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Malaria and Susceptibility to Other Infections

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**Thesis submitted to the University of London in fulfillment of the
requirements for the Doctorate of Philosophy
2012**

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Declaration

I, Aubrey Justin Cunnington, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

This thesis was completed under the supervision of Prof. Eleanor Riley and Dr. Michael Walther, and I acknowledge the following assistance in specific parts of the thesis. In the work presented in Chapters 3, 4 and 8, the review articles were written with advice and editing from Prof. Eleanor Riley and in Chapter 8, Dr. Michael Walther; in Chapter 4 additional editing was performed by Dr. Tracey Lamb. In Chapter 5 some of the laboratory work was performed with assistance from Dr. J. Brian de Souza. In Chapters 6 and 7 laboratory assistance was provided by Simon Correa and Madi Njie; extraction of whole blood RNA and PfHRP-2 assays were done together with Sarah Nogaro. In Chapter 8 advanced statistical analysis was performed by Dr. Michael Bretscher. I have acknowledged all results and quotations from the published and unpublished work of other people.

Signed:.....Aubrey Cunnington.....May 2012

Abstract

Malaria is widely perceived as immunosuppressive. Despite extensive phenomenological description, the underlying mechanisms remain poorly described. The aim of this thesis was to identify possible mechanisms by which malaria modifies host defence, and to determine the importance of these mechanisms in a translational system moving from a mouse model to human malaria.

The most frequently cited immunological consequences of malaria are: suppression of vaccine responses, susceptibility to bacterial infection, susceptibility to endemic Burkitt lymphoma, and increased HIV viral load. Of these, susceptibility to non-Typhoid Salmonella (NTS) bacteremia, associated with severe hemolysis, was the most consistent between animal and human studies. I hypothesized that hemolysis would induce the immunomodulatory enzyme heme oxygenase-1 (HO-1), which is essential for survival in malaria infections in mice, but might impair host defence against NTS.

I demonstrate in mice that malaria, chemically-induced hemolysis, or simply administration of heme, cause loss of resistance to NTS, allowing more rapid bacterial growth than in control animals. A new niche for bacterial replication is established within neutrophils, which have impaired oxidative burst and bacterial killing activity. Hemolysis and heme induce HO-1 in neutrophil progenitors in the bone marrow, and this reduces the oxidative burst capacity of maturing neutrophils whilst also causing their premature mobilization into the circulation. Inhibition of HO by the competitive inhibitor SnPP abrogates the impaired resistance to NTS infection. I observed a similar phenomenon in Gambian children with malaria, with prolonged impairment of neutrophil function, the severity of which is related to hemolysis and HO-1 induction.

In summary I have shown that hemolysis- and HO-1-mediated neutrophil dysfunction occurs in malaria and is important for susceptibility to NTS infection. HO-1 inhibition might offer a novel therapy to alleviate neutrophil dysfunction in malaria patients.

Funding

This work was funded by a Medical Research Council clinical research training fellowship awarded to Aubrey Cunnington (G0701427).

Acknowledgements

There are many people to thank for their help with the work contained in this thesis. First, I must thank my supervisors, Eleanor Riley and Michael Walther, for the time and tireless effort they have expended guiding and advising me from the conception of the studies, through funding applications, solving practical problems and critical comments on manuscripts. I must also thank them for having enough faith in me to allow me the freedom to develop my own ideas and strategies, and to get on with the work without feeling under constant scrutiny. Second, I would like to thank others who helped in the conception and planning of the studies in this thesis: my advisory board members, Nick Dorrell and David Mabey; Roberto Motterlini, who was kind enough to introduce me to the heme oxygenase field with very little offered in return; and Brian Greenwood for the benefit of his immense wisdom on numerous occasions. Third, I must thank the members of the Riley lab and other staff at LSHTM, who introduced a bumbling clinician to the ways of laboratory science: Kevin Couper, Daniel Blount, Brian de Souza, Julius Hafalla, Emily Findlay, Amir Horowitz, Carolynne Stanley, and Liz King. Fourth, I owe special thanks to David Holden and members of his lab at Imperial College London, for helpful discussions and donation of GFP-expressing *Salmonella typhimurium*. Fifth, I must thank the numerous individuals at The MRC laboratories in The Gambia who helped me to conduct my study there: the field workers, microscopists, study nurses, clinicians, health centre staff, administrators, and especially Sarah Nogaro, Muna Affra, David Conway, Lamin Manneh, Simon Correa, Madi Njie, Omar Janha, and the subjects who participated in the study. Finally, but most importantly, I thank my family for their role in the completion of this thesis. I dedicate this thesis to my wife, Marianne, and my daughters, Amelie and Elise. Without their constant support and encouragement I may never have finished! For

nearly four years they have endured the antisocial hours I kept in order to complete this thesis, they moved their lives to the Gambia for nine-months, leaving career, school and friends behind, and they have tolerated my frequent distant expressions as I pondered scientific questions whilst they were discussing what to have for dinner. It is difficult to express quite how much they have given up for me to do this research, but I want them to know that I appreciate it immensely and hope that it will have benefits for all of us in the long term.

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Glossary

| | |
|---------------|---|
| AIDS | Acquired immunodeficiency syndrome |
| AP-1 | Activator protein-1 |
| APC | Allophycocyanin |
| ARE | Antioxidant response element |
| Bax | Bcl-2 associated X protein |
| Bid | BH3 interacting domain death agonist |
| Cav-1 | Caveolin-1 |
| cDNA | complementary DNA |
| CGD | Chronic granulomatous disease |
| cGMP | Cyclic guanosine monophosphate |
| CO | Carbon monoxide |
| CoPP | Cobalt protoporphyrin |
| CORM | Carbon monoxide releasing molecule |
| CRP | C-reactive protein |
| DHR123 | Dihydrorhodamine 123 |
| DMEM | Dulbecco modified Eagle's minimal essential medium |
| EBV | Epstein-Barr virus |
| Egr-1 | Early growth response protein-1 |
| ELISA | Enzyme linked immunosorbent assay |
| ERK | Extracellular signal related kinases |
| FACS | Fluorescence activated cell sorting |
| FITC | Fluorescein isothiocyanate |
| GAPDH | Glyceraldehyde 3-phosphate dehydrogenase |
| GPI | Glycosylphosphatidylinositol |

| | |
|--------------|---|
| GSH | Sulfhydryl glutathione |
| GSSG | Glutathione disulfide |
| Hb | Haemoglobin |
| HBSS | Hank's buffered salt solution |
| HHV8 | Human herpes virus 8 |
| Hif-1 | Hypoxia inducible factor-1 |
| HIV | Human immunodeficiency virus |
| HO | Heme oxygenase |
| HSF | Heat shock factor |
| ICAM | Inter-cellular adhesion molecule |
| IFN | Interferon |
| IKK | Inhibitor of κB ($\text{I}\kappa\text{B}$) kinase |
| IRF | Interferon regulatory factor |
| JNK | c-jun N-terminal kinase |
| LB | Luria Bertani |
| LPS | Lipopolysaccharide |
| MACS | Magnetic-activated cell sorting |
| MAPK | Mitogen activated protein kinase |
| MetHb | Methemoglobin |
| MFI | Median fluorescence intensity |
| MHC | Major histocompatibility complex |
| MKK | MAPK kinase |
| MMP | Matrix metalloproteinase |
| MOI | Multiplicity of infection |
| MyD88 | Myeloid differentiation primary response gene 88 |
| NADPH | Nicotinamide adenine dinucleotide phosphate |

| | |
|---------|---|
| NF-E2 | Nuclear factor erythroid-2 |
| NFκB | Nuclear factor κB |
| NO | Nitric oxide |
| NOX | NADPH oxidase |
| NOD | Nucleotide oligomerization domain |
| NLRP | NOD like receptor family, pyrin domain containing |
| Nrf2 | NFE-2 related factor2 |
| NTS | Non-Typhoid Salmonella |
| PCR | Polymerase chain reaction |
| PE | Phycoerythrin |
| PE-Cy7 | Phycoerythrin-Cyanine 7 conjugate |
| PERCP | Peridinin chlorophyll protein |
| PfHRP2 | <i>Plasmodium falciparum</i> histidine rich protein 2 |
| PHOX | Phagocytic oxidase |
| PHZ | Phenylhydrazine |
| PPAR-γ | Peroxisome proliferator-activated receptor-γ |
| pRBC | Parasitised red blood cell |
| PSG | Penicillin, streptomycin and L-glutamine |
| Py17XNL | <i>Plasmodium yoelii</i> 17X non-lethal |
| ROS | Reactive oxygen species |
| sGC | Soluble guanylate cyclase |
| siRNA | Small interfering RNA |
| SnPP | Tin protoporphyrin |
| STAT | Signal transducer and activator of transcription |
| StRE | Stress response element |
| TBP | TATA box-binding protein |

| | |
|--------------------------------|---|
| TGF-β | Transforming growth factor-β |
| TLR | Toll like receptor |
| TNF-α | Tumour necrosis factor-α |
| TRAM | TRIF-related adaptor molecule |
| TRIF | TIR-domain-containing adapter-inducing interferon-β |
| VCAM | Vascular cell adhesion molecule |

Spelling conventions

In the scientific literature the word *heme* is now used much more frequently than *haem*, for example in *heme oxygenase*. However this trend is much less marked for the use of *hemoglobin* instead of *haemoglobin*. To try to maintain consistency between terms used in this thesis, the American spelling of this and related words (-e- rather than -ae-) has been used throughout, except for the manuscript presented in Chapter 7, where the English spelling is used due to requirements of the journal to which the manuscript has been submitted.

Chapter 1. Introduction and literature review.

Introduction

The direct and indirect burdens of malaria

The World Health Organization estimated that there were 216 million malaria infections and 655,000 deaths from malaria in 2010, with the vast majority of these occurring in the African region.¹ Other authors have suggested that this may be a substantial underestimate and that the true number of malaria death is almost twice as high.² In addition to this direct burden of disease, malaria also causes an enormous indirect burden of disease, seemingly by increasing the morbidity and mortality due to other infections.³ This is difficult to estimate directly, but is apparent from the larger-than-expected reductions in all cause mortality seen when malaria transmission is reduced.⁴⁻⁵ In a rural area of The Gambia, introduction of insecticide treated bed nets reduced overall child mortality by more than one-third, greater than the reduction in deaths directly attributable to malaria,⁴ with reductions in deaths due to causes as diverse as gastroenteritis and respiratory infections.⁶ On Bioko island, Equatorial Guinea, reducing malaria prevalence by two-thirds in a five year period using indoor residual spraying, free access to artemisinin combination therapy and long lasting insecticide treated nets, produced a reduction in all cause child mortality of almost two-thirds, remarkably achieving the Millenium Development Goal by targeting just one disease.⁵ Consistent with these ecological studies, mathematical models have predicted that the overall burden of malaria may be more than double the direct burden, depending on the transmission setting and age group considered.^{3,7} The high indirect health burden of malaria has been thought to be due mainly to increased susceptibility to other infections, although undoubtedly there are more complex factors such as its socioeconomic impact which may also influence health.⁸ Specific associations have been well described between malaria and impaired defence against other infectious diseases, most notably: Gram negative bacterial infections,⁹⁻¹¹ Human Immunodeficiency Virus (HIV),¹² and Epstein-Barr virus (EBV).¹³⁻¹⁴ These associations have been described, almost exclusively,¹⁵ with *Plasmodium falciparum* malaria, the most common of the Plasmodium species causing human malaria, and the cause of the

majority of the global morbidity and mortality attributed to malaria.¹⁶ Whether other *Plasmodium* species impair host defence in humans has scarcely been studied.

Malaria and immunosuppression

Susceptibility to death from other infectious diseases has been the most obvious explanation for the indirect burden of malaria,^{6,9,11} and has led to the suggestion that malaria causes generalized immunosuppression¹⁷⁻¹⁹. Although numerous studies have provided evidence of suppression of aspects of innate, cellular and humoral immunity by malaria, these have often been quite specific rather than generalized defects, and other studies have shown normal responses to different antigens or at different stages of infection.

The earliest observations suggestive of specific defects in immune responses caused by malaria were those relating to vaccine responses. In 1962, McGregor reported that responses to tetanus vaccination were improved in Gambian children receiving malaria chemoprophylaxis compared with children not receiving chemoprophylaxis,²⁰ and numerous subsequent studies demonstrated that responses, mainly to carbohydrate antigens, were suppressed in malaria (see Chapter 3).²¹

Later studies investigated alterations in cellular immunity and demonstrated a variety of abnormalities during and following acute malaria infection, including: T-lymphopenia,^{18,22} reduced proliferative responses to some antigens (particularly malarial antigens),^{18,22-24} reduced cutaneous delayed type hypersensitivity responses,¹⁸ and reduced levels of plasma IL-2.¹⁸ However, not all of these findings were confirmed in other studies,¹⁷ and the results must be interpreted with caution since they provide only snap shots of the function of cells in peripheral blood during infection, and not an assessment of the function of the immune system as a whole. Subsequent studies demonstrated activation and redistribution of lymphocyte subsets during acute malaria infection,²⁵⁻²⁶ suggesting that apparent cellular immunosuppression is most likely a consequence of both the timing and source of the sample during the dynamic process of migration, expansion and subsequent

contraction of lymphocyte populations, which characterises the immune response to malaria.²⁷

Abnormalities in innate immune function have been described in malaria, principally affecting monocytes, macrophages and dendritic cells. In 1999, the finding that dendritic cell maturation appeared to be compromised by co-incubation with parasitized erythrocytes²⁸ fuelled great interest in the potential role of dendritic cell dysfunction as an explanation for immunosuppression caused by malaria, unfortunately resulting in many studies with apparently contradictory results.²⁹ Studies in non-lethal rodent malaria infections generally showed normal dendritic cell function,³⁰⁻³² but abnormal maturation and function was observed in more virulent infections.^{30 33-34} However, similar to apparent variations in T-lymphocyte numbers and function, it is likely that changes in dendritic cell function also reflect the dynamic changes necessary to mount and then regulate an inflammatory response to malaria infection,³⁵⁻³⁶ and conflicting findings result, in part, from assessment of dendritic cell function at different stages in this process.

Phagocytic cell function, primarily that of macrophages and monocytes, has also been investigated in malaria. Most studies have used *ex-vivo* and *in-vitro* assessments and there has been relatively little *in vivo* investigation of the importance of these cells in explaining the susceptibility to other infections that occurs with malaria. Particular interest has focussed on the role of hemozoin, the insoluble hemin polymer produced during hemoglobin digestion inside the parasitised red cell.³⁷ Phagocytosis of hemozoin by monocytes impairs their i) oxidative burst, ii) ability to perform further phagocytosis, iii) ability to kill phagocytosed bacteria, and iv) expression of MHCII, CD54, CD11c, whilst stimulating TNF-alpha, MMP-9 and IL-10 secretion but inhibiting IL-2 and IL-12 secretion.³⁸⁻⁴² Thus hemozoin may have the potential to affect many aspects of monocyte and macrophage function, influencing direct antimicrobial activity, antigen presentation, and stimulation and polarization of the adaptive immune response.⁴³ However the relevance of hemozoin-mediated phagocyte dysfunction *in vivo* has not been clearly established. In an observational study of Kenyan children with acute malaria, the presence of hemozoin containing monocytes was associated with lower levels of systemic IL-12,⁴² but whether this simply reflects

different durations and kinetics of parasitemia, and hence the immune response, in children with hemozoin accumulation compared to those without, is impossible to establish.

Far from causing a state of anergy, there is abundant evidence that malaria drives immune activation. Plasmodium species produce a variety of innate immune system activating ligands including: glycosylphosphatidylinositols (GPIs), signalling through Toll-like receptors (TLR) 2 and 4;⁴⁴ parasite DNA, often in complex with hemozoin, signalling through TLR-9⁴⁵ and novel innate receptors.⁴⁶ Hemozoin alone signals through the NOD-like receptor containing pyrin domain 3 (NLRP3) inflammasome and parasite-derived microparticles signal through TLR 4.⁴⁷ These innate signalling pathways may augment the polyclonal B cell activation that occurs due to type 1 T-cell independent *P. falciparum* antigens.⁴⁸⁻⁴⁹ In addition, early, robust T-cell activation has been demonstrated by experimental infections in mice⁵⁰ and in sporozoite-infected human volunteers in whom expansion of interferon- γ producing effector memory CD4⁺T cells and $\gamma\delta$ T cells is observed.⁵¹⁻⁵² Consistent with early innate and cellular immune activation, elevated levels of soluble granzyme A, interferon- γ , IL-8 and IL-12p40 are detectable in plasma several days prior to microscopic detection of parasitemia in blood, coinciding with the timing of first release of merozoites from the liver.⁵³⁻⁵⁴ There may be considerable variations between individuals in these early innate responses, which influence both the rate of increase of parasitemia and the onset of symptoms.⁵⁴ In experimental infection of humans, treatment is normally commenced when parasitemia becomes microscopically detectable, and so it is not possible to examine the effect of ongoing parasitemia on subsequent activation and function of T-lymphocytes, but infections in mice suggest that there is significant redistribution of lymphocytes^{27 55} and progressive differentiation into late effector memory and effector CD4⁺ T cell phenotypes.⁵⁶

Whilst immune activation is necessary to produce an effective immune response to control a pathogen, a consequence of persistent or repetitive immune activation can be the functional exhaustion of immune cells.⁵⁷⁻⁵⁸ This has been well studied in humans with HIV, where sustained immune activation is now believed to be the most important mechanism causing immune dysfunction,⁵⁹ and many features seen in HIV

are also observed following prolonged or repeated exposure to malaria: polyclonal B cell activation,⁶⁰ hypergammaglobulinaemia,⁶¹ expansion of atypical memory B cells,⁶² and T-cell exhaustion.⁶³ In high transmission settings, where the indirect burden of malaria seems greatest,⁷ the consequences of repetitive and persistent immune activation may result in dysfunctional heterologous immune responses and explain the observed susceptibility to other infectious diseases. Furthermore, malaria infections drive the generation of regulatory T-cells, which appear not to significantly influence the early response to the malaria infection, but could conceivably alter host responses to other pathogens.⁶⁴

In summary, despite early studies often demonstrating specific immunosuppression in malaria, evidence for generalized immunosuppression by acute malaria is lacking. More likely, recurrent or persistent infections cause immunological changes due to persistent immune activation. However, as will be discussed below, there may be additional explanations for the specific associations of malaria with poor antibody responses to some vaccinations, chronic Epstein-Barr viremia and endemic Burkitt lymphoma, increased HIV viral load, and Gram negative bacteremia.

