 (Pied et al., 1991). To test whether HRF might also modulate blood stage multiplication, we deleted the *HRF*-encoding gene in *P. berghei* NK65 (*Pb*NK65). *Pb*ANKA induces cerebral malaria in susceptible mice, with lesions starting at day 5 postinfection (p.i.) and mice dying from day 7–8 p.i. (Beghdadi et al., 2008), which precludes studies on adaptive immunity. In contrast, *Pb*NK65 does not cause cerebral malaria but hyperparasitemia, leading to mouse death by severe anemia around day 25 p.i. It thus provides an opportunity to track immune responses against blood stage parasites over a longer period of time.

RESULTS AND DISCUSSION

Deletion of the *hrf* gene in *Pb*NK65 parasites causes abortive blood stage development

We generated *hrf* knockout *Pb*NK65 parasites (*Pb*NK65-*hrfΔ*) by replacing the coding sequence of *pbhrf* (PBA

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Abbreviations used: APC, allo phyco cyanine; DTR, diphtheria toxin receptor; FA, formic acid; GAP, genetically attenuated parasite; gDNA, genomic DNA; hDHFR, human dihydrofolate reductase; HPRT, hypoxanthine phosphoribosyltransferase; HRF, histamine-releasing factor; iRBC, infected RBC; p.i., postinfection; qPCR, quantitative PCR; UTR, untranslated.

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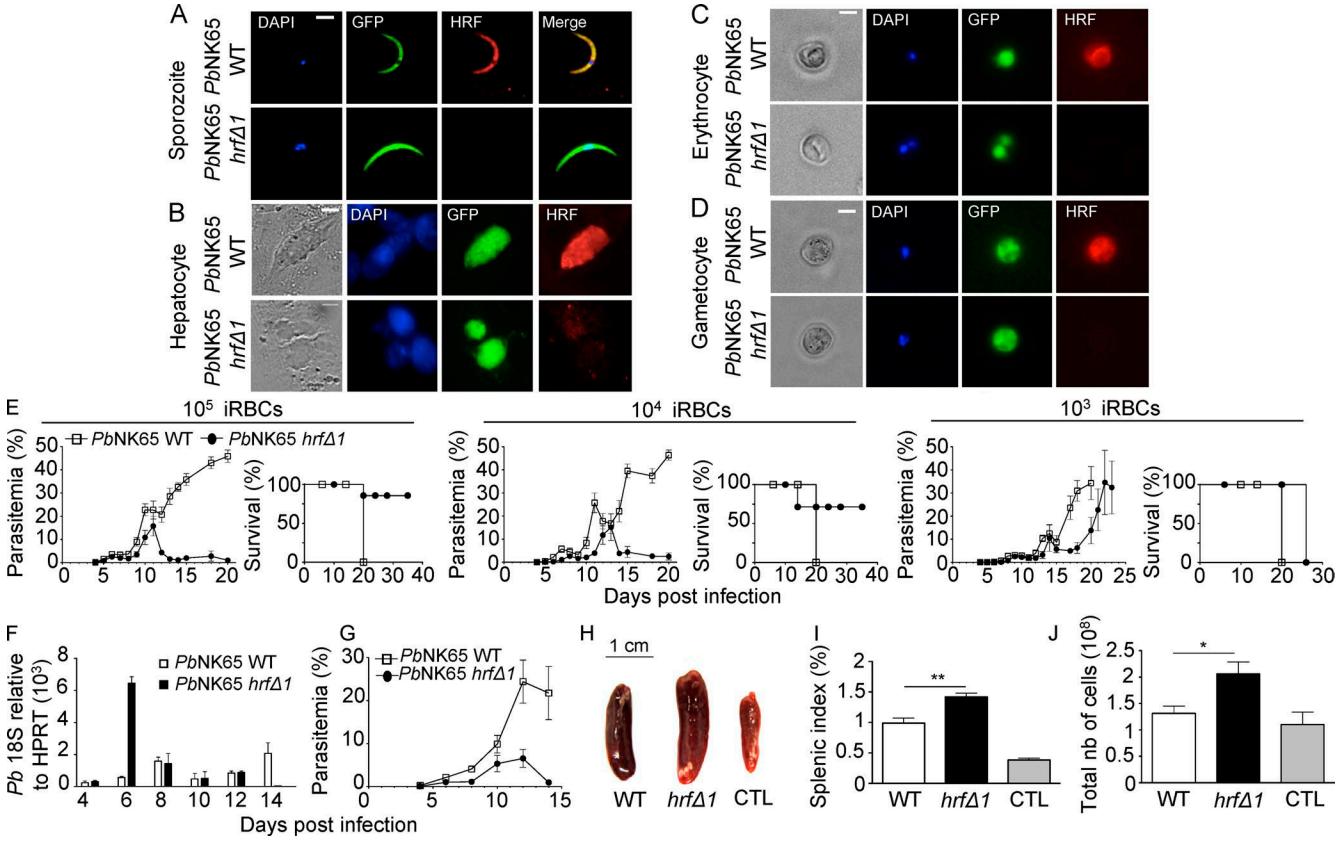

Figure 1. ***Pb*HRF protein expression and phenotype of mutant parasites.** (A–D) Anti-HRF–based immunofluorescence (red) was used to detect HRF in GFP-expressing WT or *Pb*NK65-*hrf*Δ1 sporozoites (A), liver stages obtained 48 h p.i. of HepG2 cells with sporozoites at a multiplicity of infection of 1:1 (B), infected erythrocytes (C), and gametocytes (D). Nuclear DNA stained with DAPI and phase-contrast images are shown. (A–D) Bars, 6 µm. (E) Blood stage parasitemia and survival of C57BL/6 mice (Kaplan-Meier survival plots) after i.p. injection of 10^5, 10^4, and 10^3 WT or *Pb*NK65-*hrf*Δ1–infected iRBCs measured over several days. (F and G) The kinetics of parasite load in the spleen of WT or *Pb*NK65-*hrf*Δ1–infected mice was determined by RT-qPCR analysis of *P. berghei* 18S rRNA expression relative to mouse HPRT mRNA levels (F) or flow cytometric analysis of parasitemia (G). (H) Spleen size of WT or *Pb*NK65-*hrf*Δ1–infected mice at day 6 p.i. (I and J) Splenic index (I) and total cell number (J) were compared at day 6 p.i. between mice infected with 10^5 WT and *Pb*NK65-*hrf*Δ1 iRBCs. Control: splenic index and cell number from naive mice. Error bars, SEM. Data are representative of three (A–D and H–J), six (E), and two (F and G) independent experiments with five to six mice per group. *, P = 0.029; **, P = 0.003; Mann-Whitney test. CTL, control; nb, number.

NKA_111050; UniProt accession no. A0A077XCV2) with the human dihydrofolate reductase (*hDHFR*)–selectable marker in a GFP-expressing *Pb*NK65 strain (Fig. S1 A). Two clones, *Pb*NK65-*hrf*Δ1 and 2, were selected from independent transfection experiments and verified to harbor the mutant locus by PCR (Fig. S1, B–D) and Southern blot analysis (Fig. S1 E). Using specific rabbit antibodies against recombinant *Pb*HRF (Mathieu et al., 2015), the protein was found to be expressed at all *Plasmodium* stages tested and to localize to the cytoplasm (Fig. 1, A–D), consistent with previous studies in human cells and *Pb*ANKA parasites (Bhisutthibhan et al., 1999; Mathieu et al., 2015). HRF was not detected in *Pb*NK65-*hrf*Δ1 parasites (Fig. 1, A–D), confirming both antibody specificity and successful gene knockout.

To assess the effect of *pbhrf* deletion on parasite blood stage development, C57BL/6 mice were infected i.p. with 10^5, 10^4, or 10^3 WT or *Pb*NK65-*hrf*Δ1–infected iRBCs, and parasite growth was monitored by flow cytometry. In mice infected with WT parasites, parasitemia increased steadily, resulting in severe malaria and death at approximately day 20 p.i. When using 10^5 or 10^4 infectious doses, *Pb*NK65-*hrf*Δ1 parasites multiplied like WT until day 10 p.i., reaching parasitemia of ∼20%, and were cleared from mice at day 13 or 14 p.i., respectively. Mouse survival rate after injection of 10^4 or 10^5 *Pb*NK65-*hrf*Δ1 parasites was ∼90% and ∼70%, respectively. Notably, injection of 10^3 *Pb*NK65-*hrf*Δ1 parasites did not lead to parasite clearance or mouse survival. Infection with *Pb*NK65-*hrf*Δ2, a second clone, gave similar results (Fig. S1, F and G). We concluded that infection with parasites lacking HRF can self-resolve and that parasite clearance depends on the initial parasite load and/or time to threshold parasitemia. Further experiments were performed after injection of 10^5 *Pb*NK65-*hrf*Δ1 parasites.

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To determine whether clearance of mutant parasites from the circulation could be caused by parasite retention in the spleen, the parasite load in this organ was measured by RT–quantitative PCR (RT-qPCR). After a dramatic retention of *Pb*NK65-*hrf*Δ1 parasites at day 6, parasites were no longer detected in the spleen at day 14 (Fig. 1 F), indicating that parasite clearance was not caused by retention in the spleen (Fig. 1 G). Moreover, macroscopic examination showed a more important splenomegaly at day 6 in *Pb*NK65-*hrf*Δ1–infected mice than in WT-infected mice, suggesting leukocyte infiltration in the mutant-infected spleen (Fig. 1, H–J).

IL-6, B cells, and T cells are critical for inhibition of *Pb*NK65-*hrf*Δ blood stage growth

Because HRF-deficient *Pb*ANKA sporozoites induce IL-6 production in the liver during preerythrocytic infection (Mathieu et al., 2015), we compared IL-6 levels in mouse spleens 6 d p.i with WT or *Pb*NK65-*hrf*Δ1 iRBCs. Levels of IL-6 mRNA and plasmatic IL-6 protein were higher in *Pb*NK65-*hrf*Δ1–infected mice than in WT-infected mice (Fig. 2, A and B). Given that recombinant *Pb*HRF protein is sufficient to down-regulate IL-6 expression in vivo (Mathieu et al., 2015), we tested whether clearance of mutant parasites was the consequence of elevated IL-6 by infecting IL-6^{KO} mice with mutant parasites. Mutant parasites developed normally in and eventually killed IL-6^{KO} mice (Fig. 2 C), phenocopying WT parasite behavior in WT mice (Fig. 1 E). We concluded that increased IL-6 accounts for mutant parasite clearance.

Immunostaining with leukocyte surface markers and anti–IL-6 antibodies of cells collected from the spleen at days 6 and 20 p.i. identified IL-6–producing cells as $Ly6G^+$ neutrophils at day 6 and both $Ly6G^+$ neutrophils and $CD11c^+$ DCs at day 20 (Fig. 2 D). Depletion of neutrophils (Fig. S2, A and B) or DCs (Fig. S2, C and D) in mutant-infected mice reduced splenomegaly (Fig. S2, E, F, H, and I) and cell counts (Fig. S2, G and J) compared with nondepleted mice, confirming the contribution of neutrophils and DCs in the splenomegaly caused by mutant infection.

IL-6 is known to regulate the acute phase of the immune response and major B and T cell functions (Kishimoto et al., 1992; Barton, 1997). To test whether B or T cells were involved in self-resolution of mutant infection, we infected mice lacking B cells (μS^{KO}) or T cells ($CD3^{KO}$) with WT or *Pb*NK65-*hrf*Δ1 parasites and monitored parasite development. B cell– or T cell–deficient mice were unable to control *Pb*NK65-*hrf*Δ1 blood stage multiplication and died with kinetics similar to WT mice infected with WT parasites (Fig. 2, E and F). The importance of T cells was confirmed by the normal multiplication of the mutant parasite upon mouse treatment of previously protected mice with anti-CD3 antibody (Fig. 2 G and Fig. S2 K). This indicated that B and T lymphocytes contributed to the clearance of mutant parasites.

*Pb*NK65-*hrf*Δ1 infection confers lasting protection in a species- and stage-transcendent manner

To determine whether resolved *Pb*NK65-*hrf*Δ1 parasite infection might confer protection against challenge, mutant-infected mice were challenged with 10^5 WT *Pb*NK65 iRBCs at days 20, 35, 68, 168, and 396 p.i. Mice were protected in all cases, displaying no detectable parasitemia at any time point, and survived for more than a year (Fig. 3 A). Mice challenged at days 20 and 23 p.i. with 10^5 RBCs infected with virulent *P. berghei* ANKA (Fig. 3 B) or *P. yoelii* YM (Fig. 3 C), respectively, were also protected and did not develop parasitemia. Next, we asked whether mutant-infected mice were also protected against a challenge with WT *Pb*NK65 sporozoites, the mosquito-transmitted parasite stage. Sporozoite challenge did not result in detectable blood stage infection (Fig. 3 D), and parasite genes were not detected in the blood by PCR (not depicted). To ascertain that protection indeed targeted preerythrocytic stages and not just emerging blood stage development, the livers of mice challenged with sporozoites were analyzed by RT-qPCR 40 h after sporozoite inoculation. As shown in Fig. 3 E, the parasite load was significantly lower in the liver of *Pb*NK65-*hrf*Δ1–protected mice compared with control mice. A similar protection was observed against heterologous *P. berghei* ANKA (Fig. 3 F) and *P. yoelii* YM (Fig. 3 G) sporozoite challenge. Therefore, infection with HRF-deficient blood stage *Pb*NK65 parasites induces long-lasting protection against malaria in a species- and stage-transcendent manner.

Mutant-induced immunity involves *Plasmodium*-specific IgG2c antibodies

To assess whether antibodies were involved in the anti–*Pb*NK65-*hrf*Δ1 response, anti–parasite-specific antibodies of various isotypes were quantified by ELISA in sera of mice 15 d p.i. with WT or *Pb*NK65-*hrf*Δ1 parasites. As shown in Fig. 4 A, mice infected with *Pb*NK65-*hrf*Δ1 produced higher levels of IgG antibodies than mice infected with WT parasites, essentially belonging to the IgG2c subclass and to a lesser extent to the IgG3 subclass (Fig. 4 A). Next, as shown by Western blot analysis of extracts from WT blood stages (Fig. 4 B), these antibodies recognized multiple *P. berghei* antigens in contrast to sera from WT *Pb*NK65–infected mice or from naive mice (Fig. 4 B). Interestingly, the IgG2c isotype, expressed in C57BL/6 mice in which the IgG2a heavy chain is deleted, is known to be the predominant isotype generated in antiviral antibody responses (Coutelier et al., 1987) and the most efficient IgG subclass for antipathogen FcR-mediated effector functions (Nimmerjahn and Ravetch, 2005). In most experimental mouse malaria models, parasite-specific antibodies have been shown to be predominantly skewed toward the IgG2c isotype (Ndungu et al., 2009).

Immunoprecipitation of *P. berghei* blood stage proteins with the IgG antibodies from mutant-infected mice and mass spectrometry of the immunoprecipitate revealed five *P. berghei* proteins targeted by the protective IgG response (Fig. S3, A and B). These included the vaccine candidates merozoite surface protein 1

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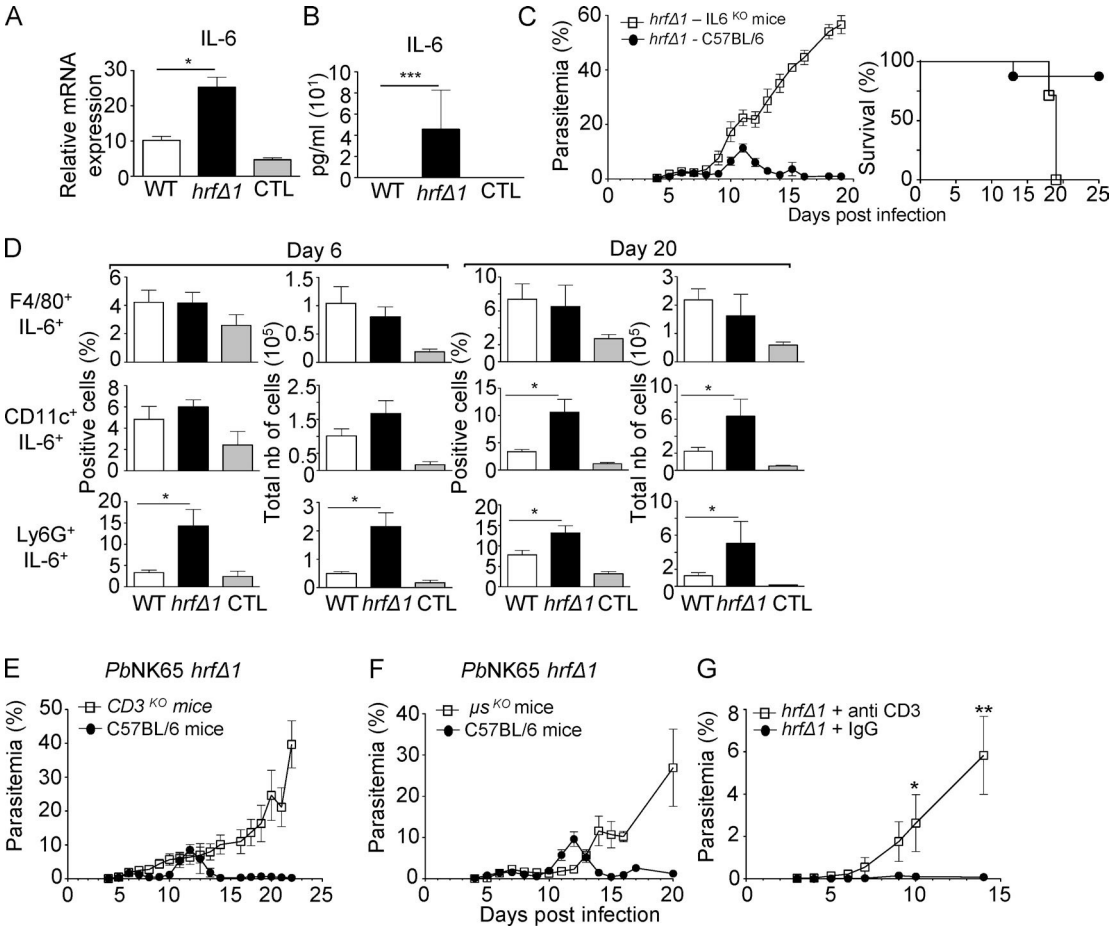

Figure 2. **IL-6 expression by neutrophils and DCs and T and B cells are essential for the clearance of *Pb*NK65-*hrf*Δ parasites.** (A and B) mRNA levels (RT-qPCR) normalized to HPRT of IL-6 in the spleen (A) and in the serum (ELISA; B) measured at day 6 p.i. from WT or *Pb*NK65-*hrf*Δ1–infected mice. Control (CTL): mRNA and sera from naive mice. (C) WT or IL-6^{KO} C57BL/6 mice were infected i.p. with 10^5 *Pb*NK65-*hrf*Δ1 iRBCs. Parasitemia and mouse survival (Kaplan-Meier survival plots: log-rank test; P = 0.0046) were followed over time. (D) Frequency and absolute numbers (nb) of IL-6–expressing splenic macrophages, DCs, and neutrophils at day 6 and day 20 p.i. from mice (five per group) infected with 10^5 WT or *Pb*NK65-*hrf*Δ1 iRBCs or from naive mice (CTL). (E and F) WT or T cell–deficient (E) or B cell–deficient (F) C57BL/6 mice were infected i.p. with 10^5 *Pb*NK65-*hrf*Δ1 iRBCs, and parasitemia was followed over time. (G) Protected mice were treated with IgG or with anti-CD3–depleting antibody 1 d prior to a challenge with WT parasites followed by two booster injections of anti-CD3 at days 1 and 3 after challenge. Error bars, SEM. Data are representative of four (A and B), three (C and E–G), and two (D) independent experiments with five to seven mice per group. *, 0.0028 < P < 0.046; **, P = 0.019; ***, P = 0.0097; Mann-Whitney test.