Malaria and antibody responses

Impaired responses to vaccination have been demonstrated in animals infected with rodent malaria parasites, as well as humans infected with *P. falciparum* (reviewed in detail in Chapter 3).²¹ In this review of the literature I found that malaria had little effect on the responses to most modern protein antigen vaccines, but seemed to predominantly affect the responses to T-independent antigens. In contrast to the extensive literature on malaria and vaccine responses, there is very little literature regarding naturally acquired humoral responses to other pathogens in individuals being repeatedly exposed to malaria. These might be particularly interesting to study, because naturally occurring responses to some pathogens such as *Streptococcus pneumoniae*, may differ from vaccine induced responses to the same pathogen,⁶⁵ and could potentially be less robust. Recent findings suggest that repeated exposure to malaria promotes atypical memory B-cell generation.^{62 66-67} In the context of HIV

infection, this atypical memory B-cell phenotype has been proposed to be functionally “exhausted”,⁶⁸ although there is still a need for clarification of the role of these cells *in vivo*. However, if these really are an exhausted B-cell phenotype, this raises the possibility that exposure to intense malaria transmission might increase susceptibility to a range of pathogens by impairing either vaccine-induced or naturally occurring humoral immunity.

Malaria, Epstein-Barr Virus and endemic Burkitt lymphoma

Endemic Burkitt lymphoma is an unusual childhood B-lymphocyte malignancy, which occurs in a restricted geographical distribution, often presenting as a disfiguring mass arising from the jaw.⁶⁹ It occurs across equatorial Africa and Papua New Guinea, in areas with holoendemic malaria and all cases are associated with EBV detectable within the tumour cells, and overexpression of the proto-oncogene *c-myc* which drives B-cell proliferation.⁶⁹⁻⁷⁰ The curious necessity for both sustained exposure to malaria (which is not closely associated with other cancers) and EBV (which is well known to promote tumorigenesis⁷¹) suggests that malaria somehow enhances the potential of EBV to cause malignant transformation.⁷⁰ Immunological mechanisms may underlie this phenomenon: malaria has been reported to impair EBV specific T-cell immunity¹³⁻¹⁴ and polyclonal B-cell activation by malaria antigens may trigger increased replication of latent EBV within these cells.^{70 72} However, it remains unclear why and how the establishment and maintenance of immunity to EBV is so impaired by malaria, or even whether this is a specific effect. A recent report suggests that replication of some, but not all, other herpes viruses may also be favoured by episodes of malaria.⁷³ Interestingly though, following a marked decline in the incidence of malaria in The Gambia,⁷⁴ impairment of T-cell control of EBV-infected B-cells¹³ is no longer apparent in children with acute malaria,⁷⁵ suggesting that repeated exposure is necessary to produce a functional deficit.

Malaria and HIV

The observation of a geographical overlap between areas of high HIV prevalence and high malaria transmission raised the obvious question of whether there are important interactions between the two infections.⁷⁶⁻⁷⁷ It was hypothesised that malaria might promote HIV replication or impair immunological control of replication, and acute malaria was found to cause modest increases in HIV viral load.¹² However, the effect of malaria on HIV viral load is similar to that of most other intercurrent infections,⁷⁸ suggesting that malaria does not have a pronounced immunosuppressive effect on the immune response to HIV. Despite this, there are many possible consequences of the reciprocal interactions between malaria and HIV, and these are reviewed in detail in Chapter 4.

Malaria and bacterial co-infection

An association between malaria and susceptibility to invasive bacterial infection has been known for almost a century,⁹ and has been repeatedly documented in different settings across Sub-Saharan Africa.^{11 79-81} This association was first described for malaria and non-Typhoid Salmonella (NTS) bacteremia,⁹ which remains the most frequent cause of malaria associated bacteremia in many studies, but also includes susceptibility to other Gram negative bacteria.¹⁰⁻¹¹ Although coincidence of two common pathologies, and shared risk factors such as the local environment, rainfall, poverty and co-morbidities (such as HIV and malnutrition) may be hypothesised to contribute to susceptibility to both malaria and invasive bacterial infection, there is compelling evidence for causality rather than merely an association.

Observations from the pre-antibiotic era provide a fascinating insight into the relationship between malaria and NTS in ways that could never now be tested in humans. Prior to the availability of penicillin, “malaria therapy” (the deliberate inoculation with blood stage malaria parasites) was widely used as treatment for neurosyphilis,⁸² presumed to work by inducing fever that killed the treponemal spirochaetes. However it was observed that malaria therapy was often associated with NTS bacteremia and bacterial meningitis, even in countries where NTS infection was very rare in healthy individuals.⁸³ Supporting the concept that the malaria was the

cause of the susceptibility to NTS infection, observations in British Guyana demonstrated that once the malaria was cured with quinine, co-infected individuals were often able to spontaneously clear NTS infection without additional treatment.

Studies of the epidemiology of malaria-NTS co-infection have clearly shown that the incidence of malaria and NTS bacteremia are strongly correlated,^{11 79 84} whereas stool carriage of NTS is not as closely related to the incidence of NTS bacteremia.⁷⁹ Where malaria transmission has declined over time, similar trends have been observed in NTS bacteremia.^{11 84} Controlling for the effects of HIV and malnutrition still reveals a robust association between malaria and bacteremia.^{10 81} The most elegant demonstration of the effect of malaria on susceptibility to bacteremia comes from recent analysis of the associations between sickle cell trait, malaria and bacteremia.¹¹ Sickle cell trait is a classic example of heterozygote advantage, providing protection against the development of malaria.⁸⁵ In Kenyan children, sickle cell trait was associated with a decreased risk of bacteraemia that was entirely dependent on the protection it afforded against malaria: as malaria incidence declined the protective effect of sickle cell trait against bacteremia was lost.¹¹ The same study also allowed the estimation that nearly two-thirds of cases of bacteraemia were attributable to the effect of malaria when malaria transmission was at its highest levels.¹¹ NTS has been reported as one of the most common causes of community acquired bacteremia in children presenting to hospital in Kenya,⁸⁶ second only to *S. pneumoniae*. However the association between malaria and bacteremia extends only to NTS and some other common Gram negative organisms but not to *S. pneumoniae* and other Gram positive bacteria.¹¹

High case fatality rates have been reported for patients hospitalised with malaria and bacterial co-infections, suggesting that mortality may be increased,^{10 87-88} but most studies have lacked statistical power to determine whether this truly exceeds the mortality associated with either bacteremia or equivalently severe malaria alone.

Whilst findings of a strong association between NTS bacteremia and malaria have been consistent, the timing and duration of susceptibility have not been so clearly established. In the earliest reports both infections were observed concurrently in

individuals who were often not treated for some time after the onset of symptoms.^{9 83} Several subsequent studies have suggested that increased susceptibility to NTS bacteremia may persist after clearance of microscopically detectable malaria infection,^{83 89-90} or that susceptibility is greater at moderate than high density parasitemia.⁹⁰⁻⁹¹ Other studies have suggested that the association is particularly strong in the case of severe malarial anemia.^{79 81 88 91-92} Since severe malarial anemia most likely occurs following prolonged or recurrent malaria infections,⁹³ the observations above might be reconciled by postulating that malaria causes susceptibility to NTS through a delayed mechanism that requires a certain amount of time to become operational following onset of either parasitemia or symptoms.

Clinical observations have prompted speculation that malaria may cause susceptibility to bacteremia through immunoparesis,¹⁰ impairment of phagocytic cell function,^{79 94-95} complement consumption,⁹⁴ or increased gut permeability.¹⁰ Since acquisition of functional antibodies against NTS also plays a crucial role in preventing infection,⁹⁶⁻⁹⁷ one may also speculate that malaria might cause susceptibility through impairment of the humoral response to NTS. However, other clinical observations suggest that an alternative mechanism, related to hemolysis, may be responsible. Malaria is not the only infectious disease to predispose to invasive NTS infection. Oroya fever, an acute form of Bartonellosis, was strongly associated with NTS bacteremia.⁹⁸ Interestingly, Oroya fever is characterised by an acute hemolytic phase,⁹⁹ and it is at this stage that susceptibility to NTS manifests clinically.⁹⁸ Hemolysis is also a characteristic feature of sickle cell disease, due to intravascular destruction of abnormal red blood cells, and sickle cell disease strongly predisposes to NTS infection.¹⁰⁰⁻¹⁰¹

Although it is not immediately obvious how hemolysis might cause susceptibility to NTS bacteremia, these clinical observations inspired investigation of the effects of hemolysis and anemia on susceptibility to NTS in mice. In seminal experiments Kaye and Hook showed that acute hemolytic anemia increased susceptibility to *Salmonella typhimurium*, whereas an equivalent degree of anemia caused by blood loss alone, did not.¹⁰² This was not dependent on the route of administration of bacteria (intravenous, intraperitoneal or by gavage) or on the method used to induce hemolysis (anti-mouse-erythrocyte serum, phenylhydrazine administration or *P. berghei* infection).¹⁰²⁻¹⁰³

Increased susceptibility was manifest by decreased survival and increased bacterial replication in liver, spleen, blood and kidneys.¹⁰²⁻¹⁰⁴ Increased susceptibility was also found following intravenous or intraperitoneal infection with *Escherichia coli* and *Staphylococcus aureus* but not two different strains of *S. pneumoniae*.¹⁰² In these experiments there was no evidence of significant impairment of the clearance of carbon particles or viable bacteria from blood, suggesting that phagocytic mechanisms were unaffected, and therefore implying that hemolysis impaired bacterial killing.¹⁰⁴ Interestingly neither administration of hemoglobin-, nor red cell stroma-enriched fractions of freeze-fractured red blood cells reproduced susceptibility to *S. typhimurium*,¹⁰² although it is not clear that the amounts administered were equivalent to the amounts likely to be generated by *in vivo* hemolysis.

Others have largely confirmed the observations of Kaye and Hook, showing that hemolysis and different rodent malaria species can induce susceptibility to infection with *S. typhimurium*,¹⁰⁵ *S. enteritidis*, *Yersinia enterocolitica* and *Listeria monocytogenes*.¹⁰⁶ These studies have also sought to explain the susceptibility caused by malaria based on dysfunction of macrophages, but crucially neither has demonstrated that increased bacterial replication *in vivo* occurs within macrophages.¹⁰⁵⁻¹⁰⁶ Furthermore, Roux et al reported that antibody mediated hemolysis caused less susceptibility than did malarial hemolysis, proposing malaria specific immunosuppression to explain this discrepancy, without demonstrating that both conditions produced equivalent degrees of intravascular hemolysis.¹⁰⁵

Tolerance and resistance

One issue not considered in detail so far, is whether malaria causes its indirect burden of disease by increasing the severity of illness associated with other infections, or by increasing the likelihood of infections occurring, or both. Another way to consider this question is using the emerging concepts of pathogen resistance and tolerance.¹⁰⁷⁻¹⁰⁸ In this context resistance mechanisms are defined as acting to limit pathogen burden, whereas tolerance mechanisms act to reduce the damage or promote survival of the host at a given pathogen load. These mechanisms need not be mutually exclusive or

even necessarily have the same effect in the context of different pathogens.¹⁰⁹ Resistance mechanisms are most obvious and include most of the defences conventionally thought to protect hosts from pathogen invasion, starting from the physical barriers of the skin and gut right through to most aspects of the innate and adaptive immune systems.¹¹⁰ Tolerance mechanisms are less well characterised¹¹⁰ but intuitively would seem to explain the common clinical scenario where one patient with bacteremia may have few symptoms besides fever, whilst another infected with the same organism may die from septic shock. Of course, the main problem in humans is determining whether these patients have the same pathogen load, and hence disentangling resistance from tolerance. One may suppose that a patient with severe malarial anemia might have reduced physiological tolerance to the additional stress of severe gastroenteritis or pneumonia, explaining how malaria might increase mortality in these conditions.⁶ However the specific examples described above suggest that malaria, particularly with repeated or sustained infection, impairs resistance to several pathogens: *P. falciparum* malaria increases EBV and HIV viral loads,^{12 14 111} and increases the incidence of bacteremia with NTS,¹¹ and - in mice - malaria permits more rapid replication of NTS.¹⁰⁵ The apparent increase in mortality in malaria-NTS co-infection also raises the possibility that tolerance is impaired.⁸⁷⁻⁸⁸

One of the few pure tolerance mechanisms clearly described in mammals is the detoxification of hemolysis-derived heme by the inducible enzyme heme oxygenase-1 (HO-1) in murine malaria,¹¹²⁻¹¹⁴ and subsequently HO-1 induction has been proposed to mediate tolerance in a sepsis model as well.¹¹⁵

Heme

Heme (ferrous (Fe^{2+}) iron protoporphyrin IX) is an essential component of heme proteins such as hemoglobin and cytochrome P450, and is indispensable to aerobic cells.¹¹⁶ The ferric (Fe^{3+}) form of iron protoporphyrin IX is termed hemin, although the term heme is also often used to describe both the ferrous and ferric forms.¹¹⁷ Heme is usually contained in the intracellular compartment, but can be released in a variety of pathological conditions, when it may incorporate into cell membranes and release iron

which can drive production of damaging reactive oxygen species (ROS).¹¹⁷⁻¹¹⁸ Situations where heme proteins are released from the cellular compartment in particularly large amounts include hemolysis (hemoglobin derived heme) and rhabdomyolysis (myoglobin derived heme).¹¹⁷ Oxidation of cell free-hemoglobin to methemoglobin allows the release of the heme moiety.¹¹² Free heme is extremely hydrophobic and intercalates into cell membranes, where it becomes susceptible to further oxidation to release free, redox-active, iron which amplifies the generation of further ROS and cell damage. The accumulation of cytotoxic cell-free heme is usually prevented by a series of defences: the plasma protein haptoglobin binds and stabilises cell-free hemoglobin, preventing its oxidation and release of heme and targeting hemoglobin to CD163 expressing cells;¹¹⁹ if haptoglobin levels are depleted by overwhelming hemolysis, another plasma protein, hemopexin binds avidly to cell free heme preventing its cytotoxic effects and targeting it to CD91 expressing cells;¹²⁰⁻¹²¹ finally, albumin and lipoproteins are able to bind heme (albeit more weakly than hemopexin) and may provide a final buffer against heme toxicity in extreme hemolysis.¹¹⁸

In rodent models of sepsis and malaria, cell free heme has been shown to contribute to tissue damage by sensitising cells to TNF- α triggered, ROS-mediated, programmed cell death.^{113 115} These dying cells may release endogenous pro-inflammatory mediators, such as high mobility group box 1 (HMGB1), which then link heme sensitised-cell death to exacerbation of the inflammatory response.¹¹⁵ In other studies free heme has been shown to activate the neutrophil oxidative burst and trigger neutrophil migration whilst preventing neutrophil apoptosis,¹²²⁻¹²⁵ to activate TLR-4,¹²⁶⁻¹²⁷ and to oxidize low density lipoprotein to cytotoxic derivatives.¹²⁸ Consequences of heme toxicity include leukocytosis,¹²⁹ and endothelial activation with upregulation of vascular adhesion molecules¹³⁰⁻¹³¹ and tissue factor.¹³² By as yet undefined mechanisms, free heme also exacerbates depletion of L-arginine, the precursor of nitric oxide, which might exacerbate endothelial dysfunction in hemolytic disorders.¹³³

Heme oxygenase

Heme oxygenase (HO) enzymes catalyze the rate limiting step of heme degradation, producing carbon monoxide (CO), iron and biliverdin (Fig 1.1).¹³⁴ Biliverdin is rapidly converted to bilirubin by biliverdin reductase.¹³⁵ Three isoforms of HO have been described (HO-1, HO-2 and HO-3).^{134 136-137} HO-1 is inducible by diverse stimuli including its substrate heme, and numerous potentially harmful stimuli (Table 1), but is also expressed constitutively in spleen and liver, presumably because of their roles in heme degradation.^{134 138-139} HO-2 is constitutively expressed by most tissues, and shows much less transcriptional regulation.^{136 138} HO-3 was identified by screening a cDNA library,¹³⁷ but subsequent studies have failed to demonstrate a functional HO-3 gene, and its relevance remains dubious.¹⁴⁰ HO enzymes have been characterized in vertebrates, invertebrates, protozoa (including *Plasmodium falciparum*¹⁴¹), plants and bacteria.¹⁴²⁻¹⁴³

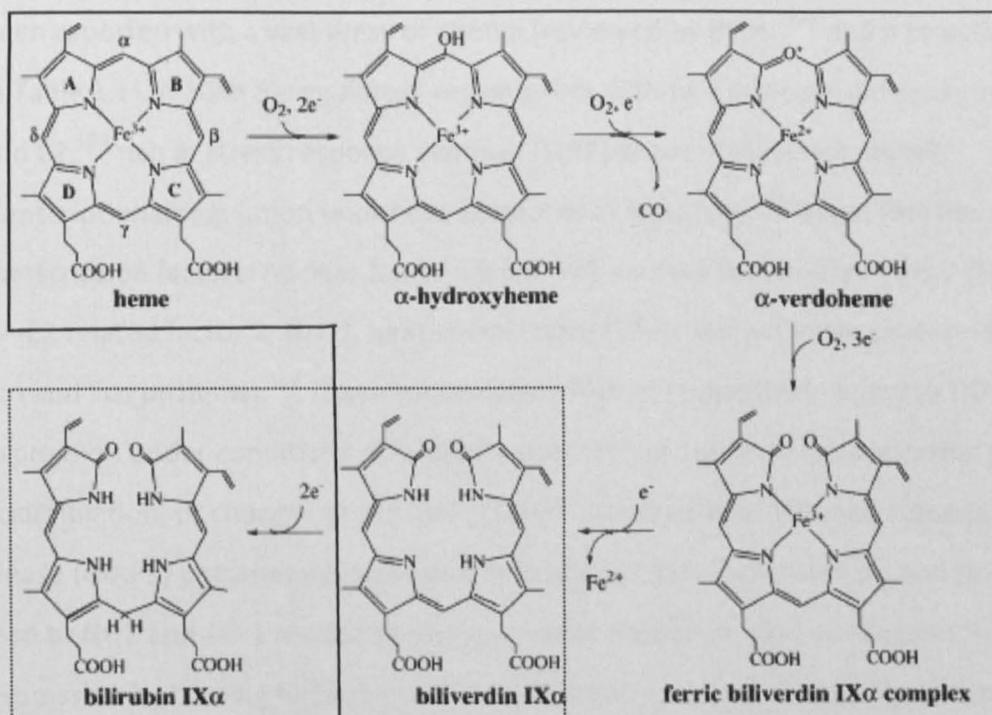


Figure 1.1 Heme degradation.

Heme is converted to biliverdin by heme oxygenase (box defined by solid line), with the liberation of CO and Fe²⁺. Biliverdin is rapidly converted to bilirubin by biliverdin reductase (box defined by dashed line). Adapted from Kikuchi et al 2005.¹⁴²

Heme oxygenase-1

Following its characterization in 1968,¹³⁴ HO was demonstrated to be induced in many tissues with constitutively low levels of activity, by hemoglobin,¹⁴⁴ trace metals,¹⁴⁵ and endotoxin.¹⁴⁶ The inducible isoform was designated HO-1.¹³⁶ Most attention focused on its role in heme metabolism but in 1987 rat HO-1 was characterised as a heat shock protein,¹⁴⁷ and subsequently HO-1 was demonstrated to be induced in skin fibroblasts by ultraviolet-A radiation, suggesting a protective function in addition to heme degradation.¹⁴⁸ The anti-inflammatory role of HO-1 was first described in 1995, attenuating the effects of carrageenin-induced acute pleural inflammation,¹⁴⁹⁻¹⁵⁰ and endotoxic shock.¹⁵¹

HO-1 is encoded by the *HMOX1* gene in humans located on chromosome 22 q12 and by the *Hmox1* gene on chromosome 8 in the mouse.¹⁵² Changes in expression have been reported with a vast array of stimuli [reviewed by Ryter,¹³⁹ and a selection listed in Table 1.1]. A 10kb 5'-regulatory region exists with two principal enhancer regions E1 and E2,¹³⁹ rich in stress response element (StRE) sequences, which permit transcriptional regulation under the control of at least four different families of transcription factors: nuclear factor- κ B (NF- κ B), nuclear factor-erythroid 2 (NF-E2; and NF-E2 related factor 2, Nrf2), heat-shock factor (HSF), and activator protein-1 (AP-1; Jun and Fos proteins).¹⁵³ These transcription factors respectively increase HO-1 expression under conditions of inflammatory and oxidative stress, abnormal protein accumulation, or changes in cell fate (growth or cell death). Mitogen activated protein kinase (MAPK) cascades are activated by many HO-1 inducing stimuli, and primarily lead to Nrf2 and AP-1 mediated transcriptional activation. Heme regulates HO-1 expression by binding to Bach1, a repressor, and causing its dissociation from the enhancer site of *Hmox1* allowing binding of Nrf-2 (Figure 1.2).¹⁵⁴⁻¹⁵⁵ Unusually, heat shock produces species-specific and possibly cell type-specific changes in HO-1 expression, probably due to differences in the sequences of the promoter heat shock elements or functional repression.^{153 156}

Rat HO-1 is a heat shock protein, whereas the heat shock response is absent in mice and is absent from most human cell lines tested except hep3B hepatoma cells,¹⁵⁷ but is

present in human whole blood leukocytes.¹⁵⁸ The human *HMOX1* has a variable length GT-rich region, (GT)_n, which negatively regulates transcription and GT_n length polymorphisms have been associated with altered susceptibility to oxidative stress,¹⁵⁹ pneumonia,¹⁶⁰ acute respiratory distress syndrome,¹⁶¹ joint damage in rheumatoid arthritis,¹⁶² and susceptibility to severe malaria.^{158 163-164} An interesting observation is that short (GT)_n repeats, which result in higher *HMOX1* expression, seem to be generally associated with better outcomes or lower risk of chronic inflammatory illnesses, whereas they are associated with worse outcome in some acute illnesses.^{158 165-166} Post-transcriptional regulation of heme oxygenase-1 protein expression by microRNAs has also been recently demonstrated.¹⁶⁷

Table 1.1 Examples of inducers of HO-1.

| Inducing Stimulus | Mechanisms |
|--|--|
| Hypoxia | Hypoxia – hypoxia inducible factor-1 (hif-1) dependent ¹⁶⁸ |
| Hyperoxia | Hyperoxia – AP1 and STAT binding ¹⁶⁹ |
| Ultraviolet irradiation | Induction of Nrf2 ¹⁷⁰ |
| Cobalt protoporphyrin | Suppression of bach1 and increased Nrf2 ¹⁷¹ |
| Nitric oxide / Carbon Monoxide | MAPK dependent activation of Nrf2 ¹⁷²⁻¹⁷³ |
| 15d PGJ₂ (15-deoxy-delta-12,14-prostaglandin J2) | Activation of Nrf2 ¹⁷⁴ |
| Mechanical stress | Activation of Nrf2 and Anti-oxidant response element (ARE) ¹⁷⁵⁻¹⁷⁶ |
| Lipopolysaccharide (LPS) | Activation of p38MAPK and NFκB ¹⁷⁷ and induction of Nrf2 ¹⁷⁸ |

HO-1 was originally described as having a “microsomal” location,¹³⁴ and is most concentrated in the endoplasmic reticulum membrane, although its presence has been demonstrated in other cellular fractions in significant amounts.¹⁷⁹ HO-1 is orientated with its active site in the cytoplasm and has a hydrophobic transmembrane segment near the C-terminus, which facilitates the oligomerization necessary for optimal function,¹⁸⁰ but which can be cleaved to allow nuclear trafficking and redistribution.¹⁸¹ Localization of HO-1 to cell surface membrane caveolae has been demonstrated in endothelial cells following LPS or hemin treatment,¹⁸²⁻¹⁸³ and there CO facilitates the inhibitory effect of caveolin-1 binding to TLR-4.

HO-1 is an essential homeostatic enzyme. HO-1 deficient (*Hmox*^{-/-}) mice exhibit partial embryonic lethality (20% of the expected Mendelian ratio of *Hmox*^{-/-} pups), endothelial damage, vascular inflammation, intravascular haemolysis, iron overload, and growth retardation.¹⁸⁴ Similar features occurred in human HO-1 deficiency,¹⁸⁵ and both showed susceptibility to oxidative stress.¹⁸⁵⁻¹⁸⁶ In fact HO-1 is a potent inducible

defence mechanism against many forms of cellular stress, and CO, biliverdin/bilirubin and iron have all been ascribed cytoprotective roles (Table 1.2).¹¹⁸ Furthermore, roles for endogenous CO in neuronal and neuroendocrine signalling (primarily from HO-2), vasorelaxation, and gut smooth muscle contractility have been described [reviewed by Wu¹⁸⁷], but will not be discussed further here.

Table 1.2. Examples of mechanisms of protection against cellular stress by HO-1.

| Cellular Stressor | Protective HO-1 product | Proposed Mechanism |
|--|-------------------------|---|
| Ultraviolet irradiation | Iron | Ferritin synthesis and sequestration of iron prevents iron catalyzed free radical reactions ¹⁸⁸ |
| Serum deprivation, staurosporine or etoposide | Iron | Iron triggers enhanced iron efflux from cells and prevents iron-catalyzed free radical reactions ¹⁸⁹ |
| Hydrogen Peroxide | Bilirubin | Antioxidant ¹⁹⁰ |
| Ischemia-reperfusion injury | Biliverdin / bilirubin | Potent antioxidant activity ¹⁹¹ (but augmented by addition of CO ¹⁹²) |
| Lipopolysaccharide | CO | Activation of MKK3/ p38 MAP kinase ¹⁹³ Inhibition of JNK signalling and AP-1 activation ¹⁹⁴ (Also see Table 1.3 and Figure 1.2) |
| Ischemia / hypoxia | CO | Suppression of extracellular signal-regulated kinase (ERK) activation and early growth response 1 (Egr-1) gene expression ¹⁹⁵ |

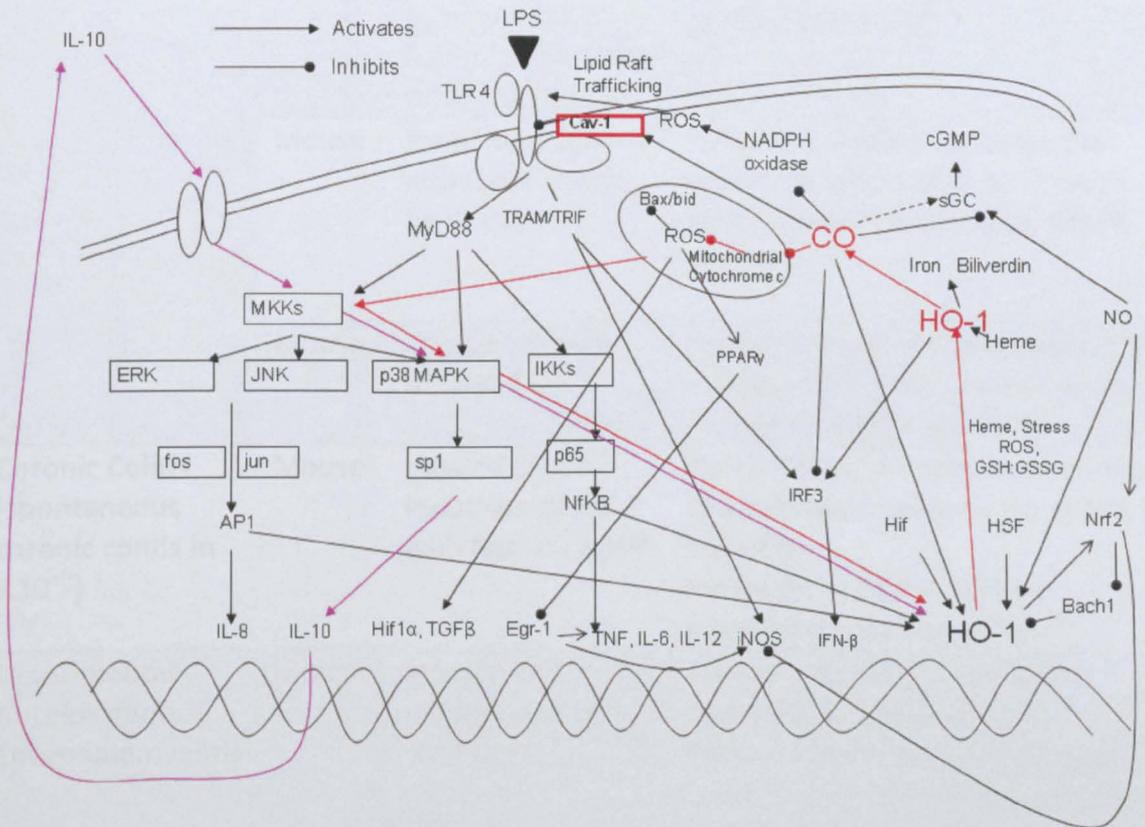
Immunomodulatory effects of HO-1

Many immunomodulatory effects have been described as a result of manipulation of HO-1 expression or activity, administration of CO and, to a lesser extent, bilirubin (Table 1.3).¹³⁹⁻¹⁹⁶ In general increased expression of HO-1 or administration of CO suppress inflammation, decrease apoptosis and reduce cellular proliferation (Table 1.4). In the *Hmox*^{-/-} mouse, there is elevated serum IgM concentration, Th1-skewed pro-inflammatory cytokine production, and abnormal distribution of B and T lymphocytes in the spleen and lymph nodes.¹⁹⁷ HO-1 influences both innate and adaptive immune responses. HO-1 induction suppresses inflammatory cytokine production and nitric oxide synthesis in macrophages, and can polarize toward the alternatively activated (M2) phenotype.¹⁹⁸ In dendritic cells, carbon monoxide inhibits TLR3/4-induced activation of interferon regulatory factor 3 (IRF3), consequent functional maturation, and the ability to stimulate CD8 T-cells to induce antigen specific autoimmunity.¹⁹⁹ CO also mediates suppression of CD4 T-cell proliferation in response to anti-CD3 plus anti-CD28 antibodies, by inhibiting IL-2 secretion.²⁰⁰ HO-1 expression in antigen presenting cells appears to be essential for regulatory T-cell function.²⁰¹ In vascular endothelial cells, induction or overexpression of HO-1 or administration of bilirubin inhibits TNF- α or LPS-induced upregulation of VCAM-1, ICAM-1, P- and E-selectin.²⁰²⁻²⁰⁴ This may be a major explanation for how HO-1 inhibits neutrophil recruitment to sites of inflammation. Recently a new mechanism by which HO-1 may modulate innate immunity has been proposed: using myeloid-specific HO-1 deficient mice, HO-1 was found to be necessary for expression of IFN- β , by forming a complex with IRF3 which was essential for its activation by TLR3/4 stimulation.²⁰⁵ This latter finding suggests that HO-1 may have immunomodulatory effects dissociated from production of its reaction products, but since CO also inhibits IRF3 activation,¹⁹⁹ it is likely that regulatory feedback loops exist.

Gram-negative bacterial lipopolysaccharide (LPS) is one of the most studied inflammatory stimuli. Induction of HO-1 prior to administration of a lethal dose of LPS prevented death and severe symptoms in rats,¹⁵¹ and inhalation of exogenous CO abrogated inflammatory cytokine production in mice exposed to a sub-lethal dose of LPS.¹⁹³ The latter observation was shown to involve activation of MAPK kinase 3

(MKK3) and p38 MAP kinase. Subsequent studies also identified CO derived from HO-1 as the downstream mediator of the protective effect of IL-10 against endotoxic shock in mice.²⁰⁶ Mechanisms which may be important in mediating the protective effect of HO-1 include: inhibition of NFκB and IRF3 activation by LPS,^{199 207} changes in organ specific iNOS expression;²⁰⁸ increased expression of peroxisome proliferator-activated receptor (PPAR-γ) and PPAR-γ dependent suppression of Egr-1 dependent procoagulant genes;²⁰⁹ inhibition of reactive oxygen species (ROS)-dependent translocation of TLRs to lipid rafts;²¹⁰ and suppression of TLR4 signal transduction by augmentation of binding of caveolin-1 to TLR4.¹⁸³ The exact mechanisms by which CO produces immunomodulatory effects have been difficult to characterise but there is an evolving concept that it alters the production of ROS and causes “oxidative conditioning” of cells which may of itself reduce inflammation,²¹¹ or protect cells from further oxidative damage and limit positive reinforcement of the inflammatory cascade.¹¹⁸ Binding of CO to cytochrome a3 of complex IV of the mitochondrial electron transport chain enhances the generation of superoxide (O_2^-) and subsequently H_2O_2 , which can function as important signal transducers,^{118 212} whereas binding to NADPH oxidase (Nox) enzymes can inhibit ROS accumulation.²¹⁰⁻²¹² Some mechanisms by which HO-1 may suppress the inflammatory response to LPS are illustrated in Figure 1.2.

Figure 1.2 Pathways mediating the effects of HO-1 on the response to LPS in a macrophage.



AP-1, Activator protein-1; Bax, Bcl-2 associated X protein; Bid, BH3 interacting domain death agonist; Cav-1, Caveolin-1; cGMP, Cyclic guanosine monophosphate; CO, Carbon monoxide; Egr-1, Early growth response protein-1; ERK, Extracellular signal related kinases; GSH, Sulfhydryl glutathione; GSSG, Glutathione disulfide; Hif-1, Hypoxia inducible factor-1; HO, Heme oxygenase; HSF, Heat shock factor; IFN, Interferon; IKK, Inhibitor of κ B ($\text{I}\kappa\text{B}$) kinase; IRF, Interferon regulatory factor; JNK, c-jun N-terminal kinase; LPS, Lipopolysaccharide; MAPK, Mitogen activated protein kinase; MKK, MAPK kinase; MyD88, Myeloid differentiation primary response gene 88; NADPH, Nicotinamide adenine dinucleotide phosphate; NF-E2, Nuclear factor erythroid-2; NF κ B, Nuclear factor κ B; NO, Nitric oxide; NOX, NADPH oxidase; Nrf2, NFE-2 related factor2; PPAR- γ , Peroxisome proliferator-activated receptor; ROS, Reactive oxygen species; sGC, Soluble guanylate cyclase; TGF- β , Transforming growth factor- β ; TLR, Toll like receptor; TNF- α , Tumour necrosis factor- α ; TRAM, TRIF-related adaptor molecule; TRIF, TIR-domain-containing adapter-inducing interferon- β .

Table 1.3. Immunomodulatory Effects of HO-1 in selected disease models.

| Disease Process / Model | Species | Manipulation | Outcome / proposed mechanism |
|--|---------|--|--|
| Endotoxemia | Rat | Induction of HO-1 by injection of haemoglobin (Hb) prior to LPS | 100% survival in Hb treated mice, due to HO-1 induction ¹⁵¹ |
| | Mouse | Protection against endotoxic shock by IL-10 | Induction of HO-1 mediates the protective effect of IL-10 through generation of CO acting on MAPK pathway ²⁰⁶ |
| | Mouse | Treatment with inhaled CO | Reduced levels of TNF- α and IL-1 β , increased IL-10, due to increased MKK3/p38 MAPK activity ¹⁹³ |
| Chronic Colitis (spontaneous chronic colitis in IL10^{-/-}) | Mouse | Inhaled CO or induction of HO-1 with hemin / CoPP | Amelioration of established chronic Th1 mediated colitis by CO or HO-1 induction. Inhibition of IRF8 and IFN- γ augmented IL-12 secretion ²¹³ |
| Experimental Autoimmune Encephalomyelitis | Mouse | Inhaled CO or induction of HO-1 with CoPP | Amelioration of disease and prevention of progression. Reduced leukocyte accumulation in brain, reduced MHC class II expression in brain APCs and reduced pathogenic CD4 cell proliferation and effector function ²¹⁴ |
| Collagen-Induced Arthritis | Mouse | Carbon monoxide releasing molecule (CORM-3) | Amelioration of established arthritis. Reduced ICAM-1 expression, reduced inflammatory infiltrate, reduced inflammatory cytokine production ²¹⁵ |
| Transplant Pancreatic islet cell allograft | Mouse | Inhaled Carbon monoxide, bilirubin or both to donors and / or recipients | Prolonged graft survival and donor specific tolerance to second graft. Accumulation of regulatory T-cells within graft ²¹⁶ |

Table 1.4. Immunomodulatory effects of HO-1 in selected cell types.

| Cell Type | Effects of HO-1 or its products |
|-----------------------|---|
| Neutrophil | Reduction of superoxide production ²¹⁷⁻²¹⁸ Delayed apoptosis ¹²⁴ |
| Monocyte | Reduction of superoxide production ²¹⁹ Downregulation of chemotaxis ²¹⁹ Delayed apoptosis ^{178 220} Reduced inflammatory cytokine response to LPS ^{178 221} |
| Macrophage | Reduced inflammatory responses to TLR ligands ^{183 193 210 222} Anti-inflammatory cytokine production ¹⁹³ Inhibition of NADPH oxidase activity ²²³⁻²²⁴ Enhanced phagocytosis ²²⁵ Inhibition of apoptosis ²²⁶ Alternative activation (M2) phenotype ¹⁹⁸ |
| NK Cells | Unknown |
| Dendritic cell | Impaired maturation ^{199 227} Impaired immunogenicity ^{199 227} Enhanced tolerogenicity and initiation of T-reg suppressive activity ^{201 228} Reduced production of reactive oxygen species ²²⁷ |
| T lymphocyte | |
| CD4 | Increased early proliferation and activation induced cell death of alloreactive CD4 cells ²²⁹ Suppression of IL-2 production and proliferation in response to anti-CD3/anti-CD28 ²⁰⁰ Resistance to apoptosis ²³⁰ |
| Regulatory | No direct influence; but regulatory function is dependent on antigen presenting cell expression HO-1 ^{201 231} |
| CD8 | Unknown |
| B lymphocyte | Possibly altered immunoglobulin class switching ²³² |

HO-1 in infectious diseases

The role of endogenous HO-1 in infectious diseases is of considerable interest, since the inflammatory response is essential to combat infection but, to limit excessive damage to the host, it must be tightly controlled and bystander damage must be limited by mediators such as HO-1.²³³ Conversely, inappropriately early downregulation of the inflammatory response could predispose to overwhelming pathogen replication, a factor not accounted for in models using endotoxaemia to simulate septic shock.