(MSP1), serine repeat antigen 1 (SERA1), and SERA2 (Bodescot et al., 2004; Putrianti et al., 2010; Alaro et al., 2013). As shown by immunoblots (Fig. S3 C) and ELISA (Fig. S3 D), only sera from protected mice recognized the recombinant MSP1-33 antigen.

Next, to test whether IgG antibodies may mediate parasite clearance via FcRγ-expressing cells, WT or FcRγ^{KO} C57BL/6 mice were infected with *Pb*NK65-*hrf*Δ1 parasites. As shown in Fig. 4 C, in contrast to WT mice, FcRγ^{KO} mice were unable to eliminate *Pb*NK65-*hrf*Δ1 parasites and phenocopied WT mice infected with WT parasites.

Antibodies and CD11b$^+$ cells from mutant-infected mice mediate protective immunity

Finally, to assess what immune effectors are important for protection, we performed passive transfer experiments. First, antibodies purified from protected mice were transferred to naive mice before challenge with WT *Pb*NK65 parasites. As shown in Fig. 4 D, parasitemia was lower in mice treated with IgG from protected mice than in normal IgG-treated mice, suggesting that antibodies alone provide partial but significant protective activity (Fig. 4 D).

Last, we asked whether CD11b$^+$ cells such as phagocytic leukocytes from *Pb*NK65-*hrf*Δ1–protected mice might be sufficient to protect naive mice from WT infection. WT C57BL/6 mice with adoptively transferred CD11b$^+$ cells from naive or *Pb*NK65-*hrf*Δ1–infected mice at day 15 p.i. were challenged with 10^5 WT parasites. As shown in Fig. 4 E, transfer of CD11b$^+$ cells from mutant-infected, but not naive, mice efficiently protected against infection. Collectively, these data suggest that

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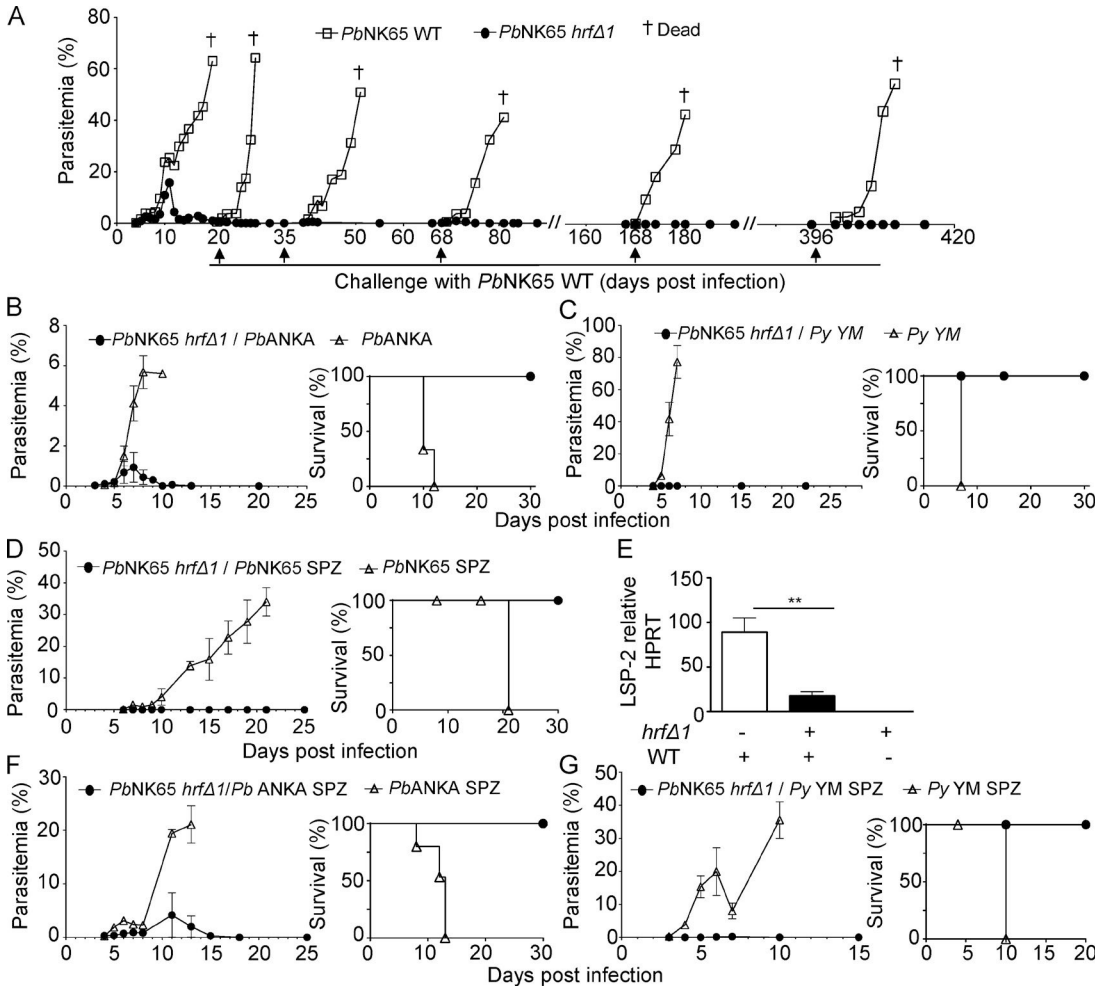

Figure 3. **Infection with HRF-deficient blood stage parasites ensures long-lasting cross-species and cross-stage protection.** (A) *Pb*NK65-*hrf*Δ1–protected mice were challenged with 10^5 WT *Pb*NK65 iRBCs at the indicated time points where control naive mice were also infected with 10^5 WT *Pb*NK65 iRBCs. (B–D) Parasitemia and survival were measured over time. Parasitemia and Kaplan-Meier survival plots of *Pb*NK65-*hrf*Δ1–protected mice challenged with 10^5 *Pb*ANKA (log-rank test; P = 0.0027; B) or *P. yoelii* YM (log-rank test; P = 0.0047; C) iRBCs at day 20 and day 23 p.i., respectively, or with 10^4 GFP-expressing WT *Pb*NK65 sporozoites (log-rank test; P = 0.0047) at day 36 p.i. (D). (E) Intrahepatic parasite development in experimental and control mice from D was assessed by RT-qPCR analysis of the liver stage–specific LSP-2 marker at 40 h p.i. of sporozoites. (F and G) *Pb*NK65-*hrf*Δ1–protected mice were challenged with 10^4 *Pb* ANKA (F) or *P. yoelii* YM (G) sporozoites at day 25 p.i., and parasitemia and survival (log-rank test; P = 0.0082) were determined over time. Naive mice infected on the same day with *Pb*ANKA (F) or with *P. yoelii* YM (G) sporozoites were used as controls. Error bars, SEM. Data are representative of two (A) and three (B–G) independent experiments with four to eight mice per group. **, P = 0.015; Mann-Whitney test. *Py*, *P. yoelii*; SPZ, sporozoites.

parasite antigen-specific antibodies and $Fc\gamma R^+$ $CD11b^+$ cells play an important part in mutant-induced protection.

This study shows that abortive blood stage infection leading to lasting protection can be achieved not just by impairing parasite intracellular growth, but also by enhancing protective immune responses. Indeed, lack of HRF and an increase in IL-6 do not affect parasite growth per se because *Pb*NK65-*hrfΔ* blood stages multiply normally in mice until day 10. Rather, IL-6, which is involved in B and T cell differentiation, boosts antiparasite adaptive responses that clear parasites. Like with previously reported blood stage GAPs that induce abortive infections, the protective response to *Pb*NK65-*hrfΔ* parasites is both solid, conferring cross-stage and cross-species immunity, and durable. We found that the protective response relies on the combination of antiparasite IgG2c antibodies and $Fc\gamma R^+$ $CD11b^+$ phagocytic cells, in particular neutrophils, which are sufficient for solid protection. Interestingly, the discovery of a B helper neutrophil population in the spleen that can act as professional helper cells for marginal zone B cells (Puga et al., 2012) highlights a neutrophil–B cell interplay that may be critical for B cell differentiation into antibody-producing plasma cells and may also contribute to inhibition of the well-known *Plasmodium* capacity to induce short-lived B cell memory (Wykes et al., 2005). Opsonic phagocytosis was also described as a protective mechanism induced by the plasmepsin-4–deficient mutant (Spaccapelo et al., 2010). Whether

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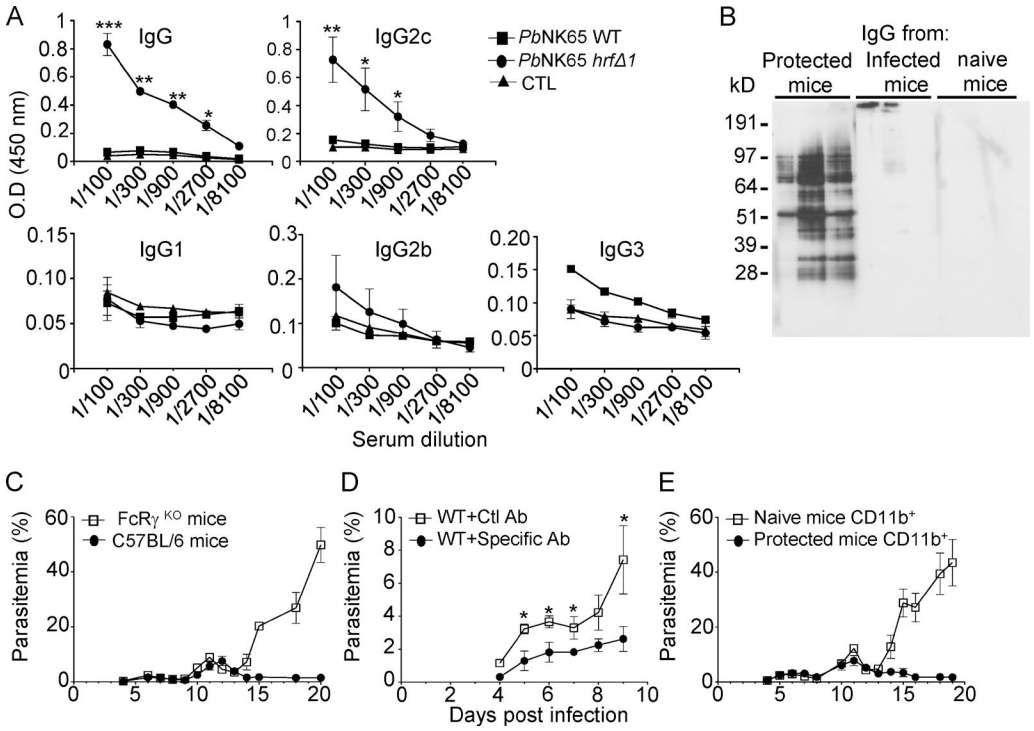

Figure 4. ***Pb*NK65-*hrf*Δ–induced immunity is T and B cell dependent and involves the secretion of *Plasmodium*-specific IgG2c antibodies.** (A) ELISA detection and quantification of antiparasite-specific antibodies of various isotypes in mouse sera at day 20 p.i. with WT or *Pb*NK65-*hrf*Δ1 parasites. Control (CTL): sera from naive mice. (B) Separated total protein extracts from WT *Pb*NK65 iRBCs were incubated with the IgG fraction from three independent mice infected with either *Pb*NK65-*hrf*Δ1 or WT parasites at day 20 p.i. or with normal mouse IgG. (C) C57BL/6 or FcRγ^{KO} mice were infected i.p. with 10^5 *Pb*NK65-*hrf*Δ1 iRBCs, and parasitemia was followed over time. (D) Purified IgG antibodies from *Pb*NK65-*hrf*Δ1–protected mice or from naive mice were injected i.p. 1 d before and 1 d after infection with 10^5 WT *Pb*NK65 iRBCs, and parasitemia was recorded over time. Ab, antibody; Ctl, control. (E) Sorted CD11b$^+$ cells from naive or *Pb*NK65-*hrf*Δ1–protected mice were transferred into WT C57BL/6 mice and immediately infected with 10^5 WT *Pb*NK65 iRBCs. Parasitemia was recorded over time. Error bars, SEM. Data are representative of three (A–D) and two (E) independent experiments with five to seven mice per group. *, 0.019 < P < 0.03; **, 0.0079 < P < 0.01; ***, P = 0.0002; Mann-Whitney test.

this represents the essential protective mechanism common to all self-resolving infections remains to be determined. Finally, although not formally demonstrated in our work, the contribution of parasite-specific CD8$^+$ cells to self-resolution cannot be precluded and awaits further investigation.

MATERIALS AND METHODS

Ethics statements. All experiments involving mice were conducted at Institut Pasteur, approved by the Direction Départementale des Services Vétérinaires' de Paris, France (permit number N° 75–066 issued on September 14, 2009), and performed in compliance with institutional guidelines and European regulations. A statement of compliance with the French government's ethical and animal experiment regulations was issued by the Ministère de l'Enseignement Supérieur et de la Recherche under the number 00218.01.

Rodents. 5–8-wk-old WT female C57BL/6J Rj and Swiss Webster (SW) mice were purchased from the Janvier laboratory. Transgenic T cell–deficient (CD3^{KO}), B cell–deficient (secretory μ chain [μs^{KO}]), Fcγ receptor–deficient (FcγR^{KO}), and IL-6^{KO} mice strains were provided by B. Ryffel (Institut Pasteur, Paris, France), J.M. Cavaillon (Institut Pasteur), P. Bruhns (Institut Pasteur), and L. Apetoh (Institut National de la Santé et de la Recherche Médicale U866, Dijon, France), respectively. CD11c-DTR (diphtheria toxin receptor)-GFP mice (Jung et al., 2002) have been used to explore the role of DCs in controlling parasite development. Transgenic mice have all been backcrossed 10 times on C57BL/6 mice from The Jackson Laboratory.

Parasites. Mice were inoculated with RBCs infected with either *P. berghei* (*Pb*) NK65 WT or mutant (*hrfΔ*) GFP-transgenic parasites. In a few control experiments, mice were infected with *P. yoelii* YM or *Pb* ANKA-GFP iRBCs, or with *Pb*NK65 or *Pb*ANKA GFP-transgenic sporozoites collected from salivary glands of infected *Anopheles stephensi*. Mosquitoes were provided by the CEPIA (Centre d'élevage, de production et d'infection des anopheles, Institut Pasteur).

Mouse infections and immunization with blood stages. Cryopreserved *P. berghei* parasites were passaged once through SW

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mice before being used to infect experimental animals. Mice were infected with blood stages of either GFP-transgenic *Pb*NK65, *Pb*NK65-*hrf*Δ1, or *Pb*NK65-*hrf*Δ2 parasites by injecting 10^5, 10^4, or 10^3 iRBCs i.p. After injection, blood samples were taken daily from the tail, and parasitemia was assessed by flow cytometry. If mice did not develop parasites after challenge, they were recorded as completely protected.

Splenic index. Spleens from uninfected and infected mice were harvested at day 6 p.i. with WT or *Pb*NK65-*hrf*Δ1 parasites. The splenic index for each individual mouse was calculated as follows: spleen weight (mg)/body weight (mg) × 100.

Sporozoite development in HepG2 cells. HepG2 cells (2–3 × 10^4/well) were plated in 8-well chamber slides (Lab-Tek) and cultured overnight in DMEM + GlutaMAX-I media (Gibco) supplemented with 10% heat-inactivated FBS (Gibco) at 37°C in the presence of 5% CO_2. WT or mutant purified *P. berghei* salivary gland sporozoites were used for HepG2 infection at a ratio of 1:1 (parasite/cells) for 36 h at 37°C with 5% CO_2 in the presence of penicillin–streptomycin–neomycin solution (Sigma-Aldrich). *Pb*HRF was detected by immunofluorescence staining as described in the next paragraph.

Immunofluorescence assays for the intracellular detection of HRF. Fixation and permeabilization of sporozoites, infected HepG2 cells, iRBCs, and purified gametocytes were performed using 4% paraformaldehyde and 0.1% Triton X-100 and blocked with 1–3% gelatin from porcine skin (Sigma-Aldrich). Thereafter, cells were incubated with specific rabbit anti-HRF antibodies (diluted 1:500; Mathieu et al., 2015) and then incubated with Alexa Fluor 568–conjugated secondary antibodies (diluted 1:500; Thermo Fisher Scientific) and 0.02 mg/ml DAPI for nuclear staining. The expression of *Pb*HRF was detected using a fluorescence microscope (AxioVert 200; ZEISS).

Preparation of total RNA and RT-qPCR analysis of mRNA. The spleens and livers of C57BL/6J mice infected with WT or *Pb*NK65-*hrf*Δ1 parasites were surgically removed 40 h p.i. or at day 2, 4, 6, 8, 10, 12, 14, and 20 p.i., respectively. Total RNAs were extracted from the spleen as well as from liver samples using the guanidinium-thiocyanate-phenol-chloroform method (all from Invitrogen). RNA was thereafter reverse transcribed by PCR (temperature profile: 65°C for 5 min, 42°C for 50 min, and 70°C for 15 min) using 100 U of SuperScript II reverse transcriptase (Invitrogen), 40 U RNase inhibitor, and 2 µM oligo(dT) 18S rRNA primer (Eurofins MWG Operon) per sample. The expression levels of diverse transcripts were analyzed by real-time RT-qPCR using Power SYBR green PCR master mix (Applied Biosystems) and various primer sets (Table S1). All reactions were performed in a real-time PCR machine (temperature profile: 50°C for 2 min, 95°C for 10 min, 40 cycles of 15 s at 95°C, and 60°C for 1 min; ABI PRISM 7000 Sequence Detection System; Applied Biosystems). The relative abundance of parasite and cytokine rRNA in the spleen was calculated using the ΔC_t method and expressed as $2^{-\Delta C_t}$. The mouse hypoxanthine phosphoribosyltransferase (HPRT) gene was used as an internal control for the variation in input RNA amounts. A no-template control was included to ensure that there was no cross-contamination during sample preparation.

Flow cytometry analysis of spleen leukocytes. Spleens were mechanically disrupted in 2 ml PBS, and cells were filtered through a 70-mm strainer (BD). Erythrocytes were lysed using Gey's solution for 5 min on ice and washed twice in PBS. Single-cell suspensions were stained for FACS analysis according to standard protocols in cold PBS containing 2% FCS and 0.01% sodium azide (FACS buffer) with the following antibodies: PE-labeled anti-CD4, PE-Cy5–labeled anti-CD45, allo phyco cyanine (APC)–labeled anti-CD8, FITC-labeled anti-CD11b, APC-labeled anti-CD11c, APC-labeled anti-Ly6G, PE-Cy5–labeled anti-F4/80, and PE-labeled anti–IL-6 antibodies (all antibodies from BD). A total of 5 × 10^5 living cells were analyzed using a four-color flow cytometer (FAC SCalibur; BD) and ProCellQuest software (BD).

In vivo cell depletion. For neutrophil depletion, C57BL/6 mice were injected with 500 µg of a rat anti–mouse neutrophil (clone NIMP-R14) provided by G. Milon (Institut Pasteur) at day 2 and day 4 p.i. with *Pb*NK65-*hrf*Δ1. For systemic DC depletion, CD11c-DTR-GFP transgenic mice were injected i.p. with 5.2 ng/g body weight diphtheria toxin (Sigma-Aldrich) in PBS at days 2 and 4 p.i. with *Pb*NK65-*hrf*Δ1. To determine whether CD3 plays a role in the antiparasitic memory response developed by protected mice, cell-specific depletion experiments were performed. C57BL/6J Rj–protected mice were injected i.p. with 20 µg anti-CD3 (clone 145-2C11) Armenian hamster IgG (eBioscience) 24 h before the infection with *Pb*NK65 WT and 48 h p.i. The cell depletion was followed and confirmed by flow cytometry. Before the infection and every day p.i., 10 µl of blood was collected from the tip of the mouse tail and analyzed to confirm neutrophil, DC, and CD3 cell depletion by FACS analysis.