Induction of HO-1 has been shown to occur in authentic and experimental sepsis.^{165 225 234-235} In the latter case, constitutive overexpression of HO-1 in endothelial cells was beneficial to survival, associated with enhanced phagocytosis of bacteria and reduced apoptosis of host cells, and HO-1 deficiency reduced survival.²²⁵ Induction of HO-1 has been shown to occur during infection with *Plasmodium* species,^{112-113 158} *Leishmania*,²³⁶ *Salmonella enterica serovar typhimurium* (*S. typhimurium*),²³⁷ *Mycobacterium tuberculosis*,²³⁸ *Rickettsia*,²³⁹ Human Immunodeficiency Virus (HIV),²⁴⁰ human herpes virus 8 (HHV8),²⁴¹ and polymicrobial sepsis,¹¹⁵ whereas *L. monocytogenes*,²⁴² *Brucella abortus*,²⁴³ and hepatitis C virus infections²⁴⁴ have been reported to downregulate HO-1 expression.

Amastigotes of *Leishmania pifanoi* induce HO-1 in murine macrophages, which appears to prevent maturation of the NADPH oxidase complex and consequent production of O₂⁻.²³⁶ Although this may be beneficial to the parasite, HO-1 induction during infections with *S. typhimurium* and *Rickettsia rickettsii* has been proposed to be beneficial to the host cell by regulating the availability of iron or preventing apoptosis,^{226 237 239} whilst CO may promote dormancy of *Mycobacterium tuberculosis*.²⁴⁵ HO-1 expression is increased in Kaposi's sarcoma (KS), and endothelial cells infected with HHV8 (the causative agent of KS) proliferate more extensively in the presence of heme.²⁴¹ *B. abortus* and *L. monocytogenes* both decrease HO-1 expression in placental trophoblast, causing increased risk of abortion in pregnant mice.²⁴²⁻²⁴³ Downregulation of HO-1 by hepatitis C virus appears to be a component of viral strategies to promote oxidative stress in the liver, thereby creating conditions which favour viral

replication.²⁴⁶⁻²⁴⁷ In patients with AIDS, HO-1 expression is increased in monocytes,²⁴⁰ but *in vitro* induction of HO-1 in monocytes and T lymphocytes suppresses HIV replication.²⁴⁸

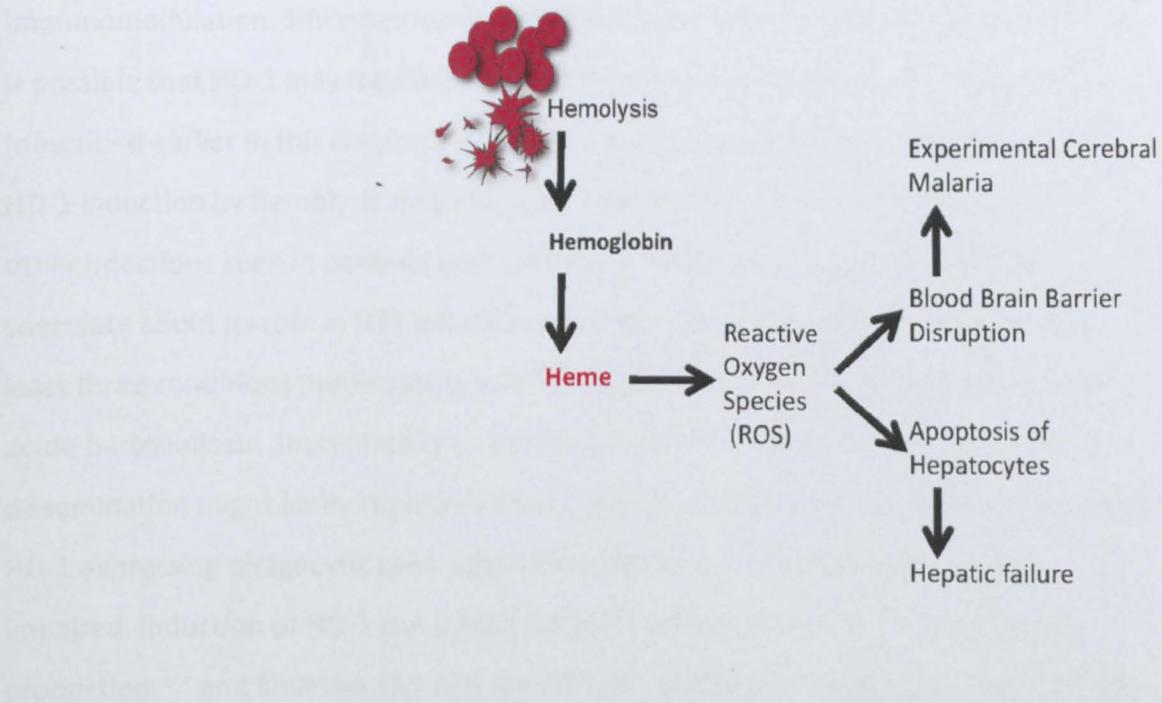
HO-1 in malaria

The malaria parasite lifecycle involves an obligatory intraerythrocytic phase during infection of the vertebrate host, and this leads to increased erythrocyte destruction through rupture at schizogony as well as increased destruction of both infected and uninfected erythrocytes by the host.²⁴⁹ The increase in erythrocyte turnover is associated with release of hemoglobin from erythrocytes and hence increased heme catabolism. Increased HO-1 activity in malaria can be demonstrated by measuring CO excretion in mice,²⁵⁰ and carboxyhemoglobin levels in humans.²⁵¹ Increased immunohistochemical staining for HO-1 occurs in monocytes and macrophages in lesions in the brain of individuals dying from cerebral malaria,²⁵²⁻²⁵³ and increased HO-1 mRNA expression is detectable in peripheral blood mononuclear cells.^{158 254} Experimental sporozoite infection in mice resulted in upregulation of liver HO-1 and if this was inhibited, the enhanced inflammatory response prevented progression to patent blood stage infection.²⁵⁵ In contrast, increased expression of HO-1 prevents experimental cerebral malaria (ECM).¹¹² Through a series of experiments it was shown that free heme was pivotal in the pathogenesis of ECM (although dependent on CD8⁺ T-lymphocytes); that methemoglobin (MetHb) was the main source of free heme generation; and that CO generated by HO-1, or exogenously administered, bound to free Hb and prevented the formation of MetHb. In additional experiments HO-1 induction was shown to be essential to protect hepatocytes from apoptosis in non-cerebral severe malaria¹¹³ and to explain the protective effect of a rodent model of sickle cell trait against severe malaria.¹¹⁴ The crucial underlying principle in all of these experimental systems was that HO-1 induction or treatment with carbon monoxide reduced the production of cytotoxic reactive oxygen species (summarised in Figure 1.3). Furthermore, in all of these situations HO-1 was shown to be a tolerance mechanism, preventing host pathology without influencing parasitemia. However three independent studies in different geographical settings examining the association

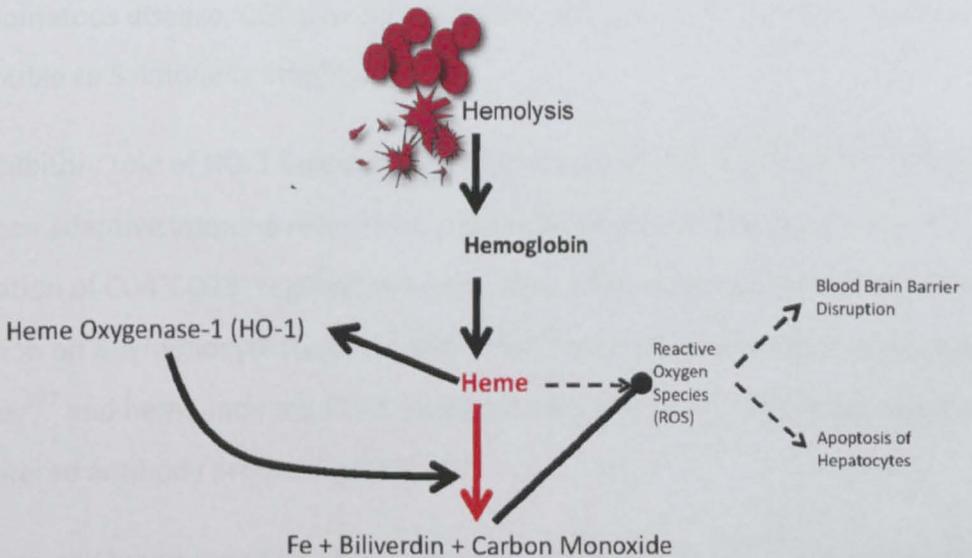
of human cerebral malaria with HO-1 microsatellite polymorphisms have shown that short GT_n alleles (associated with greater expression of HO-1) are more common in patients with severe and cerebral malaria.¹⁶³⁻¹⁶⁴ Hence the role of HO-1 in the pathogenesis of severe malaria in humans is currently unresolved,¹⁵⁸ but it is possible that either insufficient or excessive HO-1 induction might both predispose to severe malaria.

Figure 1.1. The role of heme and HO-1 in the pathogenesis of severe malaria in mice.

a. Hemolysis is Central to the Pathogenesis of Severe Malaria



b. Detoxification of Heme by HO-1 Confers Tolerance in Malaria



HO-1 as a potential immunomodulator in malaria?

HO-1 has the potential to be an important immunomodulator in malaria, but very little is known about changes in HO-1 expression in different populations of cells during the course of infection, which makes it difficult to speculate about mechanisms of immunomodulation. Since hemozoin has been shown to induce HO-1 expression,²⁵⁶ it is possible that HO-1 may mediate many of the effects of hemozoin on monocytes (described earlier in this chapter), although there is some evidence to dispute this.²⁵⁷ HO-1 induction by hemolysis may also play a role in the increased vulnerability to other infections seen in patients with malaria, and it is particularly tempting to speculate about its role in NTS infection, since hemolysis is a common feature of at least three conditions predisposing to NTS infection: malaria, sickle cell disease, and acute bartonellosis. Susceptibility to bacterial invasion through the gut mucosa and dissemination might be increased by more avid uptake of NTS by resident or circulating HO-1 expressing phagocytic cells, whilst their ability to kill Salmonella may be impaired. Induction of HO-1 can inhibit NADPH oxidase activity,²¹⁷⁻²¹⁸ suppress NO production,²²² and limit the TNF- α -IL-12/23-IFN- γ pathway,²¹³ which are all important for early,²⁵⁸⁻²⁵⁹ intermediate²⁵⁸ and late adaptive phases of control of *S. typhimurium* respectively in mice.²⁶⁰ Furthermore, humans with primary immunodeficiencies caused by genetic defects of subunits of the NADPH oxidase enzyme complex (chronic granulomatous disease, CGD), or defects in the TNF- α -IL-12/IL-23-IFN- γ pathway are susceptible to Salmonella infections.²⁶¹⁻²⁶²

The inhibitory role of HO-1 on dendritic cell maturation and function^{199 227 263} could influence adaptive immune responses in malaria, particularly by promoting the generation of CD4⁺CD25⁺ regulatory T-cells. Less is known about the effect of HO-1 induction on B-lymphocyte function, but *Hmox*^{-/-} mice have abnormal immunoglobulin profiles,¹⁹⁷ and heme-induced HO-1 expression has been reported to be associated with altered antibody production *in vivo*.²³²

Induction and inhibition of HO-1 using existing therapeutics,²⁶⁴⁻²⁶⁵ and administration of inhaled CO,²⁶⁶ are all described in humans, raising the possibility of therapeutic intervention if HO-1 has either detrimental or desirable immunomodulatory effects in malaria.

Hypothesis

Of the potential immunological consequences of malaria, one of the most striking and most clinically important appears to be the susceptibility to NTS bacteremia. Different strands of evidence from observations in humans and studies in animals point to a critical role for hemolysis in the specific susceptibility to NTS infection. Through induction of HO-1, hemolysis has the potential to produce immunomodulatory effects that may explain this susceptibility. Many of the cytoprotective effects of HO-1 converge on cellular responses to limit the production of potentially damaging reactive oxygen species, and since production of reactive oxygen species is critical for killing of NTS I decided that this would be the best mechanism to assess initially. For these reasons I hypothesised that induction of HO-1 by hemolysis would impair host resistance to NTS in malaria by impairing oxidative burst-dependent killing by phagocytic cells. This hypothesis and the resulting experimental approach is summarised in Figure 1.4.

Aim

The aim of my research was to test the hypotheses that

1. Induction of HO-1 by hemolysis impairs resistance to NTS in mice.
2. Induction of HO-1 occurs in specific cell types during malaria and impairs killing of NTS by those cells in mice and humans.
3. Induction of HO-1 can be modulated to abrogate the defect in the immune response and impaired resistance to NTS.

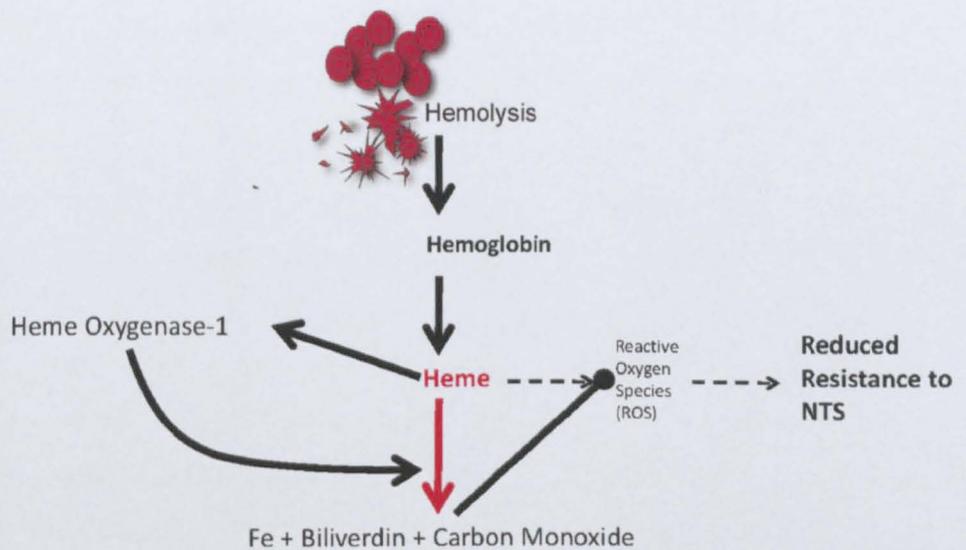
Project Outline

This project was designed to use two complementary strategies to investigate the role of HO-1 in malaria and NTS co-infection. In the first part of the project a rodent model system was used to elucidate the mechanisms by which malarial hemolysis impairs

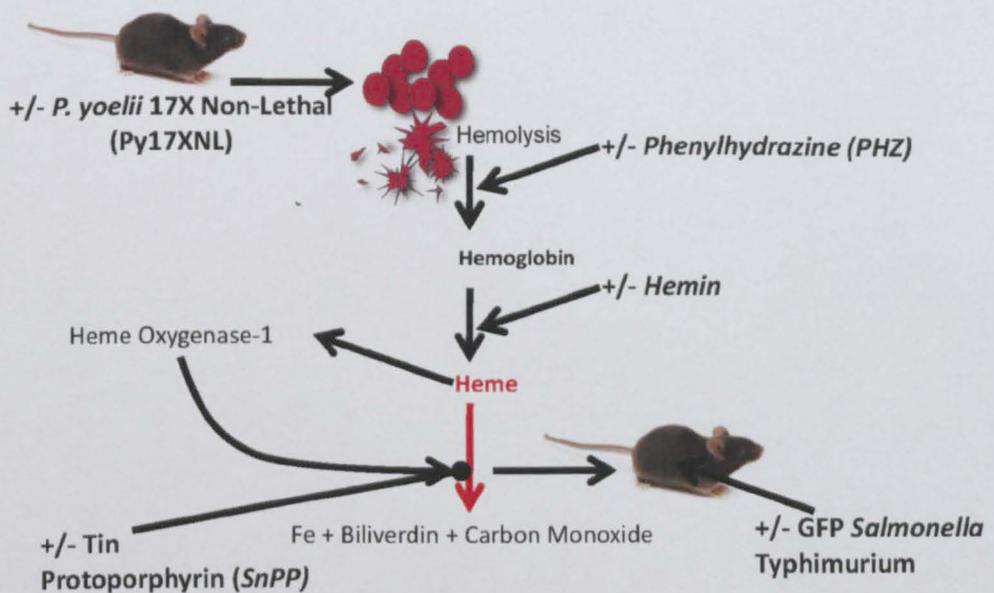
resistance to NTS co-infection. In the second part of the project blood samples were collected from Gambian children with acute malaria, and then during convalescence, to investigate whether the findings from experiments in mice would also apply in humans. The findings in humans were then used to inform additional experiments in mice.

Figure 1.4 Hypothesis and initial experimental model.

a. Hypothesis: *HO-1 confers tolerance to severe malaria but compromises resistance to NTS*



b. Experimental Model



Chapter 2. Research methods and assay validation.

Summary of the main techniques developed and optimized for studies in mice

This chapter describes the methods, optimisation and validation of the assays used for the studies presented in subsequent chapters. By necessity, this chapter includes brief discussions of results of validation studies, in order to clarify why certain methods were chosen for the studies presented later in this thesis. To avoid excessive duplication, reference is made to other chapters when relevant methodological information is fully presented as part of a research paper.

In order to assess the potential role of heme and heme oxygenase-1 in mediating some of the immunomodulatory effects of malaria, assays were needed to determine changes in HO-1 expression and activity in both tissue samples and defined cell populations. To simplify the initial phases of optimization of these assays a cell line known to have inducible HO-1 expression, RAW 264.7,²⁶⁷ was selected for *in vitro* cultivation and manipulation. After verification that the assays worked *in vitro*, assays were also verified by testing tissues or cells from mice that had been treated with HO-1 inducers.

RAW 264.7 cell culture

RAW 264.7 cells were a gift from Dr J. Brian de Souza, having been initially obtained from the European Collection of Cell Cultures, expanded *in vitro* and stored in liquid nitrogen until required for use. Under sterile conditions, RAW 264.7 cells were defrosted at room temperature, resuspended in 5ml warm culture medium (Dulbecco's modified Eagle medium (DMEM) +10% fetal bovine serum (FBS) + penicillin, streptomycin and L-glutamine (PSG) (all from Gibco)) and washed once by centrifugation at 500g for 5 minutes before aspirating the supernatant and resuspending in 7ml medium and seeding into a 25cm² culture flask. Cells were divided into two flasks in fresh medium after the first day of culture, by gentle mobilization from the flask with a sterile cell scraper, centrifugation at 500g for 5 minutes before

aspirating the supernatant and resuspending in 7ml warmed fresh medium per flask. Cells were then split in the same way every 2-3 days, when about 90% confluent. For experiments involving induction or inhibition of HO-1, treatments were performed when cells reached approximately 70% confluency and viability was assessed by trypan blue staining at the end of experiments and typically found to be 80-90% irrespective of treatment.