Detection of specific IgG antibodies and IL-6 cytokine in the serum of infected mice. To detect parasite-specific antibodies, protein extracts from blood stages obtained by saponin lysis (0.1%) of parasite pellets were sonicated in lysis buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 0.02% NaN_3, 20 mM $MgCl_2$, 1% Triton X-100, and complex protease inhibitors) and centrifuged (10,000 *g* for 30 min at 4°C). The total amount of proteins in the supernatant was measured using a Bio-Rad Laboratories protein assay. 96-well plates (Nunc-immuno plate; Thermo Fisher Scientific) were coated with 2 µg/ml *Pb*NK65 WT protein extracts in carbonate buffer, pH 9.6, for 2 h at 37°C and then saturated with 1% (wt/vol) BSA (Sigma-Aldrich). Serum samples were assayed using serial dilutions and incubated for 2 h at 37°C. Specific binding was detected

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using HRP-conjugated goat anti–mouse secondary antibody (diluted 1:2,000; Cell Signaling Technology) followed by the addition of *o*-phenylenediamine dihydrochloride substrate (Sigma-Aldrich). HCl 1N was used to block the reaction. The optical density was read at 490–630 nm. Each sample was tested against nonimmune serum and PBS as background controls. Amounts of IL-6 in the serum were analyzed following the instructions provided by the ELISA kit supplier (BD).

Western blotting. 20 µg *Pb*NK65 WT protein extract from asexual blood stages were separated by SDS-PAGE (4–12% Bis-Tris gels; BOLT mini gel system; Thermo Fisher Scientific) and transferred onto a polyvinylidene fluoride membrane (iBLOT system; Thermo Fisher Scientific). Sera from uninfected, WT, or *Pb*NK65-*hrf*Δ1 mice were added (1:1,000 dilution) and incubated overnight at 4°C. After washing the membrane with PBS + Tween 20 (Sigma-Aldrich), polyclonal anti–mouse IgGs (1:20,000; P0260; Dako) were added, and specific bands were visualized with the SuperSignal West Pico kit (Thermo Fisher Scientific) according to the manufacturer's instructions.

Adoptive transfer of IgG-specific antibodies and CD11b-positive cells. C57BL/6J mice were infected with either WT or *Pb*NK65-*hrf*Δ1 parasites as described in the previous paragraph. Specific IgGs and $CD11b^+$ cells were obtained from challenged protected mice at day 15 p.i. Immune sera were collected, and the IgG fraction was purified on an immunoabsorbent protein G–Sepharose column (BioVision). 100 µg IgGs was transferred i.p. into naive mice 24 h before WT *Pb*NK65 iRBC infection. Mice were then given 100 µg of antibody on days 3 and 6 p.i. Single-cell suspension of $CD11b^+$ cells was obtained from naive or *Pb*NK65-*hrf*Δ1–infected mice by FACs sorting of spleen and bone marrow cells stained with FITC-labeled anti-CD11b. Each mouse received intravenous injections of 2.5×10^6 $CD11b^+$ cells 1 h after WT *Pb*NK65 iRBC infection.

Generation and cloning of *Pb*NK65-*hrf*Δ parasites. For construction of the targeting vector for *pbhrf* disruption (*PbHRF*; plasmid provided by P. Smooker and K. Taylor, RMIT University, Bundoora, Australia), DNA fragments corresponding to the 5′ untranslated (UTR) and 3′UTR regions of the *pbhrf* gene were amplified by PCR using *P. berghei* NK65 genomic DNA (gDNA) as a template. These primers (Table S2) were tailed with restriction sites for ApaI, PstI, KpnI, and EcoRI to facilitate cloning into either side of the *hDHFR* cassette (de Koning-Ward et al., 2000) in pUC18 backbone. The targeting construct was integrated into the *pbhrf* gene locus by double crossover recombination, resulting in the disruption of *pbhrf* and conferring WR99210 or pyrimethamine resistance. Transfection into a GFP-*Pb*NK65 parasite strain and selection of recombinant parasite clones were performed as previously described (Janse et al., 2006). In brief, after overnight culture (37°C with 10% O_2 and 5% CO_2 at 90 rpm) of the blood of infected animals, mature schizonts were purified using a Nycodenz gradient and collected at room temperature. The electroporation mix was composed of 10^7–10^8 merozoites resuspended in 50 µl and 100 µl of human T cell Nucleofector solution (Amaxa) and 5 µl DNA (containing 5 µg of digested DNA in water). Parasites were electroporated using the U33 program of the Nucleofector electroporator (Amaxa) and immediately resuspended in PBS and injected intravenously into 3-wk-old female SW mice. Recipient mice were treated with 0.07 mg/ml pyrimethamine in drinking water or with 6 mg/kg WR99210 by subcutaneous injections, starting 24 h after electroporation. At day 6 after electroporation, the emerging parasite population was collected, gDNA was extracted, and genomic integration of the *hDHFR* cassette (within the *pbhrf* locus of GFP-*Pb*NK65 transfectants) was confirmed using specific PCR primers: (a) Apa-5′UTR *Pb*HRF-F and EcoRI-3′UTR *PbHRF*-R, (b) *HRF*5′-F and *hDHFR*5′-R, and (c) *HRF*3′-R and h*DHFR*3′-F. The first pair of primers (Apa-5′UTR *PbHRF*-F and EcoRI-3′UTR *PbHRF*-R) amplified gDNA encompassed by the ApaI and EcoRI restriction sites and inclusive of the *pbhrf* 5′ and 3′UTRs. A PCR product of 2,681 bp was indicative of *hrf*Δ, whereas a PCR product of 1,760 bp denoted a WT genotype. The second pair of primers (*HRF*5′-F and h*DHFR*5′-R) amplified DNA ouside the *hDHFR* insert (inclusive of the 5′UTR *PbHRF*) and within the 5′ region of the *hDHFR* cassette, respectively. A PCR product of 700 bp indicated a *hrf*Δ clone, whereas absence of a band denoted a WT genotype. The third pair of primers (h*DHFR*3′-F and *HRF*3′-R) amplified gDNA within the 3′ region of the *hDHFR* cassette and outside the *hDHFR* insert (inclusive of the 3′UTR *pbhrf*), respectively. A PCR product of 1,100 bp indicated a *hrf*Δ clone, whereas absence of a band indicated a WT genotype.

Statistical analysis. All data were analyzed using Prism 5.0 software (GraphPad Software). Unpaired data between two groups at a specific time point were analyzed by a Mann-Whitney test for nonparametric analysis when data did not fit a Gaussian distribution. A p-value <0.05 was considered to be statistically significant. All experiments were replicated several times as indicated in the figure legends.

Southern blotting. gDNA was obtained as follows: parasite pellets obtained by saponin lysis of iRBCs were resuspended in PBS and treated with 150 µg/ml proteinase K and 2% SDS at 55°C for 20 min. The DNA was isolated from the parasite pellet using the DNeasy blood and tissue kit (QIAGEN). The DNA was digested with EcoRV and probed with an *hrf* probe.

Mass spectrometry analysis, database search, protein identification, and statistical analysis. After the immunoprecipitation, proteins were solubilized in denaturation buffer (10 mM Tris, pH 8, and 8 M urea). Proteins were reduced, alkylated, and digested with trypsin. Tryptic peptides were analyzed by

nano LC-MS/MS (liquid chromatography coupled with tandem mass spectrometry) using a chromatograph (EASY-nLC 1000; Thermo Fisher Scientific) coupled to a mass spectrometer (Q Exactive Orbitrap). About 1 µg of each sample (dissolved in 0.1% formic acid [FA]) was loaded at 250 nl/min on a homemade C18 50-cm capillary column picotip silica emitter tip (75-µm diameter filled with 1.9 µm Reprosil-Pur Basic C18-HD resin [Dr. Maisch GmbH]) equilibrated in solvent A (0.1% FA). The peptides were eluted using a two-slopes gradient of solvent B (0.1% FA in acetonitrile) from 2–30% in 90 min and from 30–80% in 60 min at a 250-nl/min flow rate (total length of the chromatographic run was 180 min). The mass spectrometer (Q Exactive Orbitrap; Thermo Fisher Scientific) was operated in data-dependent acquisition mode with XCalibur software (version 2.2; Thermo Fisher Scientific). Survey scan MSs were acquired in the Orbitrap on the 300–1,800-m/z range with the resolution set to a value of 70,000 at m/z = 400 in profile mode (automatic gain control target at 1E6). The 20 most intense ions per survey scan were selected for higher energy collisional dissociation fragmentation (NCE 28), and the resulting fragments were analyzed in the mass spectrometer at 17,500 resolution (m/z of 400). Isolation of parent ion was fixed at 2.5 m/z, and the underfill ratio was fixed at 0.1%. Dynamic exclusion was used within 20 s. Each sample was prepared in triplicate.

Data were searched using MaxQuant (version 1.4.1.2; with the Andromeda search engine) against the Plasmodium_berghei database (22,006 entries). The following search parameters were applied. Carbamidomethylation of cysteines was set as a fixed modification. Oxidation of methionine and protein N-terminal acetylation were set as variable modifications. The mass tolerances in MS and MS/MS were set to 10 parts per million for each, respectively. Two peptides were required for protein identification and quantitation. Peptides and proteins identified with a false discovery rate <0.01% were considered as valid identification. Statistical analysis of the data was performed using Perseus, R package, MSstat, and internal tools. Two sample Student's *t* tests were used to identify significantly regulated proteins between two groups. The results were visualized on volcano plots.

Immunoprecipitation. Immunoprecipitation of *Pb* proteins from parasite extracts was performed using the Pierc Direct immunoprecipitation kit (Thermo Fisher Scientific). Before immunoprecipitation, 10 µg of purified IgG antibodies from the serum of protected, infected, and naive mice was directly immobilized onto an agarose support (AminoLink Plus Resin) using a short coupling protocol. 500–700 µg of parasite extracts was incubated with the immobilized antibody to form the immune complex with gentle end-over-end mixing overnight at 4°C. To remove nonbound material, beads were washed three times with wash buffer, and a low pH elution buffer was used to dissociate the bound antigen from the antibody. Immunoprecipitated proteins were then used for mass spectrometry analysis.

Online supplemental material. Fig. S1 shows disruption of the *pbhrf* gene in *Pb*NK65 parasites. Fig. S2 shows assessment of leukocyte depletion and the role of neutrophils and DCs in the occurrence of splenomegaly. Fig. S3 shows identification of immune sera–derived immunoprecipitated proteins. Table S1 contains a list of oligonucleotides used for RT-qPCR analyses. Table S2 contains a list of oligonucleotides used for PCR of WT and recombinant parasites. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20151976/DC1.

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The authors declare no competing financial interests.

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SUPPLEMENTAL MATERIAL

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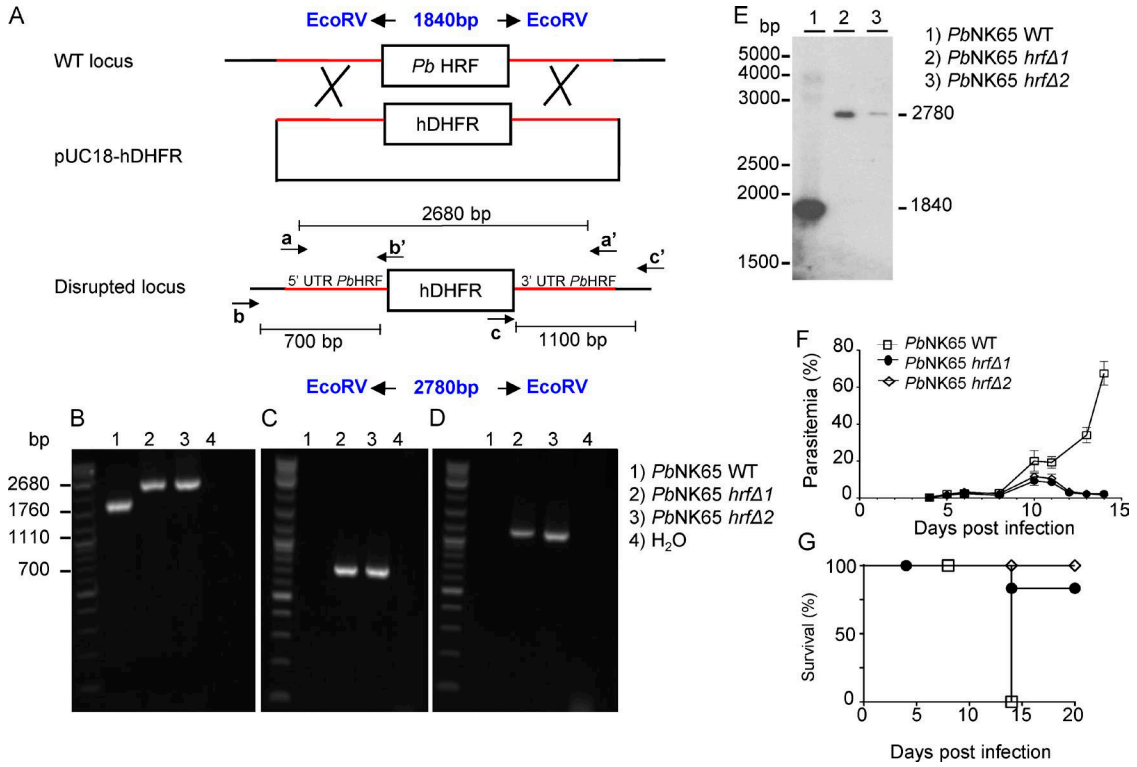


Figure S1. **Disruption of the *pbhrf* gene in *Pb*NK65 parasites.** (A) Schematic representation of the strategy used to delete the *pbhrf* open reading frame in *Pb*NK65 parasites using double-crossover homologous recombination. Red lines represent regions of homology. Successful recombination disrupts the HRF-coding sequence and replaces it with the drug resistance marker *hDHFR*. (B–D) Specific PCR primers (Table S2) were used to assess genomic integration of *hDHFR* in *Pb*NK65-*hrf*Δ clones. The primers used for PCR analysis include a + a' (B), b + b' (C), and c + c' (D), with gDNA from the following: lane 1, WT parasites; lane 2, *hrf*Δ clone 1; lane 3, *hrf*Δ clone 2; and lane 4, H_2O. (E) Southern blot analysis of the *pbhrf* locus in *Pb*NK65 WT, *Pb*NK65 *hrf*Δ1, and *Pb*NK65 *hrf*Δ2 mutant locus in *Pb*NK65 parasites. WT locus = 1,840 bp, whereas *hDHFR* insertion = 2,780 bp. (F and G) C57BL/6 mice were inoculated with either 10^5 GFP-expressing WT, *Pb*NK65-*hrf*Δ1, or *Pb*NK65-*hrf*Δ2 iRBCs, and parasitemia (F) or survival (Kaplan-Meier survival plots: log-rank test; $n = 11$; $P = 0.007$; G) was followed over time. Error bars, SEM. Experiments were replicated three times.

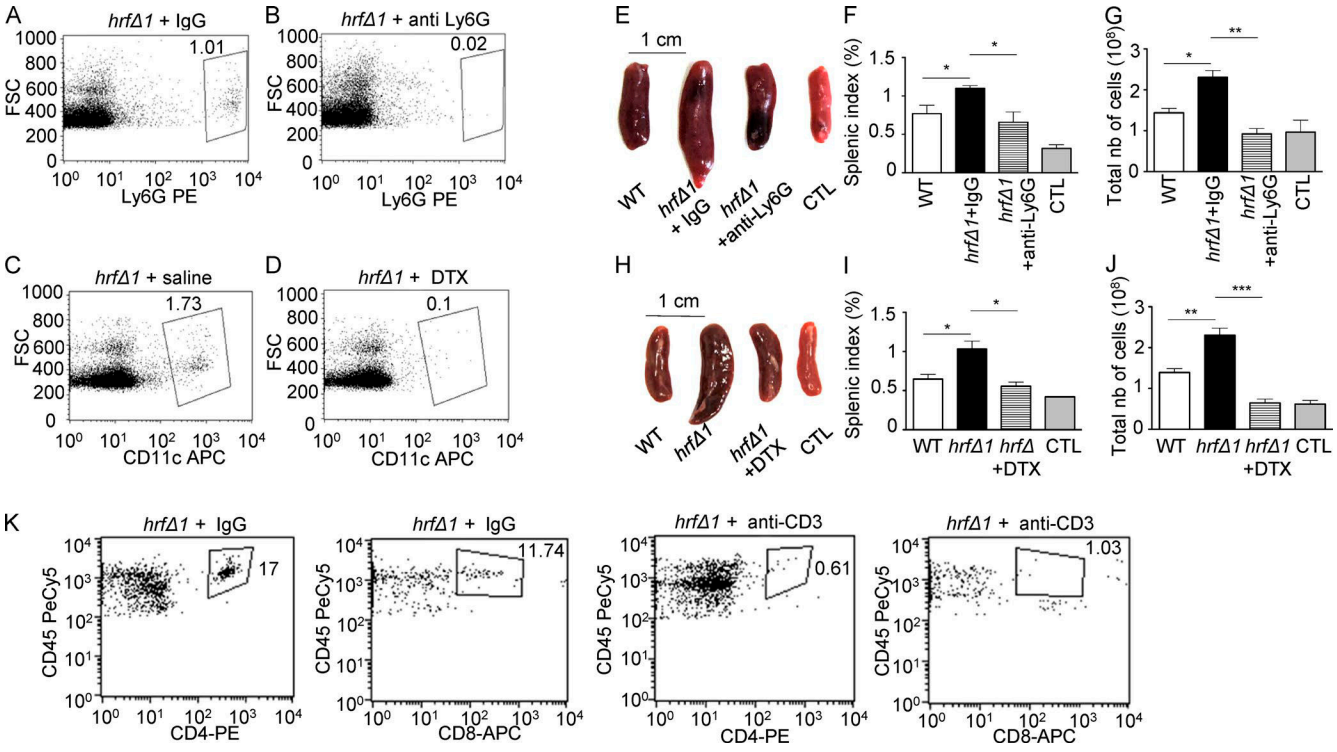
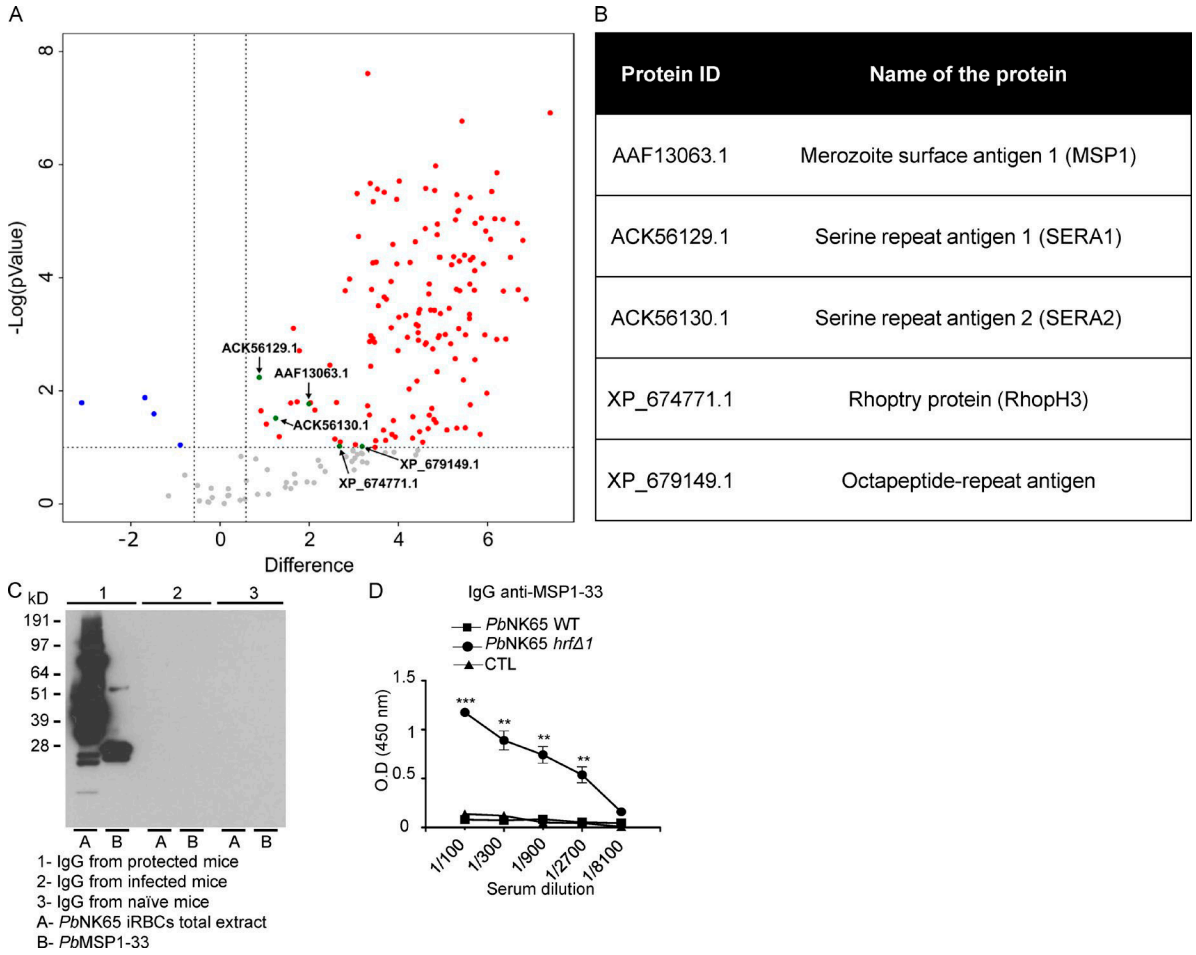


Figure S2. **Assessment of leukocyte depletion and role of neutrophils and DCs in the occurrence of splenomegaly.** (A and B) In vivo depletion of neutrophils using antineutrophil antibody (B) or normal IgG (A) was assessed by measuring at day 6 p.i. the percentage of residual Ly6G$^+$ neutrophils in spleens by FACS analysis. (C and D) Depletion of DCs was performed by injection of diphtheria toxin (DTX; D) or saline (C) into CD11c-DTR-GFP mice, and at day 6 p.i., the percentage of CD11c$^+$ cells was determined in spleens by FACS analysis. (E–J) Comparison at day 6 p.i. of splenic indexes and total number of leukocytes in WT and *Pb*NK65-*hrf*Δ1–infected mice untreated or depleted of neutrophils (E–G) or DCs (H–J). (K) Control of T cell depletion (Fig. 2 G): protected mice received anti-CD3–depleting antibody 1 d before a challenge with WT parasites followed by two booster injections of anti-CD3 at days 1 and 3 after challenge with *Pb*NK65 WT parasites. T cell depletion efficiency was assessed by FACS analysis using anti-CD4–PE or anti-CD8–APC in blood samples from protected mice that were challenged at day 15 p.i. with *Pb*NK65 WT parasites. Analysis was performed 10 d after challenge. Error bars, SEM. Data are representative of two independent experiments with five to six mice per group. *, P = 0.028; **, P = 0.015; ***, P = 0.009; Mann-Whitney test. CTL, control; FSC, forward side scatter; nb, number.



Protein ID	Name of the protein
AAF13063.1	Merozoite surface antigen 1 (MSP1)
ACK56129.1	Serine repeat antigen 1 (SERA1)
ACK56130.1	Serine repeat antigen 2 (SERA2)
XP_674771.1	Rhoptry protein (RhopH3)
XP_679149.1	Octapeptide-repeat antigen

Figure S3. **Identification of immune sera–derived immunoprecipitated proteins.** (A–D) *P. berghei* antigens recognized specifically by IgGs from *Pb*NK65-*hrf*Δ1–protected mice serum were identified by mass spectrometry. (A) Volcano plot representing results of the immunoprecipitated proteins of *Pb*NK65 parasite extract. This plot is colored such that those points having a fold change <1.5 are shown in gray, points >1.5 are in red, and points <1.5 are in blue. Green and red dots display both large-magnitude fold changes (x axis) as well as high statistical significance (−log10 of p-value, y axis). The dashed black line shows where P = 0.05, with points above the line having P < 0.05 and points below the line having P > 0.05. Statistical analysis was performed on triplicate samples. (B) Selected proteins for futher validation are in green in A, and they are reported in the table. (C and D) Evidence that protected sera actually recognize the green dot, GenBank accession no. AAF13063.1 identified as MSP1 protein was assessed by using a recombinant *Pb*MSP1-33 protein by immunoblotting (C) and by ELISA (D). Error bars, SEM. Experiments were replicated three times (six mice per group). **, 0.02 < P < 0.028; ***, P = 0.0002; Mann-Whitney test. CTL, control.

Table S1. **List of oligonucleotides used for RT-qPCR analyses**

Primer	Forward / Reverse	Sequence (5′–3′)
Pb 18S	Forward	ATTAATCTTGAACGAGGAATGGCT
	Reverse	TCAATCGGTAGGAGCGACG
Pb LSP2	Forward	GCCAAATGCTAAACCTAATG
	Reverse	TGGGTTTGTATTGTATGCAC
Pb HSP70	Forward	TGCAGCTAATCAAACTC
	Reverse	ACTTCAATTTGTGGAACACC
mu IL-23	Forward	CCACCAGGACTCAAGGACAACA
	Reverse	GCAGGCTCCCCTTTGAAGA
mu EBI3	Forward	CAGAGTGCAATGCCATGCTCC
	Reverse	GCCACACCGAGCCTGTAAGT
mu IL-12p35	Forward	TACTAGAGAGACTTCTTCCACAACAAGAG
	Reverse	GATTCTGAAGTGCTGCGTTGAT
mu IL-12p40	Forward	GGAAGCACGGCAGCAGAATA
	Reverse	AACTTGAGGGAGAAGTAGGAATGG
mu IFN-γ	Forward	AAAGGATGCATTCATGAGTATTGC
	Reverse	CGCTTCCTGAGGCTGGATT
mu IL-6	Forward	AAAGAAATGATGGATGCTACCAAAC
	Reverse	CTTGTTATCTTTTAAGTTGTTCTTCAT GTACTC
mu IL-10	Forward	GGCGCTGTCATCGATTTCTC
	Reverse	GACACCTTGGTCTTGGAGCTTATTAA
mu HPRT	Forward	CTGGTGAAAAGGACCTCTCG
	Reverse	TGAAGTACTCATTATAGTCAAGGGCA

Table S2. **List of oligonucleotides used for PCR of WT and recombinant parasites**

Oligonucleotide	Sequence (5′–3′)
ApaI-5′UTR-*PbHRF*-F (**a**)	CGCGGGCCCGCGCATTATTACCGTTGTCA
PstI-5′UTR-*PbHRF*-R	CGCCTGCAGGGCTTATGCAAGTATCGAACAA
KpnI-3′UTR-*PbHRF*-F	CGCGGTACCTTGCTACATGACGCATAAACC
EcoRI-3′UTR-*PbHRF*-R (**a′**)	CGCGAATTCTGTGAAATCGACAATGTTTTGG
*HRF*5′-F (**b**)	GCGATACAAACAAATTTATTCAGC
*HRF*3′-R (**c′**)	CGCAAGATATCAGAGCTTTTCA
h*DHFR*3′-F (**c**)	TGTTGTCTCTTCAATGATTCATAAATAGTTGG
h*DHFR*5′-R (**b′**)	TGCTTTGAGGGGTGAGCATTTAAAGC
PbHRF-5′orf-F	CCATTTGGAAATGCGGAAT
PbHRF-3′orf-R	TTTTTCTTCAAATAAACCATCTGA

Bold letters refer to the oligonucleotide's position in Fig. S1 (A and B).

Supplementary Results

5.1 HRF is important for *P. berghei* pre-erythrocytic and erythrocytic infection

Similar to previous studies performed on *Pb*ANKA strain, we examined the contribution of *Plasmodium* HRF to the phenotype of *Pb*NK65 both at the level of the pre-erythrocytic phase and at the blood stage. As depicted in Fig. 17A, mice inoculated with *Pb*NK65-*hrfΔ* sporozoites showed an initial delayed development of the parasite followed by a complete clearance during blood stage at around day 17 p.i., in a similar way as the parasite was inoculated with iRBCs (Fig. 17B). Since similar phenotypes were obtained with both sporozoites and iRBCs, the follow-up investigation was carried out using iRBCs to explore the mechanisms by which HRF shapes the immune response.

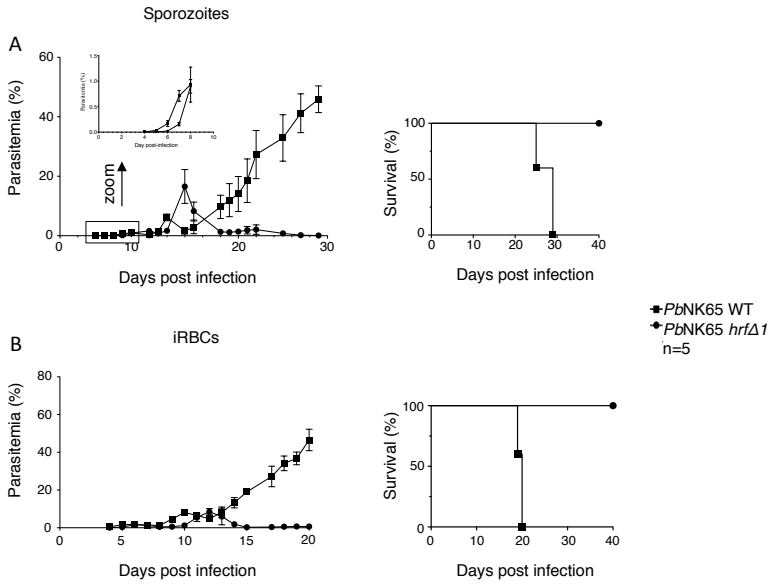


Figure 17: Marked differences in parasitaemia between WT and *hrfΔ Pb*NK65-infected mice in blood stage development. Blood stage parasitemia and survival (Kaplan-Meier survival plots) of C57BL/6 mice infected WT or *hrfΔ Pb*NK65 parasites (A) i.v. with 1000 isolated sporozoites or (B) i.p. with 10^5 iRBCs. Error bars, SEM. Data are representative of two independent experiments with 5 mice per group. (Following methods already described in the previous article (Demarta-Gatsi et al., 2016)).

5.2 Pattern of cytokines associated with *Pb*NK65-*hrfΔ* induced protection

It is known from previous studies that both $CD4^+$ and $CD8^+$ T cells play a central role in the elimination of blood-stage malaria parasites through the release of cytokines that activate other effector cells such as NK cells, macrophages and dendritic cells. In addition, Th1

cytokines IL-12, IFN-γ, and TNF-α, were shown to confer immunity against blood-stage *Plasmodium* infection (Perlmann and Troye-Blomberg, 2002). To examine whether the infection of C57BL/6 mice with either the WT or *hrf*Δ *Pb*NK65 parasite induces a particular set of cytokines, we first examined the mRNA expression of a variety of cytokines by RT-PCR in the spleen of infected mice. Spleens were analyzed at various time intervals, starting from day 2 until day 14, post infection (Fig. 18A).

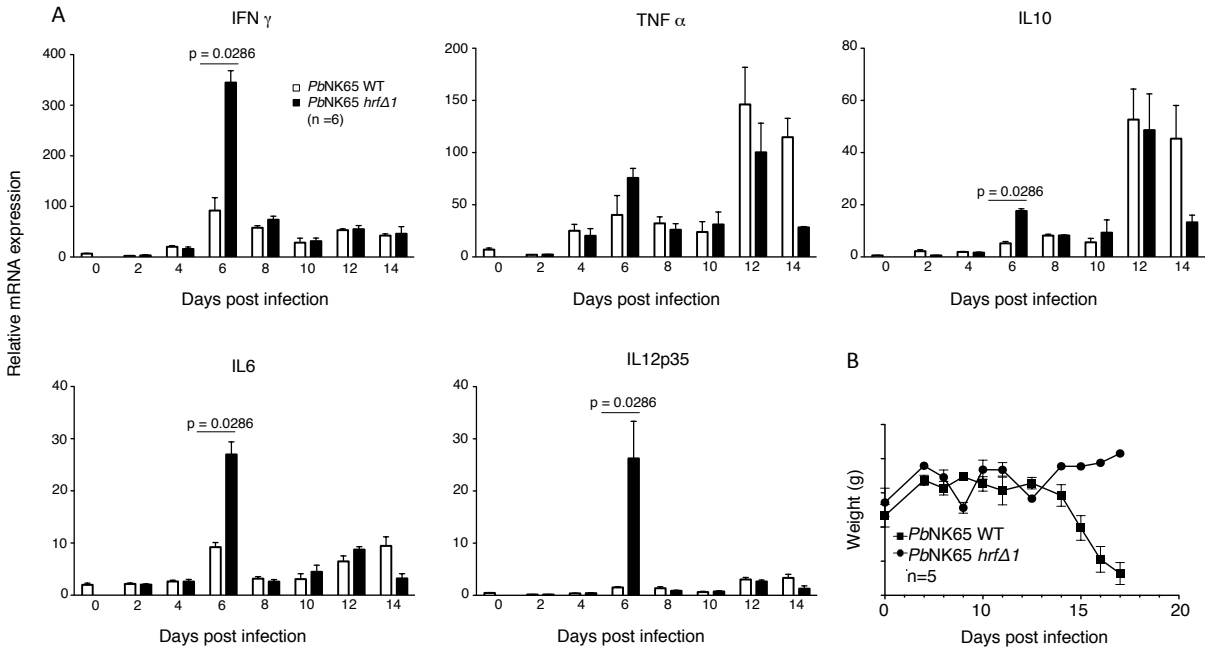


Figure 18: Immune response genes are differentially regulated by WT and *hrfΔ1* parasites. (A) mRNA levels (RT-qPCR) normalized to HPRT of cytokine production in spleen cells measured at different time points p.i., each 2 days from day 2 to day 14 p.i., from WT or *Pb*NK65-*hrfΔ* infected mice. Error bars, SEM. Data are representative of two independent experiments with 5 mice per group. (Following methods already described in the previous article (Demarta-Gatsi et al., 2016). (B) Determination of body weight during infection. C57BL/6 mice were inoculated with either 10^5 GFP-WT or *hrfΔ* iRBCs and the weight was measured over time. (Following methods already described in the previous article (Demarta-Gatsi et al., 2016)).

Analyzed samples showed that IL-23, EBI-3 (IL-27 beta subunit), IL-12p40, IL-12p35, IFN-γ, IL-6, and IL-10 mRNA expressions were all higher in *Pb*NK65-*hrfΔ* infected mice as compared to mice infected with WT parasites at day-6 p.i. (Fig. 19A, B). Higher levels of IFN-g, IL-12p70, and IL-6, as measured by ELISA, were confirmed in the plasma *Pb*NK65-*hrfΔ* infected mice as compared to WT parasite-infected mice (Fig. 19C). This burst of pro-inflammatory cytokines induced by *Pb*NK65-*hrfΔ* parasites was followed by a significant loss of weight at day 8 p.i. (Fig. 18B), which resulted first in the decrease of the parasitemia (Fig. 17B) followed by the

normalization of the weight a few days later (day 10 p.i.). The loss of weight from day 4 to day 8 represented 10% of the weight of control mice at the same age. In contrast, mice infected with WT parasites did not show any loss of weight until day 8 where this was continuously decreasing until the death of the mice (Fig. 17B). Mice that were infected with WT parasites displayed a delayed peak of TNF- α, IL-10 and IL-6 at day 12-14 post infection which in addition to high parasitemia may be associated to a drastic loss of body weight in these mice starting from day 14 (Fig. 18B).

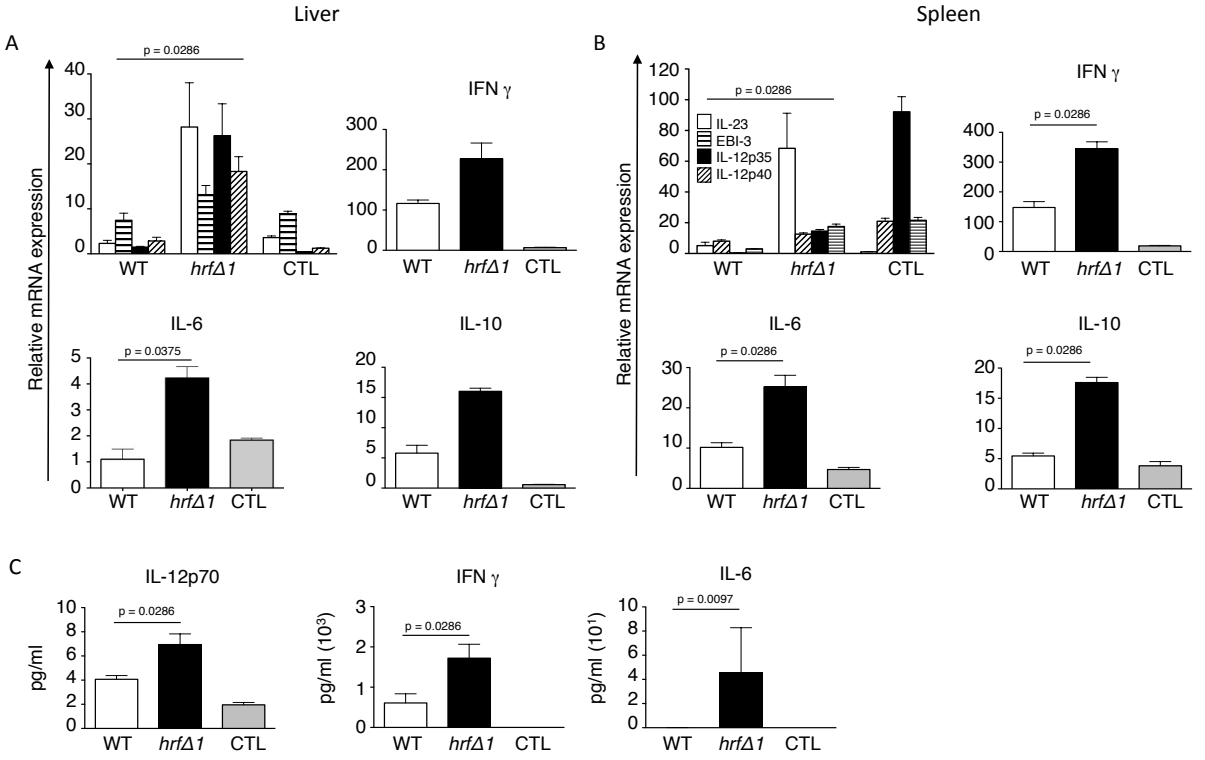


Figure 19: Cytokine production by blood-stage WT and *hrfΔ Pb*NK65 infection. A, B and C) mRNA levels (RT-qPCR) normalized to HPRT of cytokine production in liver cells (A), in spleen cells (B) and in the serum (ELISA) (C) measured at day 6 p.i. from WT or *Pb*NK65-*hrfΔ* infected mice. CTL: mRNA and sera from naive mice. Error bars, SEM. Data are representative of four (A,B and C) independent experiments with 5 mice per group. (Following methods already described in the previous article (Demarta-Gatsi et al., 2016)).

5.3 Augmented CD11b$^+$ cells during *Pb*NK65-*hrfΔ* infection

Detailed analyses of two organs (spleen and liver) mainly characterized by high parasitemia following malaria infection show differences in the innate immune cell activation (Fig. 20). As compared to the *Pb*NK65 WT infected spleen, we observed an increase in the total number of F4/80$^+$ macrophages and CD11c$^+$ CD11b$^+$ dendritic cells in the *Pb*NK65-*hrfΔ* infected spleen

(Fig. 20B), whereas we found the opposite in the infected liver (Fig. 20A). In addition, we observed no differences in the percentage and total number of Ly6G$^+$ neutrophils between WT and *hrfΔ Pb*NK65-infected spleens but increased number of neutrophils in the *Pb*NK65 WT infected liver as compared to the *Pb*NK65-*hrfΔ* infected liver. Taken together, these data suggest organ-specific innate cell activation after malaria infection.