Animals

Animal experimentation conformed to UK Home Office Regulations and was approved by Institutional ethical review (London School of Hygiene and Tropical Medicine, Imperial College London, University College London, and Royal Veterinary College London, for experiments conducted in their respective facilities). Female, 6–10 week old C57BL/6 mice were obtained from Harlan and Charles River, UK and maintained under barrier conditions. Frozen stocks of blood-stage *Plasmodium yoelii* 17X Non-Lethal (Py17XNL) were inoculated in passage mice. Blood was collected after 5-7 days and experimental animals were infected by intraperitoneal (i.p.) injection of 10^5 parasitized red blood cells (pRBCs). Parasitemia was determined by examination of Giemsa-stained thin blood smears. Mice were killed with CO₂ inhalation. Immediately after death, blood was collected by cardiac puncture into heparinised syringes and tissues were removed into ice cold RPMI and stored on ice, protected from light, until processing.

Induction and Inhibition of HO-1

RAW 264.7 cells and mice were treated with the substrate inducer of HO-1, hemin (ferriprotoporphyrin IX chloride), and the non-substrate inducer cobalt (III) protoporphyrin IX chloride (CoPP) (Frontier Scientific). CoPP induces HO-1 expression through both Bach1 and nrf-2 dependent mechanisms,¹⁷¹ allowing greater induction than that achieved by hemin alone (which is mediated through Bach1).^{155 268} HO-2 expression is generally constitutive, and not inducible by metalloporphyrins.²⁶⁹ HO enzyme activity (which is the sum of the activity of HO-1 and HO-2) was inhibited by tin

protoporphyrin IX dichloride (SnPP), a competitive inhibitor that produces minimal induction of HO-1 expression when administered alone.²⁷⁰

Metalloporphyrins were protected from light and prepared by dissolving in 0.2M NaOH, diluted to the desired concentration in PBS and buffered carefully to pH 7.5 with HCl. For some *in vivo* experiments hemin solutions were subsequently filtered through a 0.2µm acrodisc syringe filter unit (Pall Corporation) and the concentration of the filtered solution determined using a Quantichrom Heme assay (BioAssay Systems) according to the manufacturer's instructions. For experiments using RAW 264.7 cells, protoporphyrins were added directly to fresh warm culture medium (Figure 2.1a), whilst for experiments using mice, metalloporphyrins were administered by intraperitoneal injection (Figure 2.1b).

HO activity assay

HO activity assays measure the sum of enzyme activity due to HO-1 and HO-2 in the sample, based on the catabolism of heme (present in excess) detected by the equimolar generation of the heme degradation products carbon monoxide or biliverdin (measured after conversion to bilirubin). Changes in HO activity are assumed to be due to HO-1 induction, since HO-2 shows much less inducibility. It is important to note that these assays give an indirect estimate of the amount of enzymatically active HO-1 in the sample, but do not give a measure of how much heme degradation was taking place in the tissue or cells from which the sample is derived prior to the assay, since this would depend on the availability of the substrate, heme, in the tissue or cell. The reason this is important is because many biological activities of HO-1 are attributed in part to the bioactive heme degradation products.

Although there are numerous methods described to measure HO activity, the method described by Motterlini et al.²⁷¹ was selected because of its relative simplicity and because the necessary equipment was already available. In this method HO activity is assessed by the production of bilirubin, based on the assumption that heme degradation by HO is the rate limiting step in production of bilirubin and the conversion of biliverdin to bilirubin (using excess rat liver cytosol as a source of

biliverdin reductase) proceeds rapidly. Briefly: 10^{6-7} cells were harvested, washed twice with cold PBS and resuspended in 550 μ l PBS + MgCl₂ (2mM), vortexed and placed in -80°C freezer until required for the assay. Immediately prior to the assay the cell suspension was frozen and thawed 3 times (-80°C (10 min) to 37°C (5 min) water bath), vortexed then sonicated on ice for 15 seconds to completely break cells apart. In a glass tube, 400 μ l of cell suspension was added to the reaction mixture of 300 μ l PBS + MgCl₂ (2mM), 100 μ l rat liver cytosol (see below), 25 μ l hemin (20 μ M, from freshly prepared 2mM hemin stock solution), 50 μ l glucose-6-phosphate (2mM final concentration), 15 μ l glucose-6-phosphate dehydrogenase (0.5 U/ml final), followed by final addition of 25 μ l NADPH (0.8mM final concentration) and vortexing to start the reaction. 100 μ l of cell suspension was retained on ice for determination of the protein content (protein assay kit, Bio-rad) according to manufacturer's instructions. The reaction mixture was incubated at 37°C in a water bath for 1 hour, after which time the reaction was stopped by adding 1 ml of chloroform and vortexing three times to maximise extraction of bilirubin into the chloroform. Tubes were centrifuged at 900g for 5 minutes at room temperature and to ensure extraction of all bilirubin in to chloroform, samples were briefly vortexed to disturb the bottom layer, and centrifuged again at 1100g for 5 minutes. Using a spectrophotometer and quartz cuvette, the OD_{464nm} and OD_{530nm} of the lower chloroform layer were determined, after setting a blank measurement using pure chloroform. For each assay a positive control (hemin treated RAW cells) and a negative control (without cells) were run in parallel. Heme oxygenase activity was calculated as follows:

1. Bilirubin concentration in chloroform:

$$\frac{\text{OD}_{464} - \text{OD}_{530}}{40} \quad \text{nmol} \quad \epsilon = 40 \text{ mM}^{-1} \cdot \text{cm}^{-1}$$

2. HO activity in pmol bilirubin formed / mg cell protein / 60 min

$$\frac{\text{OD}_{464} - \text{OD}_{530} \times 10^6}{40 \times (\text{protein content})} \quad \text{pmol/mg/60min}$$

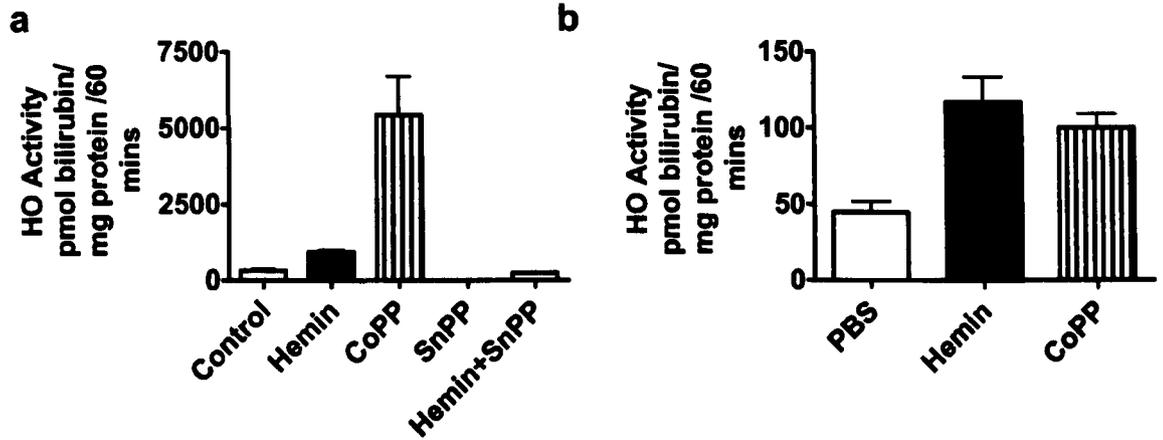
When tissue homogenates were used for the assay instead of cultured cells, tissues were disrupted by passage through a 200 μ L cell strainer, red blood cells were lysed using lysis buffer, and approx 10^{7-8} cells were washed once and resuspended in 550 μ l PBS + MgCl₂ before freezing as above.

Preparation of rat liver cytosol as a source of biliverdin reductase

Livers were harvested from male Sprague-Dawley rats (200-250 g), pooled and homogenized with a hand blender in 3 volumes of ice cold 1.15% KCl-20 mM Tris Buffer (pH=7.4). Aliquots of homogenate were transferred to polyallomer tubes and centrifuged at 5000g for 20 min, then the supernatant fraction was collected and centrifuged at 105,000g for 60 min. Following each centrifugation the turbid lipid layer was removed with a Pasteur pipette and following the last centrifugation the cytosol was removed, taking special care not to disturb the bottom microsomal pellet. The liver cytosol was aliquoted and kept at -80°C until required. Biliverdin reductase activity was confirmed by the conversion of biliverdin to bilirubin, essentially in the same way as the HO activity assay above, except that the sample volume was replaced with PBS + MgCl₂ (2mM) and biliverdin was substituted for hemin.

Figure 2.1 Validation of the heme oxygenase enzyme activity assay.

(a) HO activity was determined in lysates of RAW 264.7 cells following 24hr culture in medium alone (control) or with 20 μ M hemin, 20 μ M CoPP or 10 μ M SnPP (results are mean \pm SD of 3 samples). (b) HO activity was determined in lysates of livers of mice 24 hours after treatment with 50 μ mol/kg hemin or 20mg/kg CoPP, or PBS vehicle alone (control) (results are mean \pm SD of 2 (CoPP)- 3 (hemin and control) mice per group).

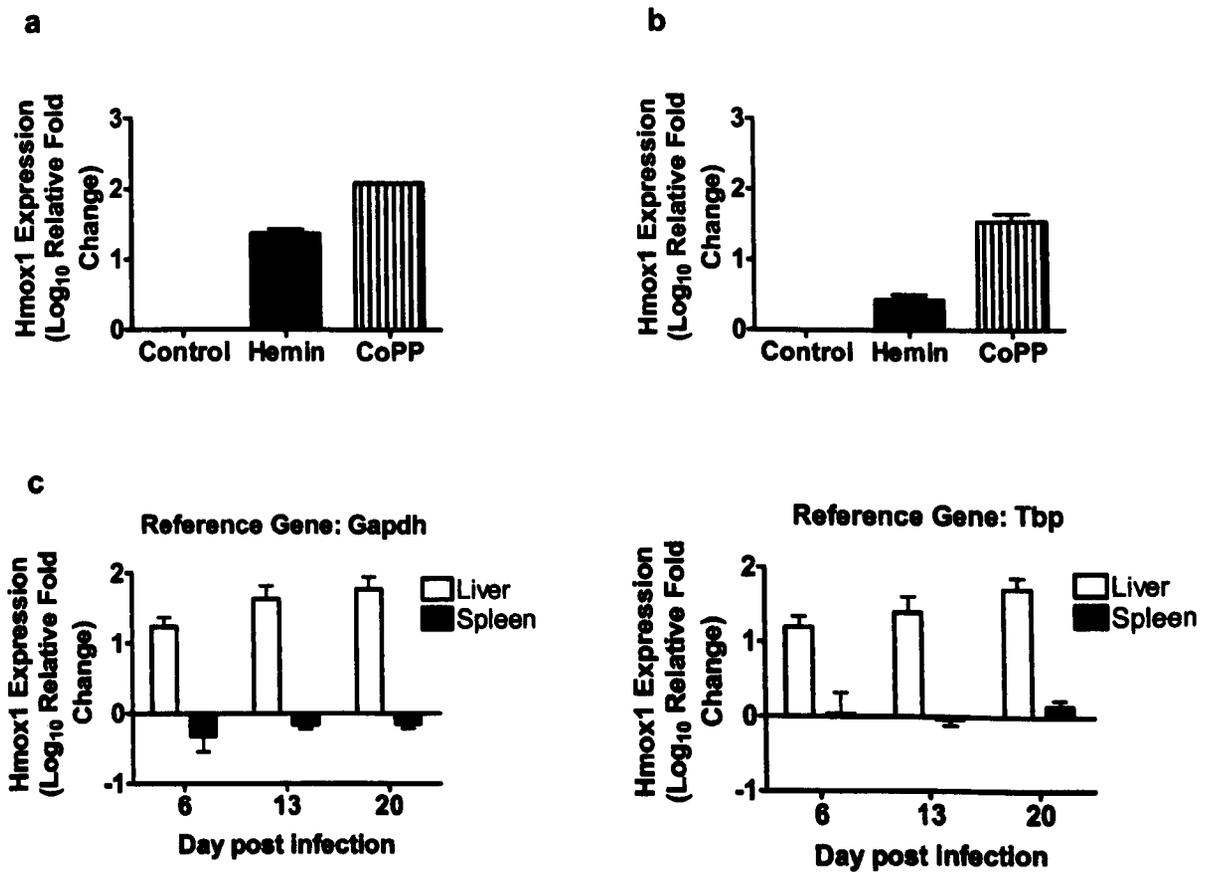


Real-time PCR analysis of gene expression

Cultured cells were harvested, washed in cold PBS and resuspended in RLT buffer (Qiagen) before immediate disruption with a tissue tearer and RNA extraction. Snips of tissues from mice were snap frozen in foil wraps in liquid nitrogen as soon as possible after removal from the animal, and were stored at -80°C before further processing. RNA was extracted from cells and tissues using RNeasy minikits (Qiagen) and genomic DNA contamination was removed using DNA-free (Ambion). RNA concentration and purity was determined by measuring absorbance at 260nm and 280nm using GeneQuant II (Pharmacia Biotech). First strand cDNA was synthesised using Superscript II reverse transcriptase (Invitrogen). Pre-validated primers for heme oxygenase-1 (*Hmox1*, Mm00516004_m1), Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*, Mm99999915_g1), and TATA Box-binding protein (*Tbp*, Mm00446973_m1) (and other genes of interest, where applicable) were obtained from Applied Biosystems. Target gene cDNA amplification was performed on the same plate as “reference” genes (*Gapdh* +/- *Tbp*), which were assumed to be invariant. Real time PCR was performed using Universal Mastermix (Applied Biosystems) and an ABI Prism 7000 Sequence Detection System. Expression was quantified by the $\delta\delta C_T$ method, whereby the difference in cycles required for target and reference genes to reach the amplification threshold in the control sample (δC_{Tc}), is subtracted from the same value calculated for the experimental sample (δC_{Te}) to give a value $\delta\delta C_T$, which can be converted to a fold change in relative expression.²⁷² In initial experiments only *Gapdh* was used as a reference gene (Figure 2.2a,b), but in later experiments in mice with malaria infection, both *Gapdh* and *Tbp* were used to provide additional reassurance that the reference gene remained truly invariant (Figure 2.2c). In the tissues tested *Gapdh* is more abundantly expressed and *Tbp* less abundantly expressed than *Hmox1* under control conditions. Good agreement was always obtained between results using either reference gene for calculation of relative gene expression.

Figure 2.2 Validation of *Hmox1* mRNA expression analysis.

Relative fold change in *Hmox1* mRNA, using *Gapdh* as the reference gene, (a) in RAW 264.7 cells following 24hr culture in medium alone (control) or with 20 μ M hemin or 20 μ M CoPP (results are mean \pm SD of 3 samples (control and hemin) or a single sample (CoPP) shown for comparison) and (b) in livers of mice, using *Gapdh* as the reference gene, 24 hours after treatment with 50 μ mol/kg hemin or 20mg/kg CoPP, or vehicle alone (control) (results are mean \pm SD of 3 mice per group). (c) Relative fold change in *Hmox1* mRNA in liver and spleen of mice at different time points after infection with *Plasmodium yoelii* 17XNL, determined using either *Gapdh* or *Tbp* as the reference gene.



Measurement of HO-1 expression by flow cytometry

At the time of starting this work, few investigators had reported analysis of cell specific HO-1 expression using flow cytometry.^{220 273-274} In order to select a suitable protocol for quantitation of HO-1 expression by flow cytometry it was decided that the assay should not only allow detection of HO-1 positive or negative cells, but also a dose response relationship should be seen between HO-1 inducers and HO-1 expression, reflecting the inducibility of HO-1 demonstrated by rt-PCR and in the HO activity assay in response to hemin and CoPP. To simplify initial experiments, RAW 264.7 cells were used. The first method attempted for detection of HO-1 expression was to use a directly conjugated monoclonal antibody (FITC-conjugated anti-HO-1, clone HO-1-2, Abcam) to stain live cells that were permeabilised with 0.1% saponin. If successful this method would have advantages of simplicity and easy separation of intracellular and surface staining steps. Briefly, cells were harvested after 24 hour treatment with HO-1 inducers, and washed twice in FACS buffer, before resuspending in a final volume of 100µL FACS buffer for 30 min. Cells were washed again and resuspended in a 1:100 solution of anti-HO-1 antibody or isotype matched control antibody (Abcam) in 0.1% saponin, and incubated for a further 30 minutes in the dark before a further wash and resuspension in 2% paraformaldehyde in FACS buffer. Unfortunately, although there appeared to be higher binding of the anti-HO-1 antibody than of the isotype matched control antibody, there was no evidence that treatment with a HO-1 inducer produced any increase in bound antibody per cell (assessed by median fluorescence intensity, MFI, Figure 2.3a), suggesting that either the antibody may not be binding to HO-1 or that the additional HO-1 induced in the cells is not accessible or not recognized by the antibody.

In case the failure to detect induced HO-1 expression was due to inadequate permeabilisation of the cells, a different method of cell preparation was assessed, using a method developed for analysis of intracellular signaling protein by flow cytometry (Chow et al cytometry 2001). After harvesting, cells were washed in FACS buffer, fixed with 2% formaldehyde at 37°C for 10 minutes, centrifuged at 500g for five minutes and resuspended in ice cold 90% methanol for 30 minutes. After this cells were washed in FACS buffer and resuspended in FACS buffer containing the anti-HO-1

antibody. Once again there was no evidence that MFI increased following treatment with a HO-1 inducer (Figure 2.3b). It was possible that the absolute change in HO-1 protein in the cell was not sufficient to give a detectable change in MFI using this antibody.

In order to attempt to amplify the signal obtained from formaldehyde-methanol fixed cells a polyclonal anti-HO-1 antibody was used, followed by a FITC-conjugated secondary antibody, as had been used by other groups^{220 273-274}. Although this would theoretically increase the fluorescence signal associated with binding to HO-1, it might also increase background fluorescence. However it is not possible to obtain a strictly isotype-matched control antibody for a commercial polyclonal antibody preparation so initially HO-1 expression in cells treated with HO-1 inducers was compared with untreated (control) cells, which seemed reasonable *in vitro* and *in vivo* in healthy mice (Figure 2.3c and 2.3d). Unfortunately, in malaria infected mice we were concerned that this might produce misleading results because inflammation may increase non-specific binding of IgG by certain cell types. For this reason, to reduce non-specific binding, cells were incubated for 15 minutes with both commercial FcBlock (BD, to prevent binding to Fc receptors) and normal mouse serum (to try to eliminate other non-specific binding), prior to addition of the anti-HO-1 antibody. To further control for residual non-specific binding every sample was divided into two aliquots, one to be incubated with anti-HO-1 and the other to be incubated with normal rabbit serum adjusted to give an equivalent total IgG concentration to the anti-HO-1 antibodies (Figure 2.4). Staining with antibodies to cell surface markers was undertaken after staining for HO-1 because the fixation-permeabilisation protocol was anticipated to cause degradation of some fluorophores.²⁷⁵ Full details of the antibodies used, and the definition of cell populations based on surface protein expression, have been published,²⁷⁶ and are reproduced in Chapter 5. The effect of increased non-specific binding during malaria infection in mice is illustrated in Figure 2.5, where failing to block non-specific binding and compare with the control antibody would lead to the incorrect conclusion that HO-1 expression is most strongly induced in granulocytes and “inflammatory” monocytes in blood during malaria infection. In fact HO-1 only seems to be induced significantly in “resident” monocytes.

Figure 2.3 Optimization of detection of cellular HO-1 expression by flow cytometry.

(a) and (b) RAW 264.7 cells, gated on FS-SS (a), were cultured for 24 hours in medium alone or with 20 μ M hemin or 20 μ M CoPP before permeabilization with 0.1% saponin (a) or formaldehyde and methanol (b) and staining with the FITC conjugated HO-1-2 monoclonal antibody or isotype matched control antibody. (c) RAW 264.7 cells were cultured for 6 or 18 hour in medium alone or in the presence of varying concentrations of CoPP before fixation / permeabilization with formaldehyde and methanol and indirect staining with the polyclonal SPA895 anti-HO-1 polyclonal antibody. HO-1 expression is quantified as the ratio of MFI in the treated sample to that in the untreated control sample. (d) HO-1 expression was induced in mice by administration of CoPP 10mg/kg 24 hours prior to harvest. HO-1 expression in all blood cells (left hand panels), and in CD11b⁺ F4/80⁺ cells (middle and right hand panels) is shown in representative examples from CoPP treated and untreated mice.

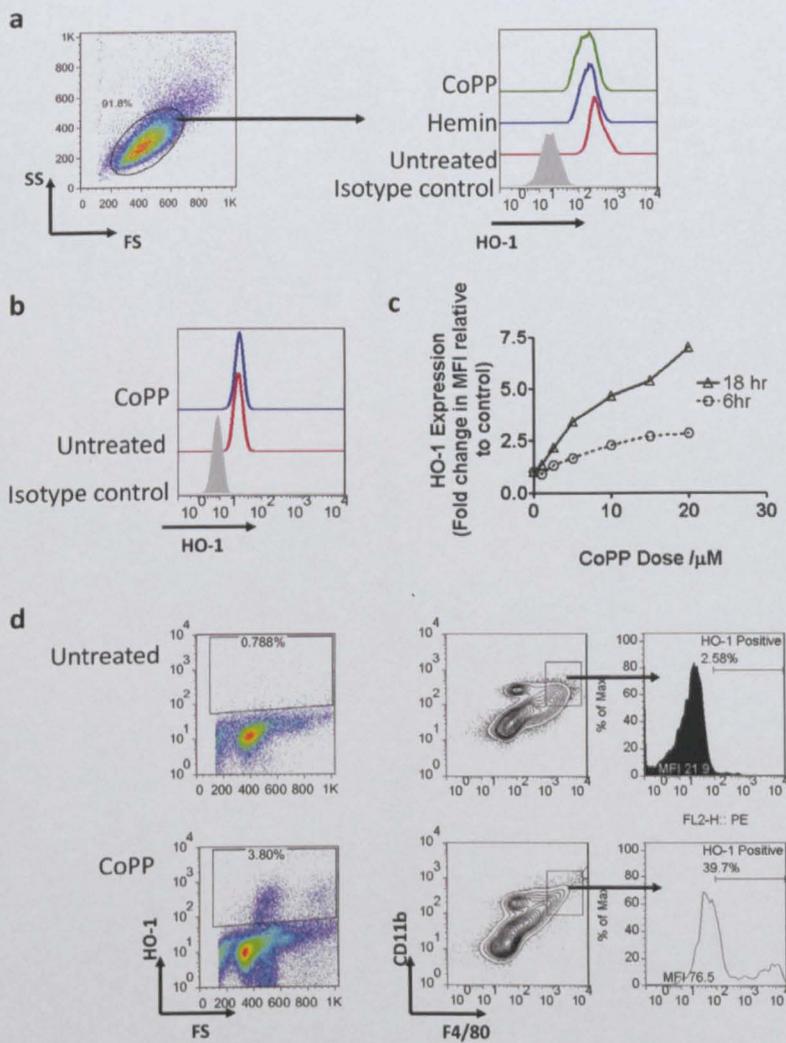


Figure 2.4 Optimized detection of HO-1 by flow cytometry in RAW cells.

RAW 264.7 cells were cultured for 24 hours in medium alone or in the presence of varying concentrations of hemin or 20 μ M CoPP before fixation / permeabilization with formaldehyde and methanol and indirect staining with the polyclonal SPA895 anti-HO-1 polyclonal antibody. HO-1 expression (right hand panel) is quantified as the ratio of MFI in the treated sample to that for the same sample stained with the control antibody (polyclonal rabbit serum).

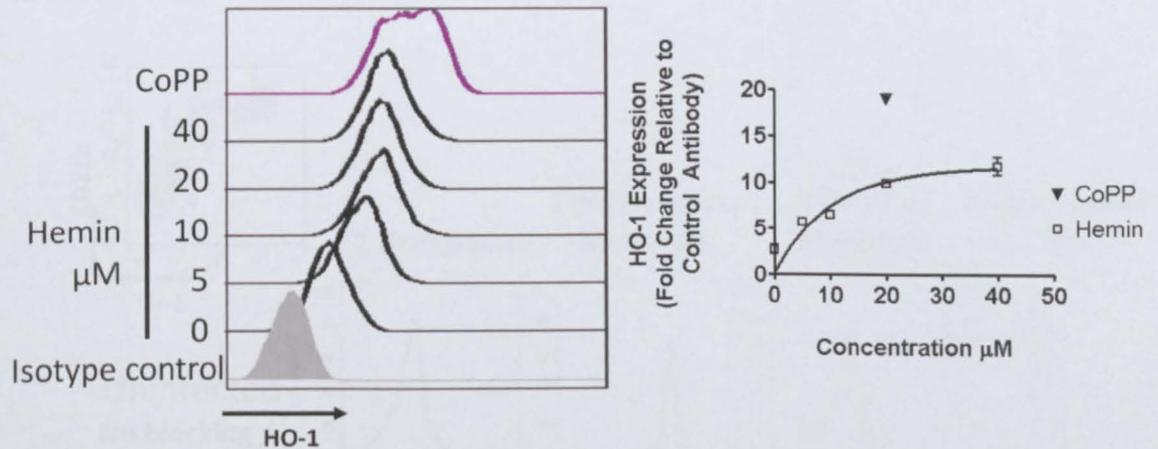
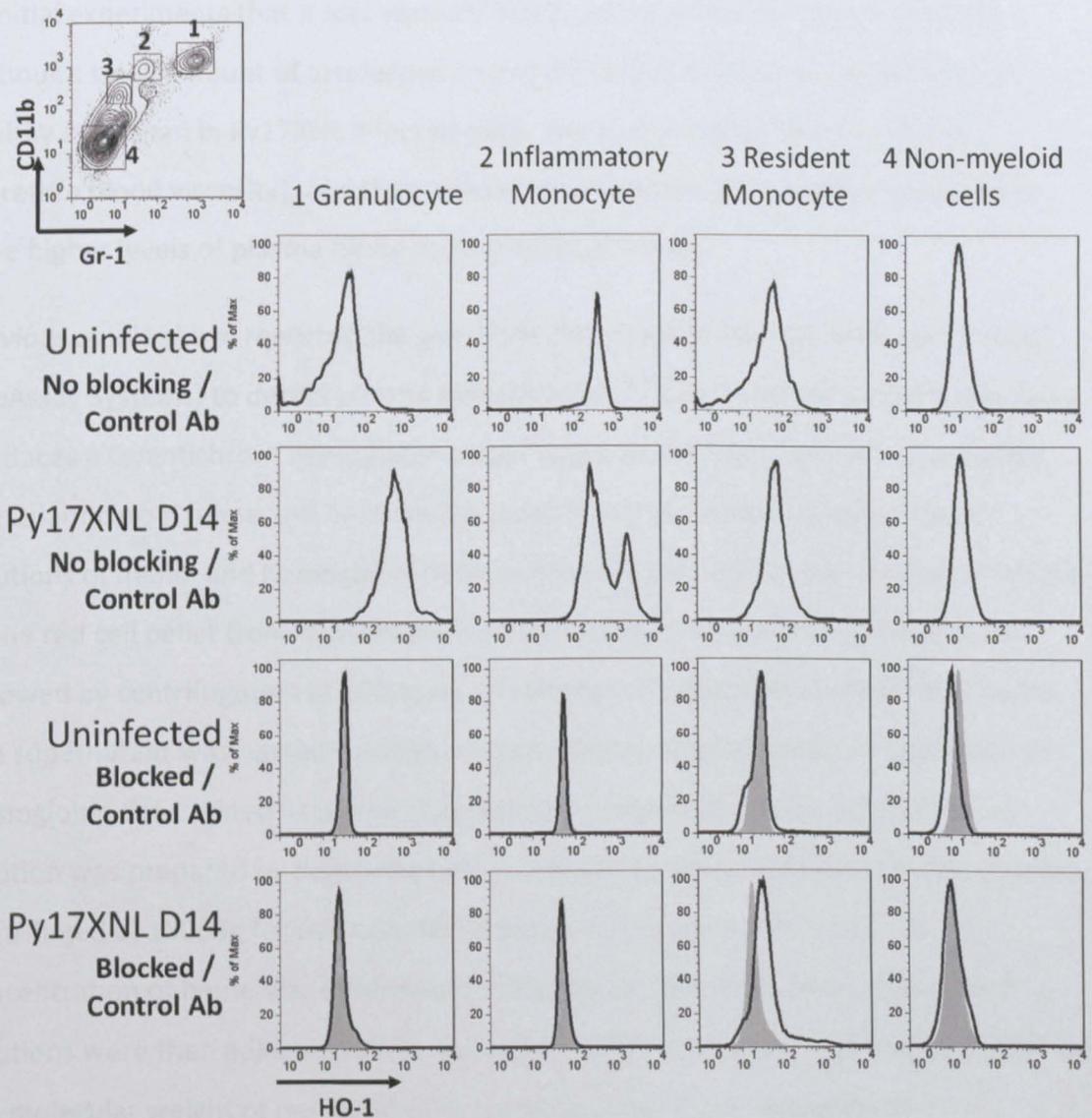


Figure 2.5. Importance of control antibody and blocking non-specific binding *in vivo*.

Blood leukocytes from uninfected and Py17XNL infected mice (day 14) were stained for HO-1 expression as in d, except that in one experiment non-specific binding was not blocked and HO-1 expression was compared with that in uninfected controls (upper two rows of panels) whilst in the other experiment, non specific binding was blocked and HO-1 expression (unfilled histogram) was compared with control antibody (filled histogram) in the sample before comparison with the uninfected control (lower two rows of panels).



Estimation of heme and hemoglobin concentration in plasma

In order to try to determine the extent of hemolysis due to malaria and the amount of heme present in plasma as a potential substrate and inducer of HO-1, plasma hemoglobin and heme concentrations were measured. Review of the literature suggested simple methods for determination of plasma heme and hemoglobin, which could be performed with relatively small sample volumes such as those obtainable from mice, included spectrophotometry²⁷⁷ and colorimetric assays.¹¹² It was obvious in initial experiments that it was very difficult to obtain blood samples from mice without a small amount of artefactual hemolysis (which was more problematic in healthy mice than in Py17XNL infected mice, due to the higher hematocrit and therefore blood viscosity), and that samples with artefactual hemolysis appeared to have higher levels of plasma heme measured by all assays.

Previous studies have reported the use of the colorimetric Quantichrom heme assay (BioAssay Systems) to detect plasma heme in mice,¹¹² and the same manufacturer also produces a Quantichrom hemoglobin assay. To assess the specificity of these assays, the Quantichrom heme and hemoglobin assays were evaluated using standard solutions of hemin and hemoglobin. A fresh hemoglobin solution was prepared by lysis of the red cell pellet from heparinised human blood in 8 volumes of distilled water followed by centrifugation at 2000g for 10 minutes to pellet erythrocyte membranes. The supernatant was passed through a 0.2µm syringe filter and the concentration of hemoglobin determined using the Quantichrom hemoglobin assay. A fresh hemin solution was prepared by dissolving hemin in NaOH as described earlier in this chapter, centrifuged at 15000g for five minutes to pellet undissolved debris and the concentration of heme was determined using the Quantichrom heme assay. Both solutions were then adjusted to the equivalent concentration of 25µM heme (based on the molecular weight of hemoglobin A, containing four heme moieties, of approximately 65,000g/mol²⁷⁸). Both solutions were then tested in both assays to determine the specificity of the assays. If the assays were specific, the heme assay should detect very little heme in the hemoglobin solution, and the hemoglobin assay should detect very little hemoglobin in the heme solution. In fact the results for each assay were very similar whichever solution was tested (Table 2.1), indicating that the

assays both measure the same thing, which is probably total heme (that is the sum of all the heme in free hemoglobin and protein-bound heme and any free heme in plasma). This meant that any artefactual haemolysis in samples would invalidate plasma heme measurements by the Quantichrom heme assay, and so these Quantichrom assays were subsequently only used to determine the concentration of pure solutions of heme or hemoglobin respectively.

Table 2.1. Comparison of Quantichrom heme and hemoglobin assays.

Hemoglobin and heme concentrations measured in standard solutions of “pure” hemoglobin (0.4g/L) and “pure” heme (25 μ M), determined by Quantichrom Hemoglobin and Quantichrom Heme Assays.

| Solution | Quantichrom Hemoglobin Assay | Quantichrom Heme Assay |
|------------|------------------------------|------------------------|
| | Hemoglobin g/L | Heme / μ M |
| Hemoglobin | 0.472 | 28.5 |
| Heme | 0.393 | 26.125 |

Results are mean of duplicate assays

Several spectrophotometric methods have been described to determine the concentration of hemoglobin in solution. One of the most accurate is the method described by Kahn et al.²⁷⁹⁻²⁸⁰ Using a NanoDrop ND1000 spectrophotometer (Nanodrop Technologies) this assay was tested using a variety of different concentrations of a standard hemoglobin solution dissolved in a final concentration of 80% normal mouse serum. Briefly, 2 μ L of the serum was used to determine absorbance at 562, 578, 598, 615 and 675nm wavelengths. Using the absorption values at 562, 578 and 598nm the concentration of plasma hemoglobin was determined using the following equation:

$$\text{Concentration of plasma Hb g/dL} = 1.55.A_{578} - 0.861.A_{562} - 0.689.A_{598} \text{ (Khan et al.}^{279})$$

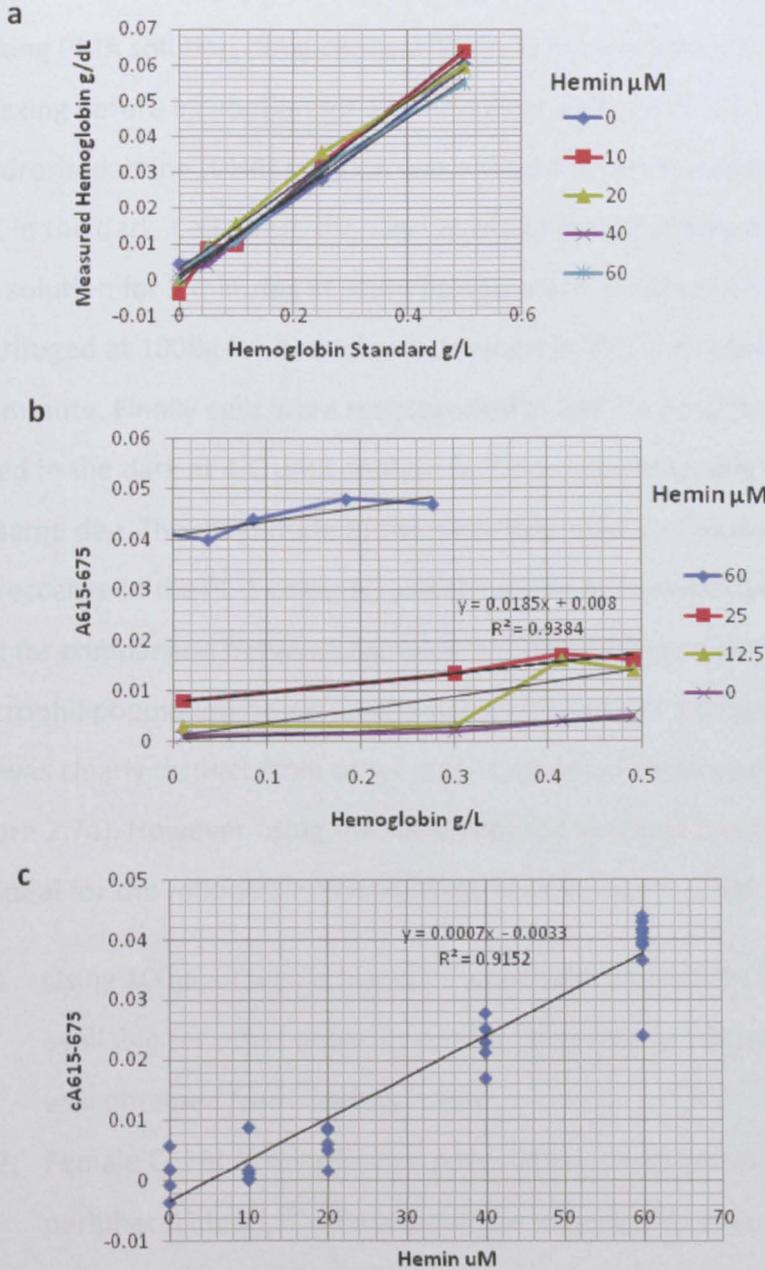
To test whether the presence of heme altered the amount of hemoglobin detected, samples were spiked with additional hemin. Estimates of plasma hemoglobin were unaffected by hemin, as shown by the series of essentially superimposed lines for measured hemoglobin vs. known hemoglobin concentrations spiked with different concentrations of hemin (Figure 2.6a).

Samples of known hemin concentration were spiked with known concentrations of hemoglobin and the absorbance difference $A_{615-675}$ was measured to determine the concentration of protein-bound heme, which is directly proportional to $A_{615-675}$.²⁷⁷ Spiking with hemoglobin produced consistent changes in the $A_{615-675}$ measurement, as shown by the essentially parallel linear relationships obtained (Figure 2.6b). It should be noted that at low concentrations of heme, values are very close to the baseline, resulting in greater variation due to background noise affecting particularly the A_{675} measurement.