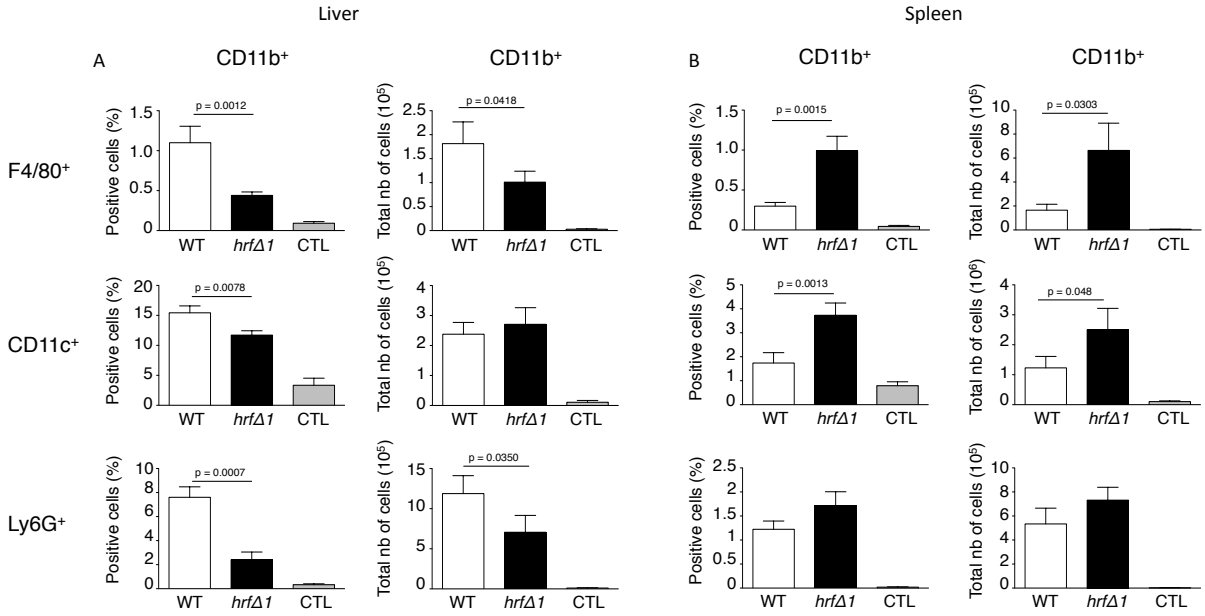


Figure 20: Innate immune cells are activated in the spleen and liver after malaria infection. (A) Liver analysis show increased levels of innate immune cells in mice infected with WT *Pb*NK65 parasites. (B) Spleen analysis show increased levels of innate immune cells in mice infected with *Pb*NK65-*hrfΔ* parasites. CTL: spleen from naive mice. Error bars, SEM. Data are representative of three independent experiments with 9 mice per group. (Following methods already described in the previous article (Demarta-Gatsi et al., 2016)).

5.4 Protection conferred by mutant parasites is dependent on effector CD4$^+$ T cells.

In order to show that the protection induced by *Pb*NK65-*hrf*Δ parasites is dependent on effector CD8$^+$ or CD4$^+$ T cells, protected mice were treated with normal mouse IgG, anti-CD8 or with anti-CD4 depleting antibody. The dose of each antibody used was set to 20 mg for anti-CD8 (clone 53-6.7, Armenian hamster IgG, eBiosceince) and 100 mg for anti-CD4 (clone GK1.5). The different blood cell populations were monitored by flow cytometry 24h after intravenous injection. Mice were challenged with 10^5 iRBCs of WT *Pb*NK65 parasites and both parasite growth and cells depletion efficacy were monitored daily by flow cytometry in blood samples (Fig. 21). The results confirmed the specific reduction of CD8$^+$ or CD4$^+$ T cells

in animals injected with anti-CD8 and anti-CD4 respectively. Interestingly, the measurement of parasitemia indicated a loss of parasite control upon treatment of protected mice with anti-CD4 antibody but not with anti-CD8 antibody. WT parasite-challenged mice treated with control IgG remained parasite free. This suggests that the activation of effector $CD4^+$ T lymphocytes but not $CD8^+$ are indispensable for clearance of malaria infection in long term immune protection. These results are further supported by previous studies in animal models where depletion or adoptive transfer of the different T cell populations highlighted the important role of $CD4^+$ T cells in the development of protective immunity against blood stages (Taylor-Robinson, 2002; van der Heyde et al., 1997).

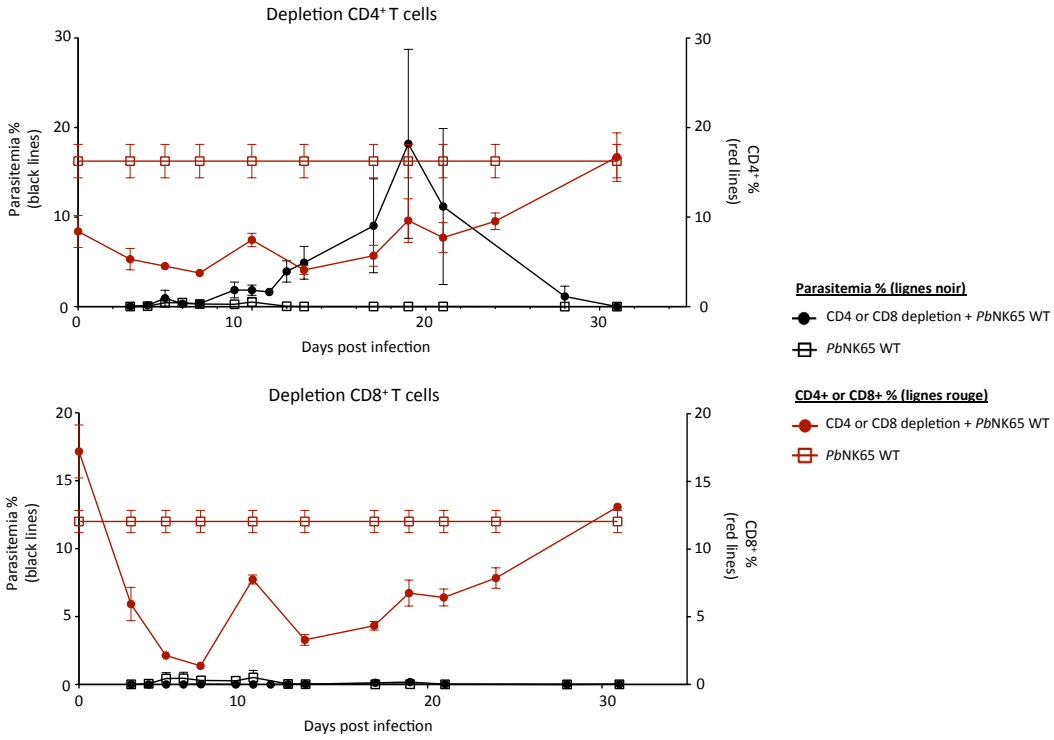


Figure 21: Influence of $CD4^+$ or $CD8^+$ T cells depletion on parasite development in protected mice. Protected mice were treated either with IgG or with anti-CD4-depletion Abs or anti-CD8-depletion Abs one day prior to a challenge with WT parasites followed by two booster injections of anti-CD4 and anti-CD8 at days 1 and 3 after challenge. Error bars, SEM. Data are representative of two independent experiments with 5 mice per group. (Following methods already described in the previous article (Demarta-Gatsi et al., 2016)).

5.5 Protection induced by *Pb*NK65-*hrfΔ* parasites is associated with down-regulation of PD-1 on T cells

As previously observed, mice lacking T cells were unable to control the parasitemia of *Pb*NK65-*hrf*Δ infected mice, suggesting that the activation of T lymphocytes may be indispensable for immune memory against malaria infection. In order to better characterize the molecular signatures of the T cell response and given that chronic malaria infection results in an increased frequency of T cells expressing surface markers of exhaustion such as programmed cell death-1 (PD-1) (Butler et al., 2012), we asked whether the self-resolving nature of *Pb*NK65-*hrf*Δ infection could be correlated to a change in PD-1 expression on the surface of effector T cells. When we analyzed PD-1 expression by flow cytometry at day 6 and day 20 p.i. (Fig. 22A, B), we observed that WT parasites induced a high proportion of $PD1^+CD4^+$ and $PD1^+CD8^+$ T cells in the spleen hinting at T cell exhaustion. In contrast, *Pb*NK65-*hrf*Δ parasites induced two-fold fewer $PD1^+$ $CD4^+$ and $PD1^+$ $CD8^+$ T cells at day 6 p.i. as compared to WT parasites, with this number decreasing to basal levels found in uninfected mice at day 20 p.i. (Fig. 22A). We further correlated PD-1 expression to the activation status of T cells using the cell surface marker CD62L: low or undetectable levels of surface CD62L are indicative of T cell activation and effector function (Oehen and Brduscha-Riem, 1998). At day 6 p.i. we observed in WT parasite-infected mice, 65% and 70% of activated $CD4^+CD62L^-$ and $CD8^+CD62L^-$ T cells, respectively, were $PD\text{-}1^+$ whereas only 40% of activated $CD4^+CD62L^-$ and $CD8^+CD62L^-$ T cells were positive for $PD\text{-}1^+$ in mice infected with *Pb*NK65-*hrf*Δ parasites (Fig. 22B). The number of $PD\text{-}1^+$ activated T cells further decreased at day 20 p.i. in *Pb*NK65-*hrf*Δ-infected mice, resetting to the basal levels found in uninfected mice (Fig. 22A, B). To investigate whether r*Pb*HRF can directly modulate the expression of PD-1 on the surface of T cells, naïve mice were injected with 100 μg of r*Pb*HRF or BSA as a negative control and 48 h later, PD-1 surface expression examined. We observed an upregulation of PD-1 on $CD4^+$ and $CD8^+$ T cells surface (Fig. 22C) and on activated $CD4^+$ $CD62L^-$ and $CD8^+$ $CD62L^-$ T cells (Fig. 22D) induced by the r*Pb*HRF. Taken together, these data demonstrate that infection with *Pb*NK65 parasites expressing *Pb*HRF is associated with an increased proportion of $PD\text{-}1^+$ T cells in mice, indicative of T cell exhaustion.

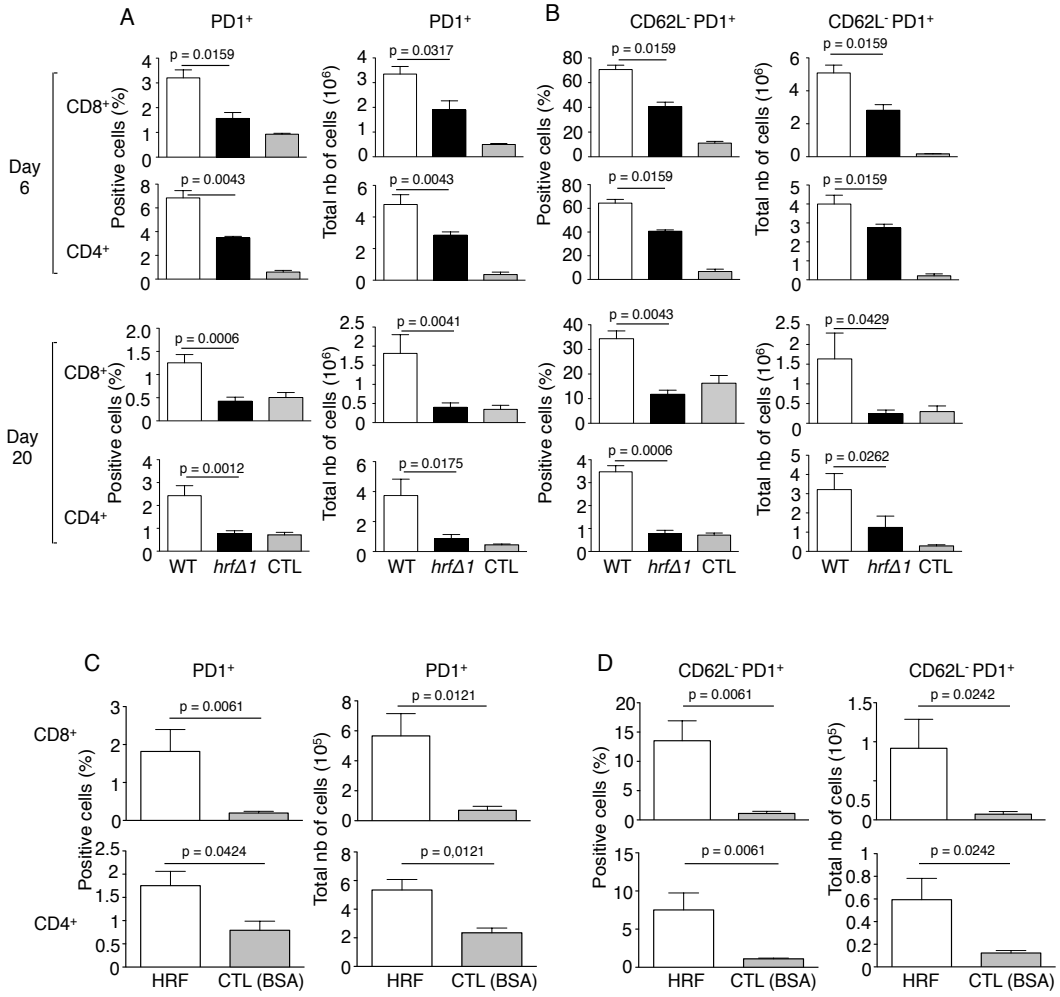


Figure 22: Protection induced by *hrf*Δ *Pb*NK65 parasites is associated with reduced induction of PD1$^+$CD8$^+$CD62L$^-$ and PD1$^+$CD4$^+$CD62L$^-$ cells in spleen. (A) Representative frequency and absolute number of CD8$^+$PD1$^+$ and CD4$^+$PD1$^+$ splenic leukocytes at day 6 and day 20 p.i. with either 10^5 GFP-WT or *hrf*Δ iRBCs. (B) Representative frequency and absolute number of CD8$^+$CD62L$^-$ and CD4$^+$ CD62L$^-$ splenic leukocytes expressing the PD1 receptor at day 6 and day 20 post infection with either 10^5 GFP-WT or *hrf*Δ iRBCs. (C) Representative frequency and absolute number of CD8$^+$PD1$^+$ and CD4$^+$PD1$^+$ splenic leukocytes 48h after *Pb* recombinant HRF administration (IV). (D) Representative frequency and absolute number of CD8$^+$CD62L$^+$ and CD4$^+$ CD62L$^+$ splenic leukocytes expressing the PD1 receptor 48h after *Pb* recombinant HRF administration (IV). Error bars, SEM. Data are representative of three (A,B) and two (C,D) independent experiments with 5 and 8 mice per group. (Following methods already described in a previous article (Demarta-Gatsi et al., 2016)).

5.6 Prior exposure to WT parasite followed by drug treatment does not hamper *Pb*NK65-*hrfΔ*-induced parasite clearance and immune protection

Results obtained from mice infected firstly with *Pb*NK65-*hrf*Δ parasites and, upon the complete clearance of *Pb*NK65-*hrf*Δ parasites, with either *Pb*NK65, *Pb*ANKA or *Py*YM WT parasites demonstrated that the sterile protection conferred by this GAP is long-lasting in a species- and stage-transcendent manner. In real field conditions, such GAP delivery would only be given to individuals who had been previously infected with WT parasites and drug treated. In endemic areas pre-exposure to WT parasites followed by antimalarial chemotherapy could induce epigenetic marks that may negatively influence anti-parasite immunity. In this situation the protective effect induced by the *Pb*NK65-*hrf*Δ mutant will not be effective. The question is to assess whether these individuals who had been exposed to WT parasites and drug cured will be equally efficiently protected following *Pb*NK65-*hrf*Δ infection as in naïve mice. To address this issue, mice were first infected with 10^5 *Pb*NK65 WT iRBCs, and when parasitemia reached around 2%, were treated for three consecutive days with 6 mg/kg WR99210 by subcutaneous injections. Once the parasites were completely eliminated from the blood stream, at day 19 p.i., mice were infected either with 10^5 WT or with *Pb*NK65-*hrf*Δ parasites iRBCs (Fig. 23). We observed that in the group of mice which have received WT parasites, parasites develop normally and the mice died around day 20 p.i.. In contrast mice, which received PbNK65-hrfΔ parasites, show a slow development of parasites which were ultimately eliminated around day 15 p.i. from peripheral blood circulation. To assess whether one single infection with the mutant parasite was enough to develop a long-lasting immune response, as we previously observed without the use of drugs, mice which received WT parasites followed by *Pb*NK65-*hrf*Δ parasites were infected with 10^5 WT iRBCs 4 weeks after the elimination of the mutant parasite from the blood circulation (Fig. 23). A control group, which has been neither infected nor treated by antimalarial drugs, was infected at the same time with 10^5 WT iRBCs. As obtained with WT parasite challenges in protected mice without drug treatment, infection with *Pb*NK65-*hrf*Δ parasites allows the development of a long-lasting immune protection despite a prior drug

treatment. This result shows that a primary infection with WT parasites followed by a drug cure does not hamper the efficacy of NK65-*hrf*Δ-induced protection.

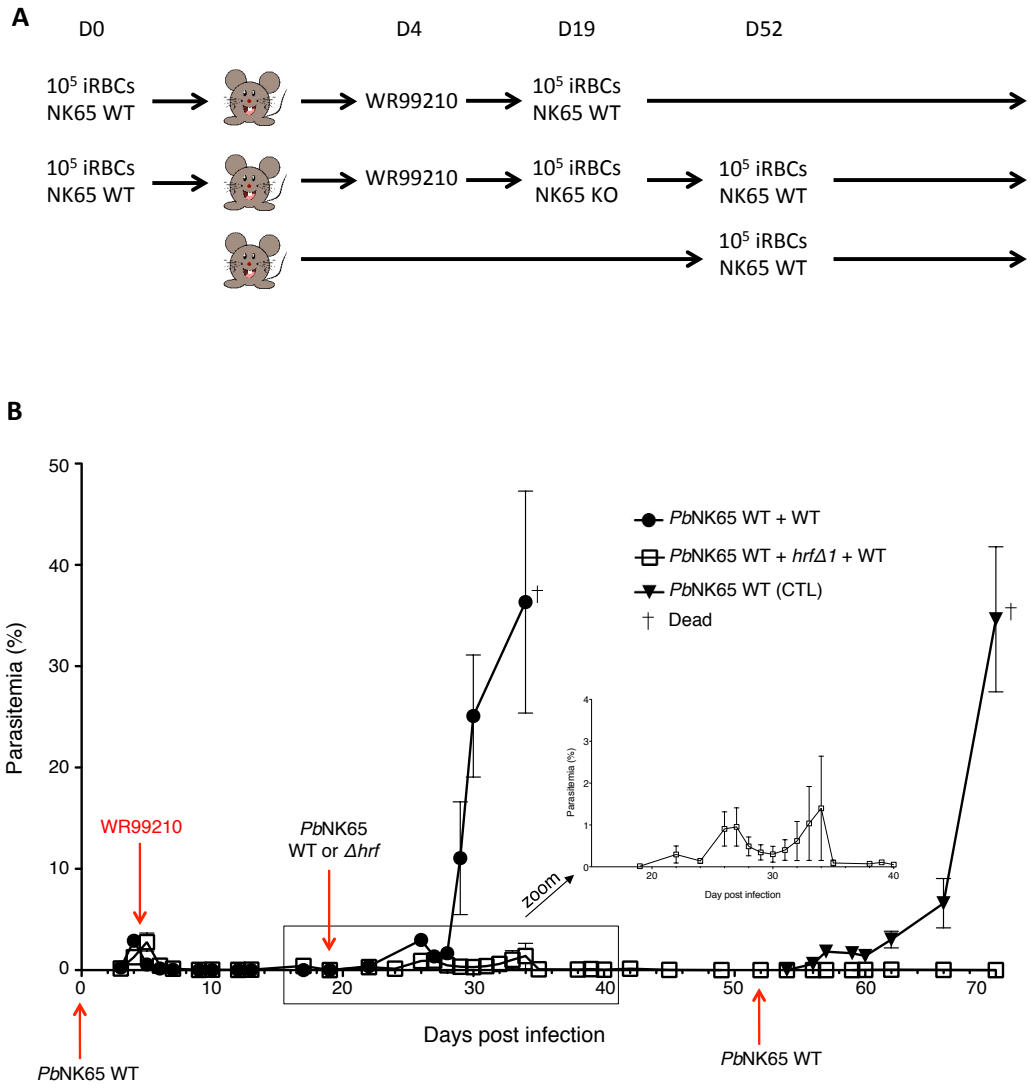


Figure 23: Drug treatment does not affect the development of long-lasting protection after infection with HRF-deficient blood stage parasites. (A) Protocol used to infect and to treat mice. (B) Blood stage parasitemia of C57BL/6 mice after i.p. injection of 10^5 WT iRBCs and treated at day 4 p.i. with 6 mg/kg WR99210 by subcutaneous injections. At day 19 p.i. infection of mice with either with 10^5 iRBCs WT or *Pb*NK65-*hrf*Δ iRBCs. Mice group infected with *Pb*NK65-*hrf*Δ parasites were subsequently, at day 52 p.i., infected with 10^5 WT iRBCs. Error bars, SEM. Data are representative of three independent experiments with 5 mice per group. (Following methods already described in a previous article (Demarta-Gatsi et al., 2016)).

5.7 *Pb*NK65-*hrfΔ* parasite-induced protection is associated with an increased number of central and effector memory B cells

The observation that *hrfΔ Pb*NK65 infected mice eliminate the parasite while they acquire a long lasting immune memory and high anti-parasite IgG Abs titers prompted us to analyse the generation of central and memory B cells. The germinal centers are the main sites where somatic hypermutations and class switch recombination occur. During the immune response, B cells express the activation-induced cytidine deaminase (AID) enzyme which acts on Ig gene loci to enhance antibody production and B cell functions. To visualize activated AID transcription in B cells, AID/YFP Tg mice were used, in which memory B cells containing populations can be divided into "central" memory (AID/YFP$^+$CD19$^+$IgM$^+$IgG) and "effector" memory (AID/YFP$^+$CD19$^+$IgM$^-$IgG$^+$) B cells. In order to investigate the generation of the immunological memory during *Plasmodium* infections AID/YFP Tg mice were infected i.p with 10^5 iRBCs of either WT or *hrfΔ* PbNK65 parasites. At day 15 p.i., mice were killed and spleen B cell populations analysed by flow cytometry (Fig. 24). From our preliminary data we observe a significant difference in the memory B cells between mice infected with either WT or *hrfΔ* PbNK65 parasites. Additionally, characterization of memory B cells populations show also significant difference in both effector and central memory B cells between the two populations of mice. As compared to the WT *Pb*NK65-infected spleen, we observed an increase in the total number of CD19$^+$AID/YFP$^+$ memory B cells and its population (IgM$^-$IgG$^+$ or IgM$^+$IgG$^-$) in the hrfΔ PbNK65-infected spleen. These preliminary results reinforce the hypothesis that the B cells are important in the immune response developed in C57BL/6 mice against the PbNK65-*hrfΔ* parasites. We can assume that the effector memory cells developed during the first infection with the PbNK65-*hrfΔ* parasites are important for the maintenance of the immune response during a long period of time, in fact challenges of protected mice with WT parasites show that the parasite is eliminated by the immune system immediately without allowing him to develop.

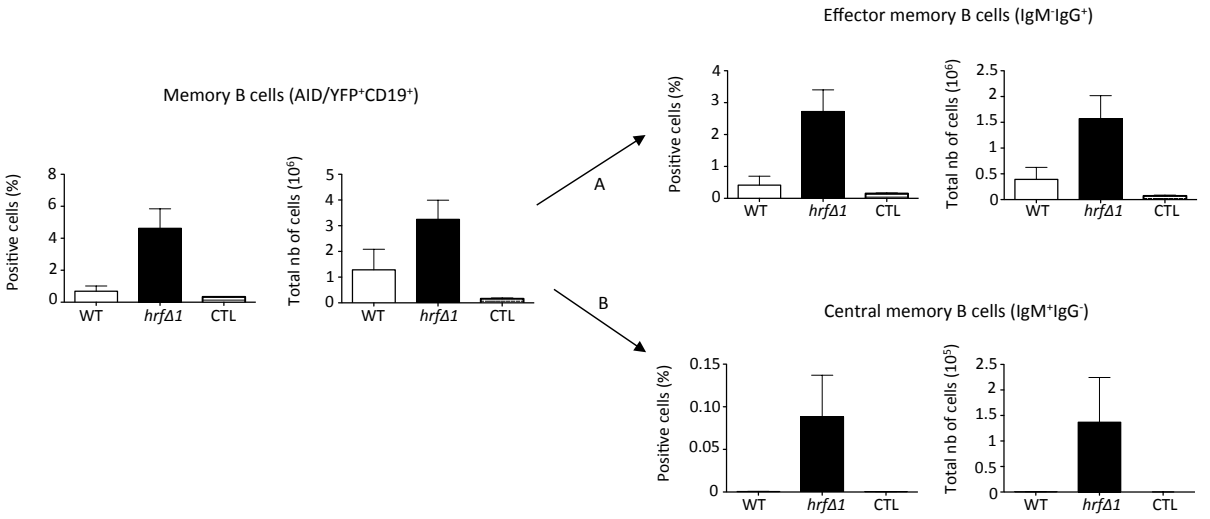


Figure 24: Frequency of memory B cells in *Pb*NK65-*hrf*Δ protected mice. Representative frequency and absolute number of AID/YFP^{+}CD19^{+} memory B cells splenic leukocytes at day 15 p.i. with either 10^{5} GFP-WT or *hrf*Δ iRBCs. (A) Representative frequency and absolute number of AID/YFP^{+}CD19^{+} memory B cells splenic leukocytes expressing IgM^{-}IgG^{+} at day 15 p.i. (B) Representative frequency and absolute number of AID/YFP^{+}CD19^{+} memory B cells splenic leukocytes expressing IgM^{+}IgG^{-} at day 15 p.i. Error bars, SEM. Data are representative of two independent experiments with 4 mice per group. (Following methods already described in a previous article (Demarta-Gatsi et al., 2016)).

Discussion

In spite of continued efforts to control the disease, malaria remains a major health problem in many regions of the world, especially sub-Saharan Africa, and new ways to control or eradicate the disease are urgently needed. Development of an effective malaria vaccine, that could greatly improve global public health by enhancing a rapid and protective immune response that completely eliminates the infection, would provide a better way of alleviating malaria. But scientists have different point of view on the best lines of research to pursue. Despite the large number of successful vaccines in use today for different diseases (such as polio, tetanus, measles, smallpox or pertussis) the development of malaria vaccine still remains a challenge for scientists. Malaria vaccine research involves the combination of vaccinology, immunology and parasitology (malariology) and there are few established theoretical principles in this field to serve as guidelines. Moreover, the differences between *Plasmodium* strains, the persistence of infection for months and the capacity of the parasite to both evade and disable the immune system by a variety of mechanisms are the major problems encountered for the development of a vaccine.

Understanding malaria pathogenesis may find its way in the investigation of possible common pathways with allergic inflammation.

Since the beginning of the humanity, the parasite has learned to live and co-evolve with the human immune system. This co-evolution has been performed through a myriad of complex mechanisms that ultimately result in the inability of the immune system to eliminate the parasite and to mediate resistance to subsequent infections. An important part in the development of an effective malaria vaccine is the understanding of the mechanisms developed by the parasite to escape the immune response or the natural immunity to malaria developed by the host. A way to address the mechanisms involved in the pathogenesis of a disease is to find out if the disease in question does not share signalling pathways associated with other pathologies. Following this line of thought we hypothesised that the *Plasmodium* parasite turns to its advantage the mechanisms involved in the allergic inflammatory response (Fig. 25).

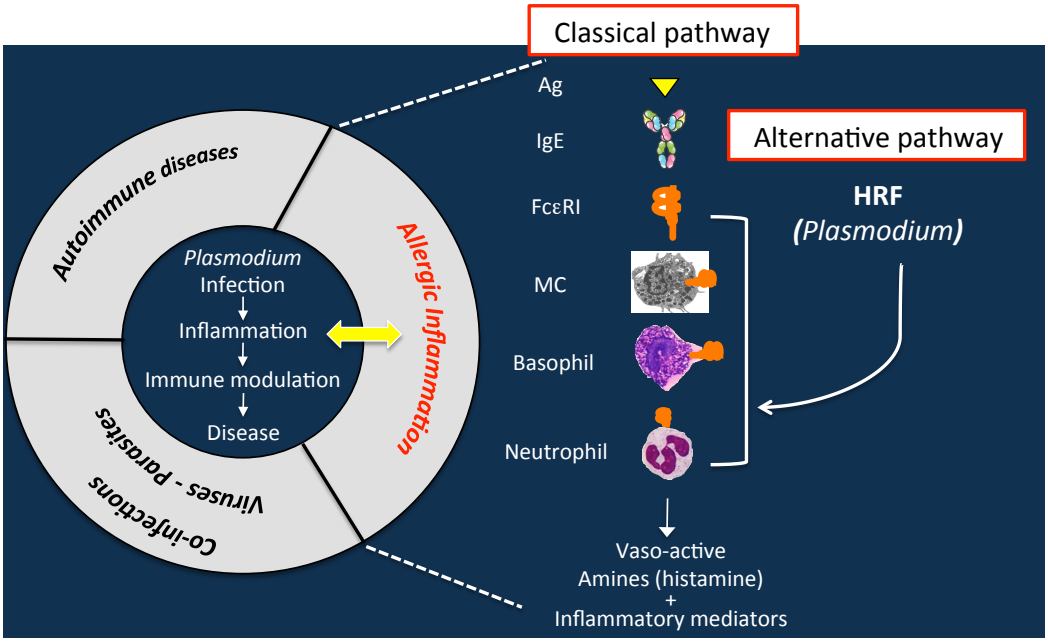


Figure 25: Hypotesis: understanding malaria pathogenesis associated with *Plasmodium* infection and allergic inflammation share common signalling pathways. The figure displays three major known pathologies: autoimmune diseases, infections diseases, and allergic responses. Indirect evidence from immuno-epidemiological studies support that an association exist between malaria pathogenesis and mechanisms involving IgE-mediated inflammatory responses. This latter consist of classical pathway which starts with the binding of antigen (Ag)-specific IgE Abs to FcεRI expressed on various leukocytes (mast cells, basophils, and neutrophils), and upon aggregation, cells will release histamine and other inflammatory mediators. Beside this classical pathway, alternative pathway independent from IgE/ FcεRI complexes, are also able to trigger mediator release. One of the prototypes IgE-independent triggers is the histamine releasing factor (HRF).

Several studies conducted in endemic areas in humans suggest a strong relationship between clinical susceptibility to malaria and severe allergic-type responses (Demeure et al., 2005; Griffiths et al., 2005; Sakuntabhai et al., 2008). Indeed, the contribution of host genetic factors to the risk of severe outcome was highlighted in different studies during *P. falciparum* infection in African populations. Large scale genetic studies in Senegal revealed that several chromosome regions were linked to clinical susceptibility to malaria. The four genome regions; 5p15-p13 and 13q13-q22 associated with clinical malaria, and 12q21-q23 and 5q31-33 associated with parasite density, evoked have been previously found to be associated to atopic disease or related phenotypes (Flori et al., 2003; Iyengar et al., 2001; Rihet et al., 1998; Sakuntabhai et al., 2008). Link between parasite density and the region on chromosome 5q31, previously found to be significantly linked to malaria parasite density and involved in the control of immunity to *P. falciparum* blood stage, have been shown to be associated to asthma/atopy related traits and to elevated IgE levels (Bouzigon et al., 2010;

Flori et al., 2003; Iyengar et al., 2001; Rihet et al., 1998; Sakuntabhai et al., 2008). Previous studies highlighted the importance of this region in immune regulation by its linkage to plasma IgE levels (Marsh et al., 1994; Meyers et al., 1994), bronchial hyper responsiveness, and to *Schistosoma mansoni* infection intensity (Postma et al., 1995) in humans and with resistance to *P. chabaudi* in mice (Hernandez-Valladares et al., 2004). The 5q31–33 region encodes for several genes mostly Th2 cytokines (IL-3, IL-4, IL-5, IL-9, IL-13) which are responsible for the differentiation of B cells that generate IgE, but also the Th1 cytokine IL-12B and other immunologically active genes such as interferon regulatory factor-1 which are strong candidates for controlling the outcome of malaria infection. The 13q13-q22 and 12q21-q23 regions contain genes, namely *PHF1153* and *STAT6*, known to increase total serum IgE levels (Chen and Khurana Hershey, 2007). Moreover, the IL-4, IL-13 and STAT6 pathways have been reported to be associated with IgE and asthma. The role of IL-4 in regulating the antibody response induced by *Plasmodium* parasite was studied using different approaches. Genetic studies on IL4 T/C single nucleotide polymorphism (SNP) in Mali (Vafa et al., 2009; Vafa et al., 2007) and in Burkina Faso (Verra et al., 2004) have highlighted the importance of the IgE antibodies induced by IL-4 pathway. Higher parasite prevalence in asymptomatic Fulani population, known to be naturally less susceptible to malarial infections than their sympatric neighbours, was correlated with the T allele, suggesting that CT genotype carriers are less successful in clearing the infection as compared to the CC genotype carriers (Vafa et al., 2007). Additionally, an increased level of IgE in the T allele group was associated to the presence of malaria parasites (Vafa et al., 2009). Burkina Faso studies in non-Fulani children correlated CT genotype carriers with higher total IgE levels with severe malaria compared to uncomplicated malaria (Verra et al., 2004).

Elevated plasma levels of IgE and anti-plasmodial IgE Abs which bind to basophils and mast cells have been associated with severity to *P. falciparum* infections in adults and children living in malaria endemic areas in different African and Asian countries (Perlmann et al., 1994; Perlmann et al., 1999; Troye-Blomberg et al., 1999a). Indeed, IgE levels were reduced amongst patients with uncomplicated malaria in comparison to those suffering from severe malaria (Seka-Seka et al., 2004) and high IgE levels were found during cerebral *P. falciparum* malaria related to the deepness of the coma compared to uncomplicated malaria

(Luty et al., 1994; Maeno et al., 2000). In a more recent study, total IgE levels tented to be higher in uncomplicated malaria than in severe cases, while the levels of *P. falciparum*-specific IgE in severe forms were higher than those found in the uncomplicated cases of the disease (Kumsiri et al., 2016), suggesting that parasite-specific IgE Abs may contribute to the severity of the disease.

During the allergic inflammatory response, the IgE Abs bind to the high-affinity receptor FcεRI and the formation of the antigen-IgE-FcεRI complex activates the cells and stimulates the release of inflammatory mediators such as histamine (Fig. 25). Release of pro-inflammatory mediators involved in allergic inflammation in the host is one of the events occurring during malaria infection (Mecheri, 2012). Significantly elevated plasma histamine concentrations, derived from basophils and mast cells (MacDonald et al., 2001), in comparison to uninfected controls ($P < 0.01$) correlate with systemic *P. falciparum* disease complications within infected children (Bhattacharya et al., 1988; Enwonwu et al., 2000; Srichaikul et al., 1976). A study conducted in a hyperendemic area of malaria in Southwest Nigeria, where chloroquine and amodiaquine treatment failed, demonstrated that treatment of children with a combination of amodiaquine or chloroquine or pyrimethamine/sulfadoxine plus chlorpheniramine, a histamine receptor type 1 antagonist reversed chloroquine resistance *in vitro* (Basco and Le Bras, 1994), and enhanced the drug efficacy *in vivo* (Sowunmi et al., 2001; Sowunmi et al., 2007; Sowunmi et al., 1997; Sowunmi et al., 1998a; Sowunmi et al., 1998b). In addition a clinical drug library screen using existing drugs for previously unknown activities identifies the nonsedating anti-histamine astemizole as an antimalarial agent able to inhibits the proliferation of three *P. falciparum* strains (3D7, Dd2 and ItG) (Chong et al., 2006). These findings strongly suggest that histamine, a major product of the allergic inflammatory cascade, plays a prominent role in malaria pathogenesis.

Moreover, a mouse model for human atopic disease (NC/Jic) was found to be susceptible to *P. yoelii* infections compared to WT mice (Ohno et al., 2001). The *Py*mr (*P. yoelii* malaria resistance) locus, able to control host survival and parasitemia after infection, was mapped on chromosome 9. In *P. chadaudi* infection, the *char1* locus (*P. chabaudi* resistance locus 1)

responsible to control host survival and peak parasitemia was mapped to the same region. Interestingly, the loci of atopic disease susceptibility (quantitative trait locus of *derm1*) on chromosome 9 are mapped exceptionally close to genes (*char1* and *pymr*) mediating parasitemia control (Kohara et al., 2001; Sakuntabhai et al., 2008). This concept has been further supported by several *in vivo* studies carried out in human and mice. Thus, atopic dermatitis in children is linked with a higher prevalence of clinical malaria attacks in Ethiopia (Haileamlak et al., 2005). In our laboratory, using a mouse model, we highlighted the crucial role of histamine in the pathogenesis of malaria disease. Firstly, by demonstrated that the allergic-type response, by the intermediary of mosquito saliva, starts immediately after the mosquito bite perturbing the early anti-parasite immune response and affecting downstream disease development (Demeure et al., 2005; Schneider et al., 2011). Secondly, by showing the importance of histamine release during malaria infection and its consequences. Indeed, mice treated with antihistamines or deficient for the H1 and H2 receptors for histamine were more resistant to ECM (Beghdadi et al., 2008). Moreover, the disruption of IgE or the α chain of FcεRI led to the resistance to the development of ECM after infection with *Pb*ANKA indicating a pathological role of IgE that acts via FcεRI to promote disease development. This study identified, in mice, neutrophils and eosinophils as alternative FcεRI-expressing cell types having a disease enhancing capacity. Although FcεRIα is essentially absent in naive mouse neutrophils, the receptor was shown to be induced during *Plasmodium* infection (Porcherie et al., 2011). The transfer of $FcεRI^{+}$, but not $FcεRI^{-}$, neutrophils to the otherwise resistant FcεRI-α-KO mice conferred ECM susceptibility highlighting the implication of $FcεRI^{+}$ neutrophils in malaria pathogenesis (Porcherie et al., 2011). Altogether, these findings demonstrate that components of the allergic cascade including IgE, FcεRI, histamine and the newly identified FcεRI-positive neutrophil population, are intimately associated with severe forms of malaria disease. This may have practical consequences on the management of malaria disease by introducing novel anti-allergic therapeutic approaches used individually or in combination with classical anti-malarial chemotherapy.

Recent studies have documented the importance of an alternative mechanism, independent of the classical pathway implicating the IgE-FcεRI complex during histamine release, where

cells are directly activated by a *Plasmodium*-derived protein called Histamine Releasing Factor (HRF) (MacDonald et al., 2001)(Fig. 25). Additionally, high levels of *Pf*HRF have been detected in the serum of mildly and severely *P. falciparum* infected Malawian children (Janse et al., 2006; MacDonald et al., 2001).

Considering these observations, we chose to provide a comprehensive description of the role of *Plasmodium* HRF during the parasite development in the host liver and during blood stages infection. To do so, we investigated (1) the influence of the *Plasmodium* HRF to modulate the immune mechanisms during malaria infection and (2) whether its expression was associated with the severity of malaria disease. For this, we generated a genetically HRF-deficient parasites (*hrfΔ*) firstly in *P. berghei* ANKA-GFP parasites and later in *P. berghei* NK65-GFP parasites, and we assessed the impact of this mutation both on the host immune response and on the severity of the resulting malaria disease. Knowing that the loss of *hrf* expression in mice results in increased spontaneous apoptosis and death during embryogenesis, we analysed the development of mutant parasites during the whole life cycle by determining by qRT-PCR or by counting the number of parasites over time. We found that the deletion of the *hrf* gene did not affect the parasite development per se, meaning that the deletion was not lethal for the parasite.