The $A_{615-675}$ was corrected for the presence of hemoglobin ($cA_{615-675}$) using the regression equation from Figure 2.6b, and $cA_{615-675}$ was plotted against the known hemin concentration to obtain a standard curve for hemin (Figure 2.6c). For subsequent experimental samples, plasma hemoglobin was first calculated, and used to calculate $cA_{615-675}$, which was then used to calculate the heme concentration in the sample using the equation of the standard curve.

Figure 2.6. Determination of hemin and hemoglobin concentrations in plasma by spectrophotometry.

(a) Relationship between measured hemoglobin and the known concentration of hemoglobin in samples of mouse serum spiked with varying concentrations of hemin. (b) Relationship between $A_{615-675}$ and the amount of hemoglobin in the sample at varying concentrations of hemin. (c) Example of a standard curve for $A_{615-675}$ corrected for hemoglobin concentration ($cA_{615-675}$) vs. varying concentration hemin standards.



Neutrophil oxidative burst and degranulation assay

In order to assess the magnitude of the oxidative burst produced by circulating neutrophils a simple whole blood flow cytometry assay was used.²⁸¹ This assay, which

measures the oxidation of dihydrorhodamine123 to its fluorescent derivative rhodamine, has been used in clinical laboratories for the diagnosis of CGD and it has recently been reported that the magnitude of the respiratory burst determined by this assay is an important determinant of survival in patients with CGD.²⁸² Initially the assay was tested on human blood to confirm that the oxidative burst was easily detected using the assay as it was originally described. Briefly, whole blood was divided into 2 aliquots each of 100 μ L, which were designated as unstimulated and stimulated. 25 μ l of working PMA solution (final concentration 1 μ M) was added to the tubes and mixed by vortexing before incubation for 15 minutes at 37°C. Next 25 μ L of working dihydrorhodamine (DHR) solution was added and incubated for a further 5 minutes at 37°C in the dark. Cells were then incubated in 2ml ammonium chloride red blood cell lysis solution for 5 minutes at room temperature, protected from light, then centrifuged at 1000g for one minute, washed in PBS and centrifuged again at 1000g for one minute. Finally cells were resuspended in 1ml 1% paraformaldehyde in PBS, and stored in the dark at 4°C until analysis by flow cytometry, which was always done on the same day. The magnitude of the oxidative burst was assessed using the rhodamine fluorescence in the FL-1 channel, and the median fluorescence intensity (MFI) was used for comparison between samples. In human blood it was easy to select the neutrophil population based on characteristic forward and side scatter properties, and this was clearly distinct from other leukocyte populations and unlysed erythrocytes (Figure 2.7a). However using the same method in whole blood from C57BL/6 mice was not ideal for the following reasons (illustrated in Figure 2.7b).

1. Using 100 μ L of whole blood for each tube limited the amount of blood that was available for other analyses on the same mouse (usually 200-400 μ L of blood was obtained from healthy mice)
2. Female C57BL/6 mice have a particularly low proportion of neutrophils in peripheral blood,²⁸³ which would be expected to decrease the relative purity of neutrophils in the forward scatter-side scatter gate
3. During Py17XNL infection of C57BL/6 mice there are many reticulocytes and parasitized erythrocytes which may clump together and are resistant to RBC

lysis, making definition of a discrete neutrophil population based on forward and side scatter properties more difficult.

4. Stimulated neutrophils from some control mice had a bimodal distribution of rhodamine fluorescence suggesting that the dose of PMA may be suboptimal in this assay

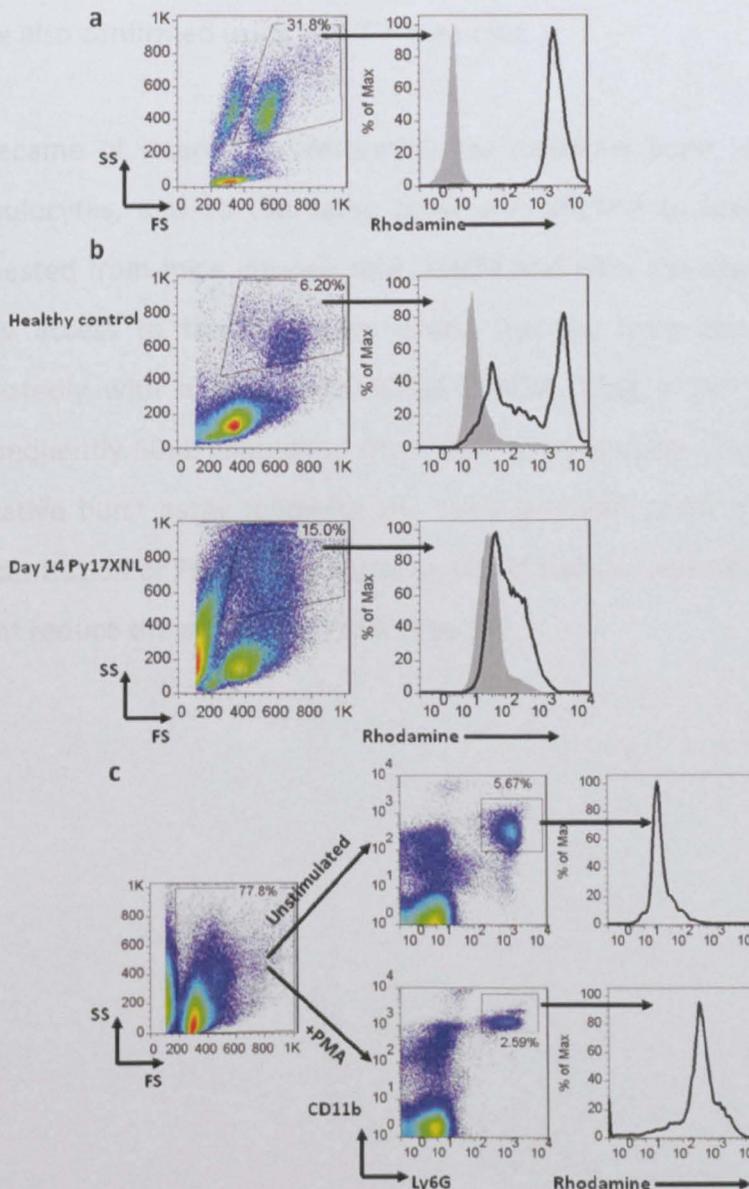
For these reasons the assay was modified to i) reduce the blood volume required, ii) to improve the specificity of identification of neutrophils, iii) to increase lysis of red blood cells, and iv) to achieve maximal stimulation with PMA. First APC conjugated anti-Ly6G and PE-Cy7 conjugated anti-CD11b antibodies were added in a cocktail along with dihydrorhodamine in the last 5 minutes incubation step of the assay. This produced a clear Ly6G and CD11b positive population of neutrophils (Figure 2.7c). An added finding was that CD11b fluorescence increased following stimulation, which reflects degranulation and fusion of CD11b within neutrophil granules with the surface membrane.²⁸⁴ This finding meant that the same assay could be used to examine two functional responses to PMA: oxidative burst response and degranulation response.

Next, to minimize the amount of whole blood required, the volume of blood used in the oxidative burst assay was reduced to 50 μ L, whilst maintaining the volumes of PMA and DHR-antibody cocktail at 25 μ L each. Using these volumes, different doses of PMA were assessed to try to achieve a maximal response (Figure 2.8a). Although this experiment did not demonstrate saturation of the response, we were reluctant to use higher concentrations because PMA is soluble at 1mg/ml in DMSO (1.6mM) and so a final concentration of 16.7 μ M PMA would also contain approximately 1% DMSO, which may itself modify cell function and viability.

In samples from mice with hemolysis (due to malaria or phenylhydrazine treatment) it was noted that the separation between Ly6G⁺ and Ly6G⁻ populations became less distinct than in healthy control mice, possibly as a result of autofluorescent, unlysed, immature red blood cells (Figure 2.8b). As an additional measure to try to remove noise from autofluorescent unlysed immature red blood cells, the length of incubation in RBC lysis buffer was extended

Figure 2.7. Optimization of a flow cytometric oxidative burst assay.

(a) The magnitude of the oxidative burst was determined by rhodamine fluorescence in neutrophils (gated by forward and side scatter properties) in 100 μ L aliquots of human whole blood following stimulation with 1 μ M PMA (unfilled histogram) and compared with unstimulated cells (filled histogram). (b) Using an identical assay procedure, clear discrimination of the neutrophil population was more difficult in mice, particularly in the context of Py17XNL infection, and PMA stimulation appeared to produce a suboptimal oxidative burst response. (c) Addition of anti-Ly6G and anti-CD11b antibodies improved discrimination of the neutrophil population and revealed upregulation of surface CD11b upon stimulation.

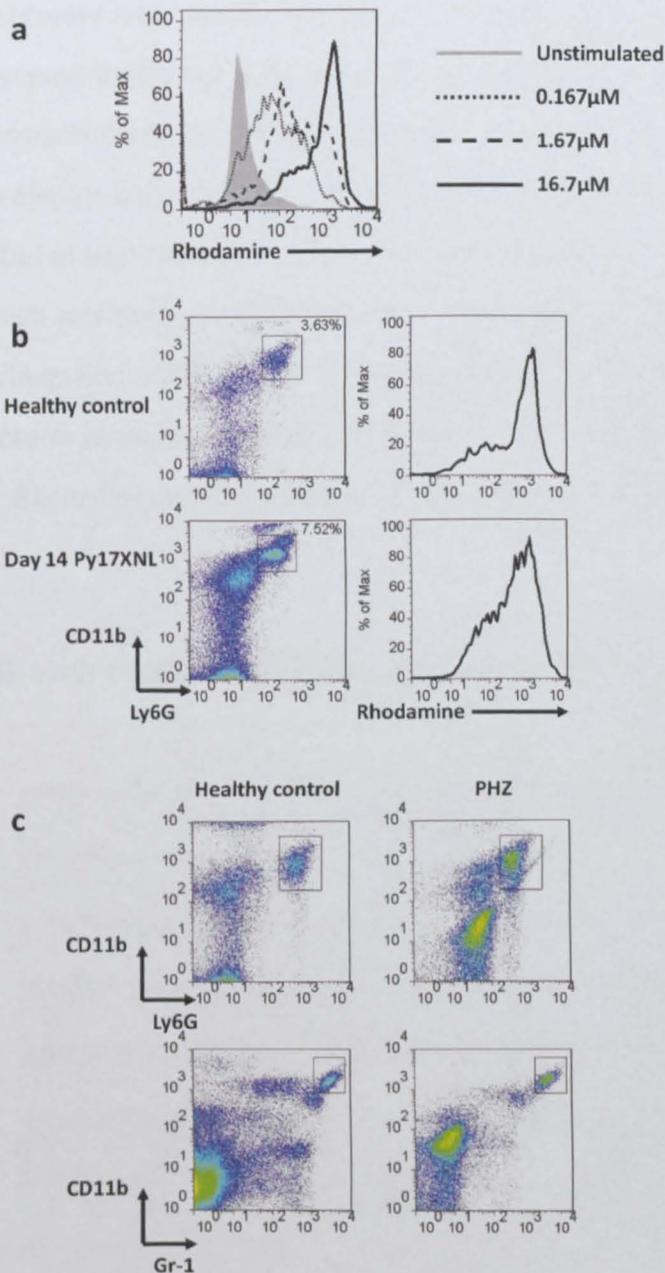


to 15 minutes. Using a FACS Calibur flow cytometer with only four fluorescent channels limited the options for using additional antibodies to define the neutrophil population because rhodamine produces strong fluorescence in the FI-1 and FI-2 channels, and if anti-CD11b is also included in the assay to assess degranulation, then neutrophils have to be defined based on a single surface marker. For this reason, staining with anti-Gr-1 was also assessed, since the typical separation between positive and negative cell populations was observed to be greater than that seen with anti-Ly6G, although separation of the Gr-1^{Hi}CD11b^{Hi} population (neutrophils) from the Gr-1 intermediate populations (which would comprise inflammatory monocytes and possibly immature neutrophils) was still not ideal (Figure 2.8c). In practice, where there was difficulty separating populations on the basis of anti-Ly6G staining, results were also confirmed using anti-Gr-1 staining.

It became of interest to determine the oxidative burst response of bone marrow granulocytes, and so the same assay was applied to bone marrow. Femurs were harvested from mice into ice cold DMEM and after shaving off bone at both ends to allow access to the medullary cavity, marrow from one femur was flushed out repeatedly with a volume of 500 μ L DMEM using a 1ml syringe and 25G needle. Subsequently 50 μ L aliquots of the 500 μ L bone marrow suspension were used for the oxidative burst assay following the same protocol as for blood but using 1/10th the concentration of PMA, since it was assumed that the absence of large numbers of RBCs might reduce the amount of PMA required.

Figure 2.8 Further optimization of the oxidative burst assay.

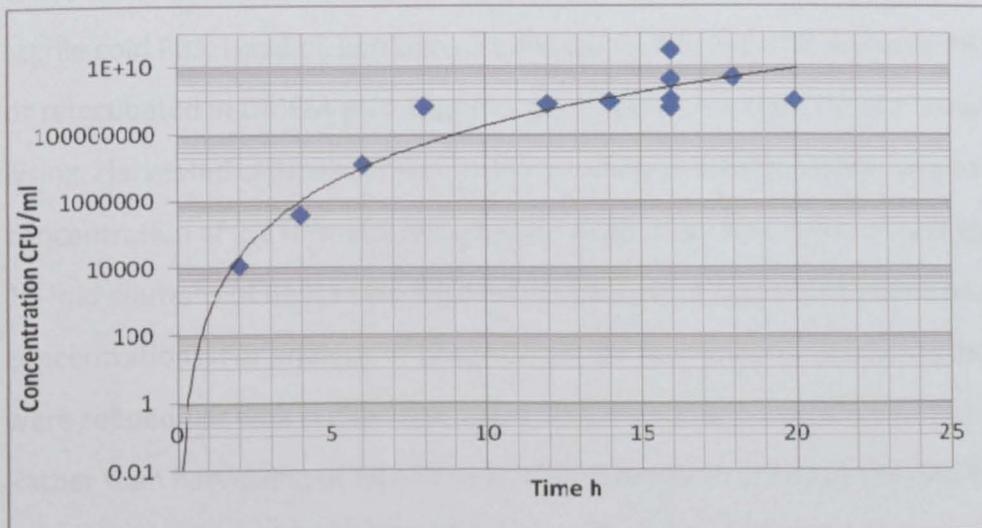
(a) The volume of blood used in the assay was reduced to 50 μ L and the concentration of PMA was titrated upwards to try to achieve maximal stimulation. (b,c) In samples from Py17XNL- infected and PHZ treated mice, separation of Ly6G positive and negative populations of cells in stimulated samples appeared less distinct than in healthy mice, whereas Gr-1^{hi} cells were more clearly separated from the majority of other cells (c).



***Salmonella enterica* serovar typhimurium**

Salmonella enterica serovar Typhimurium pfpv 12023 (hereafter referred to as *S. typhimurium*) constitutively expressing Green Fluorescent Protein (GFP) was a kind gift from Prof. David Holden (Imperial College London, UK). In order to ensure an adequate and consistent supply of *S. typhimurium* for use in each series of experiments, *S. typhimurium* was grown to late log-phase in Luria-Bertani (LB) broth supplemented with 50µg/ml carbenicillin (to maintain selection of the GFP-carbenicillin resistance plasmid) at 37°C. Viable bacterial concentration was monitored by dilution culture on LB agar at different time points during growth of the bacteria in broth (Figure 2.9), and 500µl aliquots were typically collected at 14-16 hours of incubation and stored at -80 °C in 10% glycerol. Viable bacterial concentrations in the aliquots were reconfirmed before use in each experiment. Aliquots were thawed to room temperature, then 100µl of the aliquot was mixed with 900µl sterile PBS and centrifuged at 16000g for 7 minutes. 900µl of supernatant was carefully aspirated leaving behind the pellet of bacteria, which was then resuspended with addition of a further 900µl sterile PBS and repeat centrifugation at 16000g for 7 minutes. 900µl of supernatant was aspirated and then the bacteria were resuspended in the desired final volume of reagent for the experiment. Bacterial concentrations in stock aliquots were between 10^8 - 10^9 CFU/ml.

Figure 2.9. Growth curve for *S. typhimurium* in LB broth.



Phagocytosis and killing assays

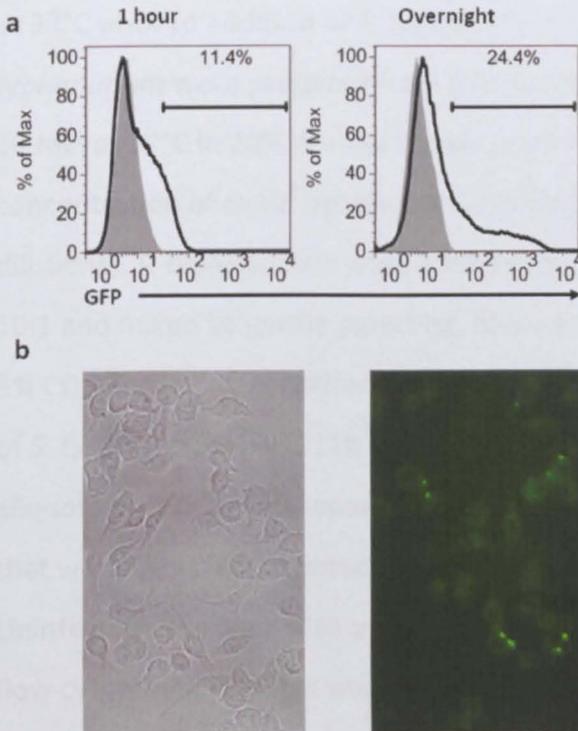
To assess the ability of phagocytic cells isolated from mouse blood to kill *S. typhimurium in vitro* we used a modification of the classical gentamicin protection assay.²⁸⁵ The principle of this assay is that Salmonella and phagocytic cells are co-incubated at a high ratio (multiplicity of infection, MOI) of bacteria to cells and after allowing time for phagocytosis, the remaining extracellular bacteria are killed by addition of a high concentration of gentamicin. The medium is then changed and further incubation proceeds in the presence of a lower concentration of gentamicin which should prevent any replication of bacteria released into the extracellular environment, but should not kill intracellular bacteria. At different time points the medium and extracellular bacteria can be washed off, and viable intracellular bacteria enumerated by culture after lysis of the cells. In addition, the presence of GFP expressing bacteria in cells can be assessed by flow cytometry or fluorescent / confocal microscopy.