Inoculation of *Pb*ANKA WT or *hrfΔ* sporozoites was implemented either via intravenous or intradermic injection, ensuring that a consistent number of sporozoites enters mouse circulatory system, or via mosquito bite. The later involves saliva effects, that include anti-platelet, anti-coagulant, vasodilatory and anti-platelet mediators and was shown to induce more likely a development into blood stage parasitemias than their intravenous inoculated counterparts. We also studied the influence of HRF in the development of the parasite during direct injection of RBCs previously infected with WT or HRF mutant parasites in mice. Our results showed that infection with sporozoites was characterized by a delay in blood stage development of mutant parasites as compared to *Pb*ANKA WT parasites. This delayed development resulted in clinical immunity but without sterile immunity.

Sporozoite infectious capacities are dependent on three characteristics: host cell traversal, host cell invasion and gliding motility, all critical for parasite proceeding development within

the liver. Therefore, cell traversal assay was done to deduce a potential role of *Plasmodium* HRF in mediating sporozoite locomotive capacities. Incubation of hepatic cells (HepG2) with *Pb*ANKA WT or *hrfΔ* sporozoites *in vitro* did not display any detectable defect in gliding motility or host cell traversal and invasion. A comparative qualitative analysis between *Pb*ANKA WT and *hrfΔ* parasite gliding properties was also observed via intravital (*in vivo*) microscopy. Injection of *Pb*ANKA WT and *hrfΔ* sporozoites in the tail vein was performed to compare the parasite ability to cross the sinusoidal barrier. The number of moving sporozoites and respective percentage within the liver parenchyma did not show any reduced gliding motility in mutant parasites compared to WT parasites, confirming the previous *in vitro* results and demonstrated that HRF is not important during the entry into hepatocytes. However, the impaired development of HRF mutant liver stages *in vivo* is likely to induce some parasite death, as suggested by intravital imaging studies. This resulted in a 10^{2-} to 10^{3-} fold reduction in the numbers of merozoites initially released in the blood, and an extension of the time between sporozoite injection and detectable parasitaemia. This prolonged liver-stage development influenced the clinical outcome of infection by decreasing the frequency of ECM as was observed in mice infection by mosquito bite sporozoite inoculation. Indeed, in these conditions, 100% of the mice that received *Pb*ANKA WT parasites develop ECM and died on day 7 or 8 p.i., while only 10% of mice infected with *Pb*ANKA-*hrfΔ* parasites showed signs of cerebral complications.

These first results, using *Pb*ANKA strain, demonstrated the importance of the *Plasmodium* HRF during malaria infection and the development of the pathogenesis of cerebral malaria. In order to confirm and consolidate our results on the role of *Plasmodium* HRF in parasite and pathogenesis development we decided to extend these observations to a murine parasite strain *Pb*NK65, which causes a lethal infection in C57BL/6 mice that is less acute than *Pb*ANKA, and anemia and mortality occur within 20-25 days p.i. allowing us to explore the immune mechanisms in more detail over a longer period of time. Mice infection with *Pb*NK65 WT or *hrf*Δ sporozoites shown, as previously observed with *Pb*ANKA, a delay in blood stage parasite development between mutant and WT parasite confirming a prolonged liver-stage development of *Plasmodium hrf*Δ. Moreover, infection with *Pb*NK65-*hrf*Δ sporozoites results at day 17 p.i. in complete blood stage clearance of the parasite, in

contrast to *Pb*ANKA-*hrf*Δ sporozoite infection that despite the development of a clinical immunity (no ECM) the parasite develops normally without being eliminated at the blood stage. More interestingly, differently from what was observed during *Pb*ANKA-*hrf*Δ iRBCs infection where there was no difference between WT and mutant parasite development and pathology, the infection course with the *Pb*NK65-*hrf*Δ iRBCs gave rise to a particular phenotype characterized by an initial growth similar to that of the WT parasite, followed by a clearance of the parasite at day 12 p.i.. This phenotype was reminiscent of an infection with the WT *P. chabaudi chabaudi* parasite strain, which mimics a similar pattern of an initial growth followed (around day 12) by a tight control of the parasite. This phenotype was well documented in terms of the mechanisms involved in the anti-parasite immune response. Indeed, parasite elimination was preceded by a Th1 response with a marked IFN-γ production during acute infection followed by a switch to a Th2 response crucial for the total clearance of the parasite (Muxel et al., 2011).

HRF, a key regulator of IL-6 production: a prototype example of how a parasite gene product directly modulates host cytokine responses (Fig. 26).

A more detailed analysis showed that the impairment in the development of *Pb*ANKA-*hrfΔ* parasites in liver stages was associated with an early rise in systemic IL-6 at 40 hours post infection from neutrophils and inflammatory monocytes. The importance of IL-6 was confirmed by injection of anti-IL-6 antibodies or infection in IL-6-deficient mice during *Pb*ANKA-*hrfΔ* infection that rescue WT phenotype. In parallel, injection of human recombinant IL-6 in mice infected with *Pb*ANKA WT parasite shows a decrease in parasite load in the liver. Additionally, we demonstrated the inhibitory effect of HRF on LPS-induced IL-6 cytokine production confirming a direct role of parasite HRF in the down-regulation of anti-parasite cytokines. Previous studies have shown that in addition to the cellular mechanisms involving both $CD8^+$ and $CD4^+$ T cells (Crispe, 2014; Van Braeckel-Budimir and Harty, 2014), the production of type I IFN responses (Haque and Engwerda, 2014; Liehl et al., 2014) has an important role in parasite killing within hepatocytes. Additionally, IL-6 with IL-1 and TNF-α have been involved in induction of acute phase proteins in the liver (Gabay and Kushner, 1999; Gauldie et al., 1987; Gruys et al., 2005; Heinrich et al., 1990; Ramadori et al., 1988), some of which being known to interfere with malaria parasite development or the

outcome of disease (Aggrey et al., 2013; Pied et al., 1989). Consistently, addition of recombinant IL-6 during *P. yoelii* infection in *in vitro* hepatic cultures resulted in a specific dose-dependent inhibition of parasite development exerting its action during both the early phase of infection and during the subsequent maturation of the schizonts. These observations support the hypothesis that production of IL-6 during liver stage could be important for the outcome of the disease (Nussler et al., 1991; Pied et al., 1991). Consistent with these reports, our data also highlight the link between the early rise of IL-6 induction and the B-cell-activation at 40 h p.i. induced by mutant parasites. One of the features in malaria infection is the induction of short-lived B-cell memory resulting in the short-lived nature of many anti-malarial antibody responses (Dorfman et al., 2005) and the increase of the frequency and absolute number of apoptotic cells (T cells, B cells, and macrophages) in spleen (Helmby et al., 2000). Accordingly, studies using lethal *P. yoelii* YM infection have reported that malaria parasites interfere with the immune response by disabling B-cell differentiation, possibly by inducing apoptosis (Wykes et al., 2005). In our murine malaria model, the increase in B-cell population in the liver of mice infected with mutant parasites reflects the possibility that the HRF molecule could interfere with B-cell function and memory possibly via the down-regulation of IL-6 production. Despite the observation that recombinant murine HRF stimulates proliferation and increased serum Abs production in splenic B cells both *in vivo* and *in vitro* (Kang et al., 2001), recent studies have shown that *Plasmodium* HRF could inhibit B cell functions. Indeed murine splenic B cells were shown to incorporate more efficiently and faster the parasite protein than the human ones, indicating that *Plasmodium* HRF could be responsible for the altered immune response, thus allowing the parasite to escape the immune response (Calderon-Perez et al., 2014). This result is supported by structural studies on *Plasmodium* HRF, highlighting some differences between parasite and mammalian protein structure, which could impact the functionality of the protein (Hinojosa-Moya et al., 2008).

Interestingly, the production of IL-6 observed in mice infected with the *hrf*Δ *Pb*NK65 parasite confirms what was previously observed with *Pb*ANKA-*hrf*Δ where IL-6 strongly suppressed the liver-stage development. More importantly, in the *Pb*NK65 model, the elevated IL-6 production during blood stage in the absence of HRF expression seems to be important in

establishing parasite clearance and acquisition of sterile immunity during *Pb*NK65-*hrf*Δ infection. Based on two main observations: first, a marked upregulation of IL-6 expression induced by *Pb*NK65-*hrf*Δ parasites found in spleen and plasma at day 6 p.i. as compared to WT parasites, and second, a normal development of *hrf*Δ parasites and acquisition of the WT phenotype when the mutant parasites were inoculated to the IL6-^{KO} mice allow us to conclude that the IL-6 pathway, a component of innate immunity, plays a central role in controlling *Pb*NK65-*hrf*Δ blood stage parasitemia. In support of our findings, Prakash and colleagues, by studying the clusters of cytokines that determine the severity of the disease during *P. falciparum* infection in endemic areas of central India showed that the induction of serum IL-6 was inversely correlated with the disease severity (Prakash et al., 2006). In addition, co-infection with *Schistosoma mansoni* followed by *P. knowlesi* in baboons showed that animals with chronic schistosomiasis were protected from severe malaria by an increase of IL-6 levels compared to animals not infected with (Nyakundi et al., 2016). In an effort to identify the cellular source of IL-6 during *Pb*NK65-*hrf*Δ infection that was negatively regulated by HRF, examination by intracellular cell staining flow cytometry analysis showed that neutrophils are the main cell targets. Nonetheless, how IL-6 affects parasite growth inside erythrocytes, and the contribution of other cytokines such as IL-12 or TNF–α to the deficient growth of the *Pb*NK65-*hrf*Δ mutant parasite, remain open questions. IL-6 is a cytokine with well-defined pro- and anti-inflammatory properties. Among the biological activities, IL-6 plays a role in T cell stimulation (Tormo et al., 2012), B cell proliferation and differentiation (Hirano et al., 1986), monocytes/macrophages differentiation (Chomarat et al., 2000) and neutrophils activation (Borish et al., 1989). Additionally, IL-6 deficiency can lead to dysfunctional innate and adaptive immunity against many infections (Hoge et al., 2013; van der Poll et al., 1997). For example, IL-6 was shown to play a protective role in many bacterial, viral and fungal infections (Imanishi, 2000). Consistently, IL-6-deficient mice are significantly more susceptible to some fungal infections like candida (Basu et al., 2008) and herpes simplex virus type 1 infections. In this later infection, IL-6 ensures survival of infected mice, but does not substantively affect herpes simplex virus replication (LeBlanc et al., 1999).

HRF mutant parasite infection is associated with potent effector immune mechanisms (Fig. 26).

The reasons for the abortive infection and subsequent protection by *Pb*NK65-*hrf*Δ parasites are likely multifactorial. Analysis of cytokine levels at early time points (day 6 p.i) indicated an increase of several pro-inflammatory cytokines, such as IL-12 cytokine family including IL-12p35, IL-12p40, IL-23, and Ebi3, IFN-γ and IL-6, in the spleen, liver and plasma of *Pb*NK65-*hrf*Δ-infected mice indicating the development of a Th1 response at early time points following infection. In support of our results, administration of IL-12 antibody to mice infected with *P. chabaudi chabaudi* AS resulted in the elimination of the parasite with an increased cell numbers of NK and $CD4^+$ T cells that enhance IFN-γ production (Mohan et al., 1997). A similar mechanism operates in a distinct experimental model where administration of recombinant IL-12 before inoculation of rhesus monkeys with *P. cynomolgi* provides 100% protection through an IFN-γ-dependent antiplasmodial mechanism (Hoffman et al., 1997). A parallel can be made in the human situation since during *P. falciparum* infection, children with mild malaria infection have higher levels of plasma IL-12 than children with severe malaria infection, and the levels of IL-12 are inversely correlated with parasitemia and numbers of malaria pigment-containing neutrophils (Luty et al., 2000; Perkins et al., 2000; Prakash et al., 2006). Additionally, an increase in IL-12 levels during the acute phase of uncomplicated *P. falciparum* malaria is thought to reflect an early and effective immune response regulated by proinflammatory Th1 cytokines (Prakash et al., 2006). Furthermore, IL-12 production by macrophages in response to infection mediates resistance to *Trypanosoma cruzi (Aliberti et al., 1996)*. Studies regarding strategies toward cancer immunotherapy showed that optimal target-cell clearance required DC support and IL-12 production allowing the development of $CD4^+$ cytotoxic T lymphocytes (Nelles et al., 2014). Knowing that in cancer cells the IL-12 production is almost non-existant and that the HRF is implicated in cancer development (Li et al., 2001; Tuynder et al., 2002), assumption can be made that HRF maybe responsible for the inhibition of IL-12 production, function that during malaria infection allows the parasite to escape the initial immune response.

Some evidence suggests that IFN-γ have a protective role during malaria infections. Indeed, enhanced IFN-γ protective immunity was observed during *P. chabaudi* and *P. yoelii 17XL*

infection in IFN-γ-deficient mice where absence of cytokine production increases the severity of the infection resulting in death or require additional time for the resolution of the infection in comparison to WT mice (van der Heyde et al., 1997). Mice infected with *P. chabaudi* and treated with neutralizing anti-IFN-γ Ab failed to suppress acute infection developing high levels of parasitemia and administration of IFN-γ to intact C57BL/6 mice slightly decreased and partially delayed parasitemia (Meding et al., 1990). In humans, the protective capacity of IFN-γ during malaria infections appears to be related to the timing of IFN-γ production. Early appearance of IFN-γ after infection is correlated in humans and animal models with protection against the development of clinical symptoms of malaria as CM (D'Ombrain et al., 2008; Villegas-Mendez et al., 2012). The Fulani ethnic group in Mali present elevated levels of IFN-γ suggesting a protective role for IFN-γ against malaria (McCall et al., 2010; Prakash et al., 2006). Moreover, IFN-γ is a key mediator of inflammatory immune responses induced primarily by IL-12 and its secretion is essential for control of intracellular pathogens (such as *Mycobacteriul tuberculosis* or *Listeria*) and tumours. Indeed, it was demonstrated that during Ebola infection, a disease that at the beginning of the infection present the same symptoms of malaria, the administration of IFN-γ in mice profoundly inhibits Ebola virus infection of macrophages reducing morbidity and serum viral titres (Rhein et al., 2015). Once adaptive immunity is initiated IFN-γ secreted by $CD4^+$ T cells is critical for optimal activation of cytotoxic $CD8^+$ T cells (Janssen et al., 2003), induction of B cell class-switching to the cytophilic IgG2a antibodies (Su and Stevenson, 2000; Xu and Zhang, 2005) and induction of inflammatory monocytes in bone marrow that migrate into spleen (Sponaas et al., 2009) or expression of a number of proteins such as FcγRI on macrophage surface enhancing their phagocytic abilities (Waddell et al., 2010), all of which perform vital roles in the control of *Plasmodium* infection.

At day 6 p.i. analysis also show that the parasite load in the spleen was higher in mice infected with *Pb*NK65-*hrf*Δ parasite than the ones infected with WT parasite. Moreover, macroscopic examination of spleens showed a splenomegaly in mice infected with *Pb*NK65-*hrf*Δ confirmed by quantitative cell analysis. The importance of the spleen in elimination of parasites and in the development of the immune response during malaria infection was demonstrated in different studies both in humans and in experimental murine models.

Several studies in the African population showed that an enlarged spleen was more frequently seen among the Fulani compared to the Dogon (Bereczky et al., 2006; Greenwood et al., 1987; Oomen et al., 1979). Indeed, during acute *P. falciparum* infection patients with splenomegaly accelerate the clearance of iRBCs compared to patients with normal spleen sizes (Looareesuwan et al., 1987) and splenectomised patients show an increase in parasitemia (Bachmann et al., 2009), demonstrating the crucial role of spleen in parasite clearance. It is known that the first lines of defence against blood stage in spleen are the red pulp macrophages and the splenic marginal zones DCs able to capture malaria antigens and activate T cells. During *P. chabaudi* infection two population of DCs responsible for the activation of $CD4^+$ T cells in spleen with different kinetics. At the beginning of the infection splenic $CD8^+$ DCs cells or macrophages through the production of IL-12 induce the proliferation of $CD4^+$ T cells and their production of IFN-γ (Th1 response) (Ing and Stevenson, 2009; Sam and Stevenson, 1999a; Sam and Stevenson, 1999b). The switch of the immune response from Th1 to Th2 starts at the peak of infection when $CD8^+$ DCs cells undergo apoptosis in the spleen and $CD8^-$ DCs cells activated $CD4^+$ T cells to produce IL-4 and IL-10 (Helmby et al., 2000; Sponaas et al., 2009). These mechanisms allow the generation of a robust splenic immune response to the parasite, including prominent germinal centre formation and generation of a long-lived memory B and $CD4^+$ T cell responses as well as long-lived plasma cells and protective antibodies (Del Portillo et al., 2012).

Additionally, we show that immunological effectors are required to clear *Pb*NK65-*hrf*Δ parasites and induce protection. In concordance with findings on previous protective blood stage mutants (Aly et al., 2010; Spaccapelo et al., 2011), *Pb*NK65-*hrf*Δ-induced protection was T cell- and B cell-dependent, with the involvement of two types of effectors. Indeed, this mutant parasite developed normally in T- and B-cell deficient mice. By reflexion, these findings raise the question why WT parasites develop normally within the host environment without being cleared. Two key findings were presented in the present work: first, no trace of anti-parasite antibodies could be detected at any time of the infection course with the WT parasite and the B cell compartment seems to be completely non functional, and second, a significant proportion of $CD4^+$ $CD62L^-$ and $CD8^+$ $CD62L^-$ cells, which are memory effector T cells, have their surface PD-1 expression upregulated during infection with WT parasites in

contrast to *hrf*Δ parasites. These two combined mechanisms may explain why the WT parasite remains unaffected by the host immune system.

During *Pb*NK65-*hrf*Δ infection antibodies of the IgG2c subclass were dramatically increased in *Pb*NK65-*hrf*Δ-infected mice. A clear evidence of the association of parasite clearance and the subsequent sterile immunity was provided by the strong antigen-specific antibody response demonstrated by immunoblot and by ELISA assays. Among these antigens, MSP-1, MSP-8, SERA-1, and SERA-2 which are known to elicit antibodies associated with protection in people living in malaria-endemic areas (Banic et al., 1998; Okech et al., 2001) were identified by mass spectrometry. Moreover, BALB/c mice infected with *P. Yoelii* YM-*sera2*Δ but not SERA-1 mutant parasites, showed a clearance of the parasite at day 28-30 p.i. compared to the lethal WT parasite which killed mice within 6-8 days indicating that SERA2 during malaria infection is an important antigen and that its inhibition or blocking could help parasite elimination (Huang et al., 2013). In contrast, in a distinct murine model of malaria using NMRI mice (originally from Swiss strain but maintained as an inbred strain), namely during infection with *P. berghei*, SERA1 and SERA2 seem not to play a vital role *in vivo*, since loss-of-function parasite lines progressed normally through the parasite life cycle (Putrianti et al., 2010). These murine models highlight inconsistencies regarding the GAP phenotypes depending both on the parasite and the mouse strain. In order to gain more insight into the role of genes of potential interest, novel studies must be carried out to design GAPs using the same parasite line tested in the same mouse strain. We found that secreted antibodies from *Pb*NK65-*hrf*Δ protected mice were able to reduce parasitemia following a passive transfer to naïve mice but were not able to completely clear the infection. In addition $Fc\gamma R^{-/-}$ mice were unable to clear infection by mutant parasites and $CD11b^{+}$ cells, most likely macrophages and dendritic cells, were necessary and sufficient for adoptive transfer of immunity. These results suggest that secreted antibodies are important for the protective immunity to *P. berghei* but the apparent lack of complete protection suggests that these antibodies need to act in concert with “primed“ $Fc\gamma R^{+}$ effector cells such as monocytes, macrophages, NK cells, and others that are existing in *hrf*Δ parasites-infected but not in naive mice. This picture of antibody-dependent cellular cytotoxicity involved in protection was earlier reported in the case of the plasmepsin-4-deficient mutant (Spaccapelo et al.,

2010) and in host resistance to blood stage *P. berghei* XAT infection (Yoneto et al., 2001). *In vivo* experiments using rodent malaria parasites transgenic for *P. falciparum* $MSP1_{19}$ in mice also transgenic for human Fc-receptors (FcRs) after three i.p. inoculations of functional humans anti-$MSP1_{19}$ IgG1 Abs effectively suppressed a lethal blood stage challenge with *P. berghei.* In contrast in non transgenic mice, human anti-$MSP1_{19}$ IgG1 Abs where not protective suggesting that FcγRI recruitment is crucial for parasite elimination (McIntosh et al., 2007). Several *in vitro* studies have indicated the biologic relevance of the FcγRII and IgG subclasses in resistance to high-density parasitemia by showing the importance of ADCI mechanism during protection against blood-stage *P. falciparum* parasitemia (Bouharoun-Tayoun et al., 1990; Bouharoun-Tayoun et al., 1995; Druilhe and Perignon, 1994; Shi et al., 1999; Zhou et al., 2015). The ADCI function is triggered by the binding of the IgG-merozoite complex to monocytes via the FcγRII receptor (Bouharoun-Tayoun et al., 1995), and correlates with malaria-specific IgG (Bouharoun-Tayoun and Druilhe, 1992; Shi et al., 1999). Human studies also showed the capacity of FcγR to mediate potent antimalarial immunity (McIntosh et al., 2007). Studies on the Fulani population demonstrate a relation between FcγRs polymorphisms and susceptibility to malaria, which is associated with higher antibody levels (Cherif et al., 2016; Modiano et al., 1996). A recent study on the allelic frequencies of rs396991 (FCGR3A) and rs3933769 (FCGR2C) between the Fulani and Dogon populatations in Mali revealed differences in these allelic frequencies suggesting that the genes may contribute to the interethnic variability in malaria susceptibility seen among the Fulani and their sympatric neighbours. For example, the rs396991 mutant allele occurs at a higher frequency in the Dogon, who are more susceptible to malaria relative to Fulani, suggesting that this SNP may play a role in malaria pathogenesis (Cherif et al., 2016). However, further studies will be needed to determine the possible roles of these SNPs in malaria pathogenesis. Additionally, a study performed in Kenyan infants, residing in an area of high malaria transmission, provides evidence that genetic polymorphism of FcγRIIa is associated with protection against high-density *P. falciparum*. Indeed, infants with FcγRIIa-Arg/Arg131 genotype, which binds IgG1 or IgG3 Abs, were less likely to have repeated high-density *P. falciparum* infections during the first year of life suggesting a protective effect of this FcγRIIa genotype. Moreover, the study showed that infants with FcγRIIa-His/Arg131 genotype were

at greater risk for high-density *P. falciparum* infection compared to infants who were at low risk (Shi et al., 2001). The clinical importance of FcγRIIa polymorphism has been evaluated during encapsulated bacterial infections where FcγRIIa-His/His131 genotype was associated to protection, whereas the poorly IgG2-binding allotype FcγRIIa-Arg/Arg131 was associated with increased susceptibility to these pathogens (Musser et al., 1990; Platonov et al., 1998; Yee et al., 2000).

One of the characteristic features of malaria infection is the inability to generate an acquired protective immunity suggesting that memory T cells develop inadequately or their maintenance is not assured. Using our mutant strain of parasite, we observed a dramatic difference with the WT parasites in modulating the expression of PD-1 on the surface of both $CD4^+$ and $CD8^+$ T cells. PD1 and its ligand PD-L1/PD-L2 belong to the family of immune checkpoint proteins that act as co-inhibitory factors transmitting an inhibitory signal into the T cell, which reduces cytokine production and suppresses T-cell proliferation (Day et al., 2006; Hofmeyer et al., 2011; Wherry, 2011). The PD1/PD-L1 pathway represents an adaptive immune resistance mechanism that is exerted by tumour cells in response to endogenous anti-tumour activity. Moreover, T cells exhaustion by upregluation of PD1 expression of cell surfaces was described in humans in the context of chronic viral infection such ad HIV and hepatitis (B and C) (El-Far et al., 2008; Kim and Ahmed, 2010; Wherry, 2011). Interruption of PD1/PD-L1 binding, with monoclonal antibodies against PD1 and PD-L1, has shown to rescue tumours and HIV-infected cells from this state of anergy or 'exhaustion' (Porichis and Kaufmann, 2012; Swaika et al., 2015). Examining the expression of PD-1, an inhibitory receptor expressed on T cells, we found that throughout the infection period starting from day 6 p.i., the absence of HRF was associated with a significantly reduced expression of this receptor. This suggests that this marker, found early during infection (day 6 p.i.) and lasted throughout the infection period (day 20 p.i.), may have an influence on both clearance of primary infection and induction of lasting protection. Moreover using the *P. berghei* recombinant HRF protein we demonstrated the direct involvement of HRF in the upregulation of PD-1 receptor. Interestingly, an involvement of PD-1 in malaria blood stage control has already been reported: in *P. falciparum* infections, higher expression of PD-1 was associated with T cell dysfunction and the blockade of PD-1 ligand in a murine model of

infection rapidly cleared blood-stage malaria in a B- and T- cell dependent manner resulting in enhanced parasite control (Butler et al., 2012). In the *P. chabaudii* rodent model of chronic blood stage infection (Good et al., 2013), parasite-specific protective $CD8^+$ T cells undergo significant PD-1-dependent exhaustion (up to 95% reduction), which exacerbates acute blood stage infection and drives chronic disease. A parallel that can be made with our results is that our mutant parasite allows a rapid clearance of the parasite, a down-regulation of PD-1 expression and the establishment of a long lasting memory which are all dependent on both B and T cells.

After confirming the importance of T cell responses during parasite clearance such as helper function for B cells to produce Abs that are essential for parasite clearance or production of cytokines able to amplify the phagocytosis and the parasitocidal response of the innate immune system, we wanted to determine the importance of T cells for the maintaining of the immune response and to identify which population of T cells are implicated in the memory. Depletion of CD4 or CD8 T cells in protected mice followed by *Pb*NK65 WT re-infection showed no differences in parasitemia in the control group and in CD8 depleted mice. In contrast, all of CD4 depleted mice had detectable parasitemia (until 30%) indicating the contribution of $CD4^+$ T cells to long term immunity. Studies performed in both mice and humans have shown the importance of $CD4^+$ T cells in parasite-specific memory (Brake et al., 1988; Vinetz et al., 1990). Depletion of $CD4^+$ or $CD8^+$ T cells in mice during *P. yoelii* 17X infection and adoptive transfer into a naïve mice showed that mice which received $CD8^+$ T cells failed to control the infection in contrast to mice which received $CD4^+$ T cells which were able to control the infection, suggesting that $CD8^+$ T cells are not responsible for blood-stage immunity (Vinetz et al., 1990). Additionally, parasite-specific memory $CD4^+$T cells were found in the blood of human volunteers deliberately exposed to very low doses of malaria parasites with very few or no parasite-specific antibodies (Pombo et al., 2002; Roestenberg et al., 2009).

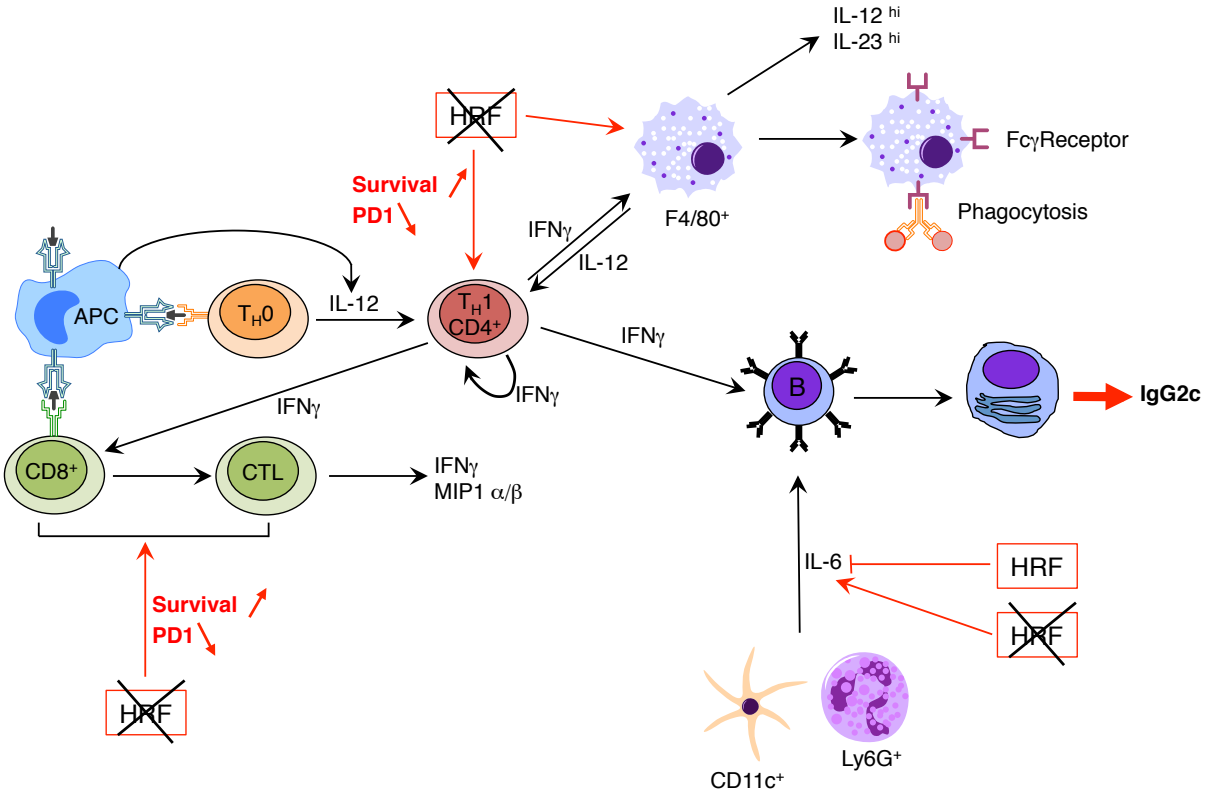


Figure 26: Hypothetical mechanisms induced by *Pb*NK65-hrfΔ parasites involved in parasite killing and development of protective immunity. Parasite Ag presentation to both $CD4^+$ and $CD8^+$ T cells by APC cells. IL-12 production which polarizes activated $CD4^+$ T cells toward Th1 response. $CD4^+$ helper T cells through the production of IFN-γ induce three types of cellular events; (1) activation of CD8 cytotoxic T cells and their production of IFN-γ, (2) activation of macrophages that increase FcγR expression on their surface and the production of other cytokines, and (3) the Ig switching of B cells, previously activated by IL-6, to produce IgG2c Abs. These Abs promote ADCI of iRBCs by FcγR expressing macrophages. Elimination of *Plasmodium* HRF exerts its inhibitory effects at 3 levels; (1) decrease of $CD4^+$ and $CD8^+$ T cell survival due to the increase on PD1 expression, (2) decrease of spleen macrophage number, and (3) inhibition of IL-6 production with subsequent reduction in B cell proliferation and abrogation of ADCI.

HRF mutant parasites as vaccine candidates

The use of whole parasites to induce protection against malaria blood stage infection has a long history of inventive approaches. Early studies in the 1940s using *P. knowlesi* or *P. falciparum* infection in monkeys showed that high doses of killed parasites along with complete Freund's adjuvant provided protection against blood stage challenge (Pombo et al., 2002). In the 1980s, radiation-attenuated derivatives of *P. berghei* or *P. yoelii* blood stage parasites which initiate self-resolving infections in immuno-competent hosts, were found to be protective (Miyagami et al., 1987; Waki et al., 1982). Nevertheless, production and use of this live attenuated parasite presents different problems such as the large scale production

and the maintaining of the efficacy after storage. More recent work has demonstrated that in humans, repeated infections that were initiated by ultra-low doses of *P. falciparum* and rapidly cured by appropriate drug treatment, induced protective immunity. The resulting protection was associated with $CD4^+$ and $CD8^+$ T cell proliferative responses (Fell et al., 1994), nitric oxide synthase activity and IFN-γ production, but did not appear to involve parasite-specific antibodies (Pombo et al., 2002). Likewise, low doses of killed *P. falciparum*-infected red blood cells in the adjuvant CpG-ODN demonstrated strong protective efficacy, mainly dependent on $CD4^+$ T cells, IFN-γ and NO (Pinzon-Charry et al., 2010). Nonetheless, human studies are highly regimented and require the use of rodent and ape models for initial validation.

Given the advances in genetic manipulation techniques for rodent malaria parasites, the identification of GAP vaccines against infections by pre-erythrocytic and blood stage malaria infections has become more feasible. Some of the pre-erythrocytic GAP studies have been extended to human malaria parasites: for example, in 2013, *P. falciparum* Δ*p52*Δ*p36* GAPs, that arrest in pre-erythrocytic stages, were tested in human adult volunteers (Spring et al., 2013); and they conferred partial protection, similar to the equivalent murine model *P. berghei* GAP *Pb*Δ*p52*Δ*p36* (Annoura et al., 2014). However, few candidates for blood stage GAP vaccines have been explored. For example, *P. yoelii* lacking genes involved in the purine salvage pathway (Aly et al., 2010; Ting et al., 2008) or *P. berghei* lacking an aspartic protease, plasmepsin-4, involved in hemoglobin degradation (Spaccapelo et al., 2010) and a merozoite surface protein 7 (Spaccapelo et al., 2011) displayed various degrees of attenuation, from limited peak parasitemia to delayed blood stage growth. Crucially, all these mutants generated self-resolving infections that induced strong and lasting immunity. Protection was effective against both erythrocytic and pre-erythrocytic stages of the parasite as well as across *Plasmodium* species (Aly et al., 2010; Spaccapelo et al., 2011; Spaccapelo et al., 2010), and lasted up to at least one year (Spaccapelo et al., 2010). Moreover, both antibodies and T cells (Aly et al., 2010), as well as antibody-dependent cellular cytotoxicity in which antibodies function by enhancing clearance of infected erythrocytes by splenic macrophages (Spaccapelo et al., 2011; Spaccapelo et al., 2010), were shown to be involved in protection. Interestingly, *P. berghei* plasmespin-4 KO parasites were shown to be more rapidly cleared

and induced stronger protective immunity than WT parasites treated by pyrimethamine (Spaccapelo et al., 2010). Taken together with our observations, we conclude that regardless of the specific effect of the genetic mutation, self-resolving, protective blood stage GAPs induce similar immune responses that are B- and T-cell dependent (Aly et al., 2010; this work) and rely on opsonized phagocytosis (Spacapello et al., 2010; this work). Importantly, this protection is stage-transcendent against homologous and heterologous challenges. In contrast there are no evidence of heterologous protection using liver stage GAPs. Furthermore, it was shown that *Plasmodium* blood stage infection can suppress, through modulation of DCs maturation and their capacity to activate $CD8^+$ T cell responses against the liver stage, inhibiting the establishment of a protective response during the liver stage leaving the host susceptible for the next infection (Ocana-Morgner et al., 2003).

Overall, we speculate that early during blood stage infection, protective mechanisms are induced by low amounts of parasites, which might be progressively overwhelmed, and possibly replaced by inhibitory immune mechanisms caused by further parasite multiplication. The balance between protective and inhibitory mechanisms might depend on peak parasitemia (*i.e.,* parasite load), or more importantly, on the parasite growth rate after the initial antigen stimulation in the host. One can then assume that any genetic modification that causes a growth defect would tip the balance towards protective mechanisms, resulting in parasite clearance before inhibitory mechanisms can be fully engaged. We further propose that PD-1 expression might constitute a crucial marker of protective *vs.* inhibitory responses and needs to be further exploited for both antimalarial drug and vaccine design. Future work should continue to dissect the different protective mechanisms induced by self-resolving blood stage GAP infections, with an emphasis to develop an effective multi-stage malaria vaccine. In order to achieve this aim it would be interesting to compare, using the same murine *Plasmodium* strain, the phenotypes of all the blood stage GAPs developed until now. This first step could help for the choice of a second mutation in order to develop a double mutant parasite maybe more performing in the development of the immune response. The last step in the vaccine development will be the construction of a *P. falciparum* mutant parasite, for example *Pf*3D7-*hrf*Δ and the assessment of mutation on the parasite viability.

Beside the whole parasite vaccine approach, we could also propose to use *Plasmodium* HRF as a subunit vaccine since anti-*Plasmodium* HRF Abs may mimic *Plasmodium-hrf*Δ induced protection. Nevertheless, the evidence does not support this hypothesis. First, we cannot hide the high sequence homology that exists between the human and the parasite HRF protein, even if structural differences have been highlighted. It cannot be excluded that this homology might generate cross-reactive Abs against human HRF, thus causing autoimmune reactions. Second, a recent study showed that immunization of mice with *Plasmodium* HRF resulted in a weak protective capacity is against malaria infections (Taylor et al., 2015). Taken together these observations strongly support the fact that, at least in the case of HRF, whole organism approach should be used to generate sterile immunity.

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Résumé:

De études récentes suggèrent une forte relation entre susceptibilité au paludisme et réponse allergique. En effet, des niveaux élevés d'histamine plasmatique et tissulaire ont été associés à la sévérité de la maladie chez l'homme infecté par *P. falciparum* et dans de nombreux modèles animaux. Histamine releasing factor (HRF), une protéine pro-inflammatoire libérant l'histamine au cours des processus allergiques, est produite par le parasite au cours des infections palustres modérées et sévères, suggérant l'implication du HRF dans l'altération des réponses immunitaires et dans la pathogenèse. Les objectifs de ce travail consistaient à évaluer le rôle de la protéine parasitaire HRF dans le développement de la réponse immunitaire et à déterminer si son expression est associée à la sévérité de la maladie en étudiant deux parasites murins, *Pb*ANKA et *Pb*NK65, déficients pour la protéine HRF (*hrfΔ*). Les souris infectées avec des sporozoïtes *Pb*ANKA-*hrfΔ* ont montré une diminution de la fréquence du neuropaludisme associée à un déficit du développement des parasites mutants au cours du stade hépatique et à une augmentation précoce systémique d'IL-6. En outre, l'infection par des parasites *Pb*NK65-*hrfΔ* a confirmé l'importance de la protéine HRF dans la virulence du parasite. En effet, l'infection par les parasites *Pb*NK65-*hrfΔ* est caractérisée par l'élimination du parasite qui conduit à une protection durable et au développement d'une mémoire immunitaire caractérisée par une augmentation d' IL-6, une diminution de l'expression de PD-1 sur les cellules T et une amélioration de la phagocytose dépendante des anticorps. La protéine HRF est le premier gène de parasite *Plasmodium* dont l'effet direct sur la réponse immunitaire de l'hôte est démontré.

Mots clés: *Plasmodium*, Histamine releasing factor, GAP, IL-6, immunité protectrice, vaccin

Abstract:

Recent findings have raised the hypothesis that clinical susceptibility to malaria may be related to allergy-type response. In human infection with *P. falciparum*, as well as in murine models of malaria, increased levels of histamine have been shown to be associated with disease severity. Histamine releasing factor (HRF), shown to be implicated in the release of pro-inflammatory histamine during late-phase allergy, was demonstrated to be produced by the parasite during mild and severe malaria infections suggesting that *Plasmodium* HRF may affect host immune responses and contributes to the pathogenesis. The objectives of this work were to evaluate the role of *Plasmodium* HRF in the development of the immune response and to determine whether its expression is associated with the severity of malaria disease by studying two HRF-deficient (*hrfΔ*) murine parasites (*Pb*ANKA and *Pb*NK65). Infection with *Pb*ANKA-*hrfΔ* sporozoites showed a decrease in the frequency of ECM due to the impairment of the development of the mutant parasites in liver stages as a consequence of the up-regulation of IL-6. Infection with *Pb*NK65-*hrfΔ* parasites confirmed the importance of HRF in enhancing the virulence of the parasite. Indeed, *Pb*NK65-*hrfΔ* infection results in parasite clearance leading to a long-lasting protection and immune memory as reflected by an up-regulation of IL-6, a down-regulation of PD-1 expression on T cells and in the enhancement of Ab-mediated phagocytosis. HRF is the first parasite gene which directly modulates the host immune response.

Keywords: *Plasmodium*, Histamine releasing factor, GAP, IL-6, protective immunity, vaccine