In order to validate this assay we initially assessed phagocytosis and killing of *S. typhimurium* using RAW264.7 macrophages. Cells were seeded at a density of 1×10^6 cells per well in 12-well tissue culture plates, 18 hours before use to allow formation of monolayers. *S. typhimurium* were added at a MOI of 10:1 and incubated for 25 min at 37 °C and 5% CO₂. Plates were washed three times with sterile PBS and then re-incubated in DMEM + 100µg/mL gentamicin for 1 hour. Cells were washed again three times with sterile PBS, and then cells were either harvested by scraping from wells into sterile cold PBS, lysed by addition of 1.0% sterile Triton X-100 in sterile PBS for 10 min, or re-incubated in DMEM plus 10µg/mL gentamicin overnight before harvesting or lysing. Harvested cells were fixed by the addition of 40% formaldehyde to give a final concentration of 2% formaldehyde before analysis by flow cytometry (Figure 2.10a). 10-fold dilutions of lysates were plated on LB agar to determine viable bacterial concentrations. For analysis of phagocytosis by fluorescent microscopy 1×10^5 cells were seeded per well in chamber slides, and the same procedures were followed. Rather than harvesting or lysis of cells, they were fixed *in situ* at the end of incubation using 4% paraformaldehyde for 15 minutes. They were then washed three times with PBS, air dried and mounted in Fluoromount. Images were acquired using a Zeiss

Axioplan2 microscope with HB50 fluorescent lamp and images were obtained with a Retiga 2000R camera (QImaging) and analysed using Volocity 5.5.1 software (PerkinElmer) (Figure 2.10b). At the 1 hour time point (representing phagocytosis) the average (of three replicates) percentage of cells containing bacteria determined by fluorescent microscopy was 16.6% and the average number of viable bacteria was 2.0×10^5 per well, which are both in relatively good agreement with the % GFP⁺ cells determined by flow cytometry (Figure 2.10a). After overnight incubation in this experiment bacterial growth was so heavy on the lowest dilution plate prepared, that discrete colonies could not be accurately counted, and flow cytometry analysis showed not only an increase in the proportion of GFP⁺ cells but also many cells with very bright GFP fluorescence (Figure 2.10a) suggesting numerous intracellular bacteria.²⁸⁶

Figure 2.10. Infection of RAW cells with *S. typhimurium*.

a) Representative flow cytometry histogram showing GFP fluorescence of RAW cells following incubation with (black line) or without (filled) *S. typhimurium* for 1 hour (phagocytosis) or overnight. b) Representative phase and fluorescent microscopy images showing RAW264.7 cells containing GFP-expressing *S. typhimurium* (bright green).



The bacterial phagocytosis and killing assays were modified for assessment of function of neutrophils and monocytes from whole blood of mice. Neutrophils have a relatively short survival *in vitro*²⁸⁷, so to prevent results being confounded by cell death, phagocytosis was assessed as before after 45 minutes, but bacterial killing was assessed after 2 hours in culture. Mice were killed by carbon dioxide inhalation and blood was collected immediately after death by sterile cardiac puncture. Red blood cells were lysed with 2ml ammonium chloride lysis buffer per 100 μ l of blood, incubated for 5 minutes at room temperature, and then cells were washed with 10ml PBS after centrifugation at 1000g for 5 minutes. After centrifugation at 500g at 4°C for a further 5 minutes the resulting cell pellet was resuspended in 180 μ L MACS buffer

(prepared according to manufacturer's instructions) and then CD11b⁺ cells were isolated using anti-CD11b magnetic beads (Miltenyi Biotec) according to the manufacturer's instructions. CD11b⁺ cell fractions were collected in 15mL tubes and washed twice in ice cold DMEM before resuspension in 1mL DMEM without antibiotics, and determination of the cell concentration. Cells were distributed into flat bottom 96-well plates at 10⁵ /well in 180uL volume or into 8-well chamber slides at 2x10⁵/chamber in 360ul volume. Cells were allowed to rest in the wells for 10 minutes at 37°C prior to addition of *S. typhimurium*. In order to maximize phagocytosis, *S. typhimurium* were prepared from frozen stock as described above, and opsonized for 30 min at 37°C in 20% normal mouse serum (Southern Biotech) before adjustment to a concentration of 6x10⁷ opsonized *S. typhimurium*/ml (confirmed by plating of serial dilutions). *S. typhimurium* were added to the wells containing CD11b⁺ cells at a MOI of 10:1 and mixed by gentle pipetting, followed by incubation for 15 minutes at 37°C and 5% CO₂. To control for differences in autofluorescence of cells and for surface binding of *S. typhimurium* to CD11b⁺ cells, which might occur in the absence of phagocytosis, aliquots of cells were prepared that were either uninfected with *S. typhimurium*, or that were fixed by suspension in 2% formaldehyde prior to addition of *S. typhimurium*. Uninfected cells were also used to determine purity of CD11b⁺ cells when analysed by flow cytometry, and this was typically around 98%. After the initial 15 minutes incubation, gentamicin was added to give a final concentration of 100µg/ml and cells were reincubated for 30 min at 37°C and 5% CO₂. At the end of this time, plates were centrifuged for 2 min at 1000g and cells were gently washed twice with warm medium to remove non-phagocytosed bacteria.

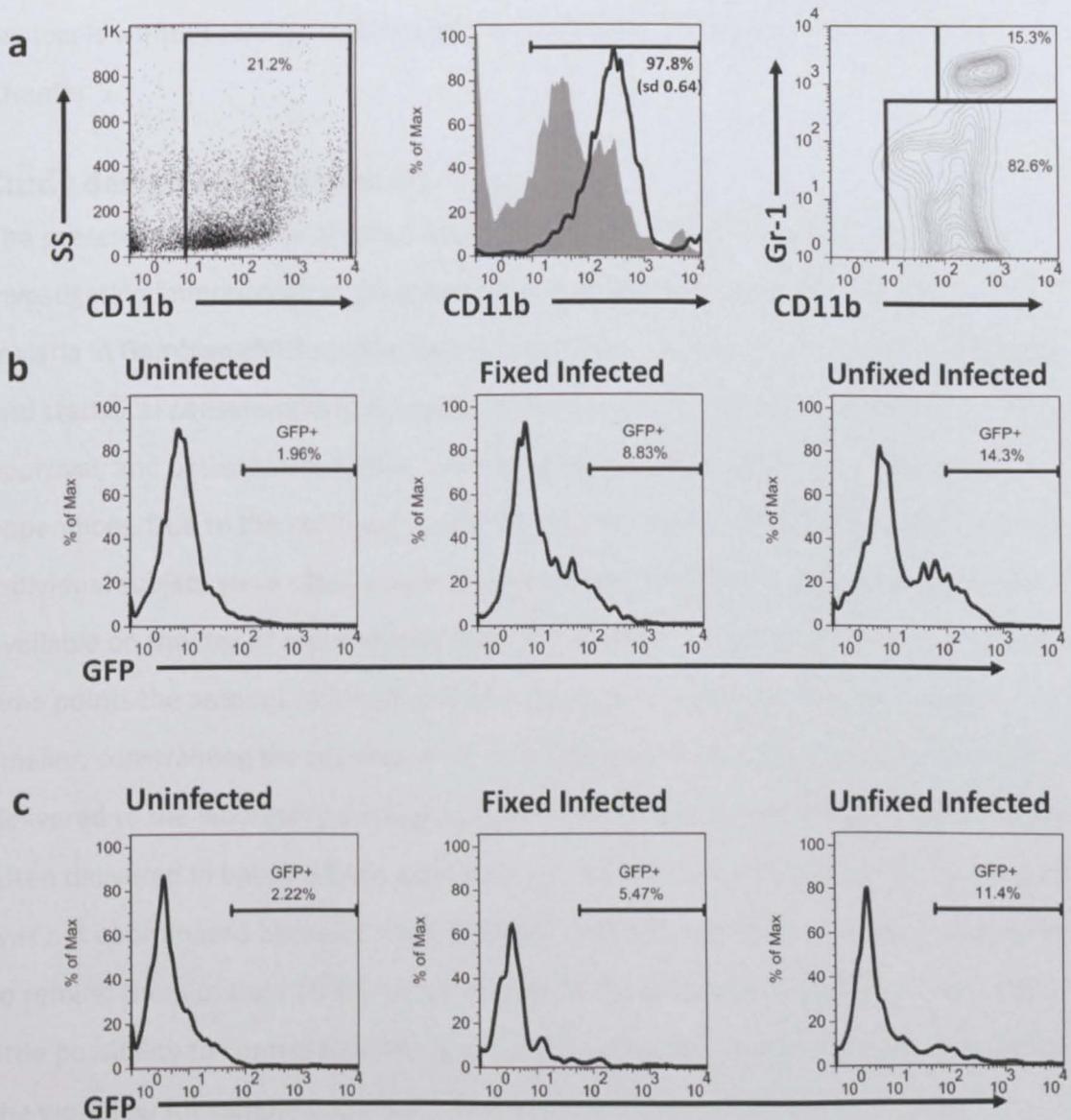
To assess phagocytosis by quantitative culture, cells were lysed *in situ* by addition of 1% Triton X100, and 10-fold dilutions were plated on LB agar plates. To assess phagocytosis by flow cytometry, plates were placed onto ice for 30 minutes to facilitate removal of any plastic-adherent cells, followed by gentle scraping with a bent pipette tip prior to aspiration of the well contents, and resuspension in 2% formaldehyde for 15 min. These cells were then resuspended in staining buffer containing APC anti-Gr-1 and PE-Cy7 anti-CD11b and incubated at room temperature for 15 min, before further washing in PBS and resuspension in PBS with 4%

paraformaldehyde. Cells were analysed by flow cytometry, and divided into neutrophil (Gr-1^{Hi}CD11b⁺) and monocyte (Gr-1^{Lo/-}CD11b⁺) populations (Figure 2.11a) within the CD11b⁺ cells. The percentage of cells phagocytosing *S. typhimurium* was calculated by setting a gate defining the GFP⁺ population using uninfected cells, then subtracting the proportion of GFP⁺ cells in the fixed infected sample from the proportion of GFP⁺ cells in the respective unfixed infected sample (Figure 2.11b,c). To assess phagocytosis by confocal microscopy, cells in chamber slide wells were fixed *in situ* with 2% formaldehyde for 15 min at room temperature, before washing gently twice with PBS plus 5% fetal bovine serum. After this cells were thoroughly dried in air at room temperature and then mounted in confocal matrix (Micro Tech Lab) containing 4',6-Diamidino-2-Phenylindole (DAPI). Analysis of the slides is described in detail in Chapter 5.²⁷⁶ To assess bacterial killing by CD11b⁺ cells, after washing off the medium containing 100µg/ml gentamicin, cells were reincubated at 37°C and 5% CO₂ in DMEM containing gentamicin 10 µg/ml for a total of 2 hours. After this time, viable bacteria in each well were enumerated by quantitative culture in the same way as for assessment of phagocytosis.

Further details of methods used for *in vivo* experiments with *S. typhimurium* infection are described in Chapter 5.

Figure 2.11. Flow cytometric analysis of phagocytosis of *S. typhimurium*.

(a) Definition of CD11b⁺ cells in blood by flow cytometry (gated region, left hand panel), purity (middle panel) of MACS-isolated CD11b⁺ cell fraction (unfilled histogram, mean percentage purity displayed) compared with unselected cells (filled histogram), and definition of cell populations within the CD11b⁺ cell fraction (right hand panel) as neutrophils (Gr-1^{Hi}CD11b⁺) and monocytes (Gr-1^{Lo/-}CD11b⁺). (b) Gating to assess phagocytosis of GFP-expressing *S. typhimurium* in monocytes. (c) Gating to assess phagocytosis of GFP-expressing *S. typhimurium* in neutrophils.



Summary of the main techniques developed and optimized for studies in humans

These studies were undertaken at the Medical Research Council (UK) laboratories in The Gambia. Technical assistance was provided by two laboratory technicians, Madi Njie and Simon Correa. Some assays were performed in conjunction with another PhD student, Sarah Nogaro, when samples from a single subject were used by both investigators and the assay results were important for both projects. The methods developed and described in this chapter were used in the study reported as a research paper in Chapter 6. To avoid duplication, methods performed according to standard protocols without further modification, are described in the methods section of Chapter 6.

Study design and constraints

The present study was embedded within the final year of a six-year study platform investigating immunological, parasitological and clinical aspects of mild and severe malaria in Gambian children. Recruitment, sample collection, clinical case definitions and statistical considerations are described in Chapter 6. Scientific and ethical approval, and patient information, consent and record forms are provided as appendices. Due to the multi-purpose nature of the study platform, samples from an individual subject were often used for three different studies if sufficient blood was available on the day of presentation (day 0). Sometimes this meant that at subsequent time points the amount of blood available for each study might be considerably smaller, constraining the number of assays that could be performed. Samples were delivered to the laboratory throughout the working day by fieldworkers. Samples were often delivered in batches from each of the three recruitment sites, but their delivery was not coordinated between sites. Although subjects were given appointment cards to remind them of their follow-up visits, due to the voluntary participation there was little possibility to control the timing of sample collection. In consequence, managing the workflow for sample processing in the research laboratory was extremely challenging, and sometimes several batches of samples were simultaneously being processed for several different assays. Due to the limitations of sample volumes,

numbers of staff, and equipment, and loss of viability of fresh samples over time, assays were developed with special attention to how they could be made time and resource efficient, and to the order in which they should be prioritised.

The aim of this study was to assess neutrophil function (oxidative burst, degranulation, phagocytosis and bacterial killing) and its relationship to hemolysis and HO-1 induction, during acute malaria and convalescence. Fresh, live cells were required for neutrophil function, degranulation, phagocytosis and killing assays, and fresh cells were ideally required for analysis of intracellular HO-1 expression. It was obvious that performing these assays would be the main demand on the available blood sample volume and also on laboratory time. In order to ensure that the most complete and highest quality data set was available, assays had to be prioritized. The combined oxidative burst and degranulation assay was judged to be most important because i) it would be the most convincing way to determine if a similar mechanism of neutrophil dysfunction occurred in both mice and humans, ii) it could be performed with the smallest volume of blood (and therefore was most likely that it would be possible to perform this assay for every sample), and iii) it required the least time to perform (and therefore would be easiest to perform numerous times within a day if necessary).

The intracellular HO-1 expression assay was considered the next most important, because i) it might provide a mechanistic explanation for any neutrophil dysfunction, ii) after fixation of cells subsequent staining could be done at a later stage in the day, and iii) it required a relatively small sample volume.

The bacterial phagocytosis and killing assay was given lowest priority, because i) it required the largest volume of blood (meaning that it may not be possible in all subjects) and ii) it was labour intensive. In all cases assays were optimized to try to minimize the effects of these constraints as described below.

Neutrophil oxidative burst and degranulation assay

Following on from the assay used to determine oxidative burst and degranulation in mice,²⁷⁶ it seemed logical that similar modifications to the original assay described by Richardson *et al*²⁸¹. could also be applied for analysis of neutrophils in children with

P.falciparum malaria. Because every patient's blood samples were to be shared between three studies, it was essential to minimize the amount of blood required for this assay. Briefly, three 50 μ L aliquots of blood were split into separate FACS tubes labeled 'unstained', 'unstimulated' and 'stimulated'. To each tube, 50 μ L of PMA (final concentration 1 μ M) or PBS was added. Samples were vortexed and incubated for 15 minutes at 37°C in a water bath. Next 25 μ L of PBS (unstained) or staining cocktail (DHR plus PE-Cy7 anti-CD11b plus APC anti-CD15, unstimulated and stimulated tubes) was added and incubated for 5 minutes at 37°C in the dark. Red blood cells were lysed with 2ml of ammonium chloride lysis buffer (5 min at room temperature, shielded from light) before centrifugation at 1000g for 3 min and washing in PBS before resuspension in 300 μ L PBS containing 1% paraformaldehyde. All samples were stored at 4°C, protected from light, and analyzed in a single batch on the same day as samples were collected. Data was acquired using a using a 3 laser/9 channel CyAn™ ADP flowcytometer with Summit 4.3 software (Dako). Compensation for spectral overlap between rhodamine and the fluorophores was performed for each batch of samples, by mixing stimulated but unstained cells with cells from tubes stained with DHR only, PE-Cy7 anti CD11b only or APC anti-CD15 only, and using forward scatter-side scatter characteristics to gate on the neutrophil population. The initial gating strategy is shown in Figure 2.12a.

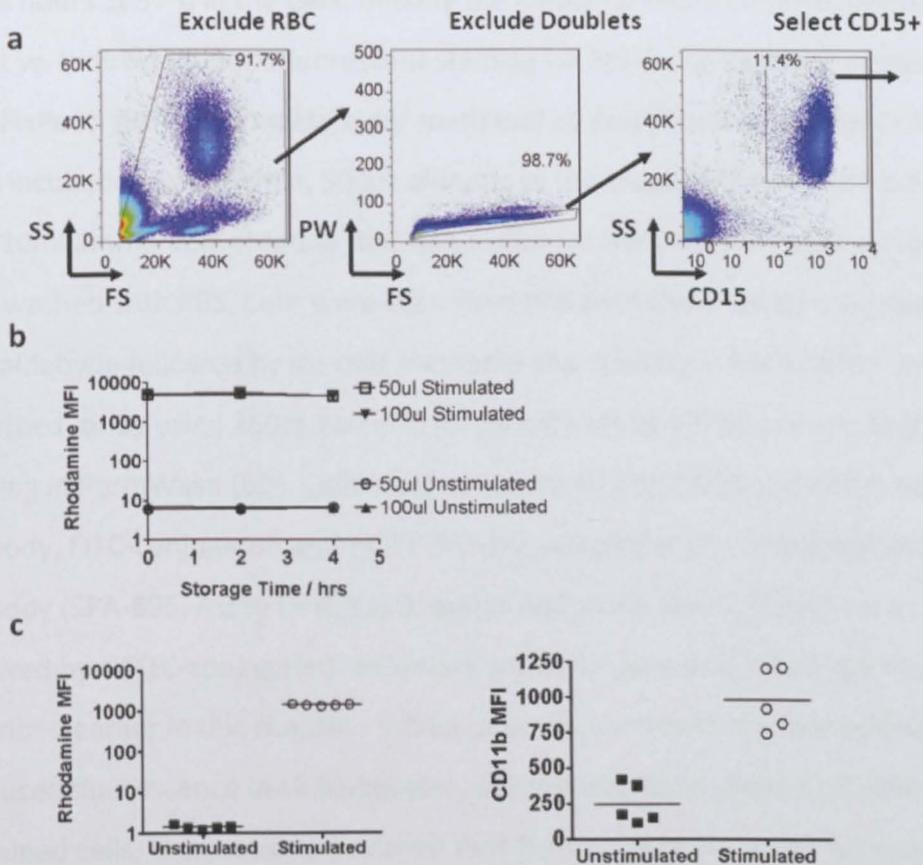
Sample collection criteria for the study specified that all samples must arrive at the laboratory within 2 hours of collection from the patient. To facilitate sample management in the laboratory, samples were processed in batches where possible, trying to allow no more than 4 hours from sample collection until the oxidative burst assay was started. To validate this strategy we assessed the effect of storage on the magnitude of the oxidative burst of healthy donor blood. Blood was collected into Sodium Heparin tubes identical to those used for patient samples and placed in ice in an insulated box which was then placed outdoors in direct sunlight to simulate the conditions of samples being collected in the field. The oxidative burst assay was performed on samples after 0, 2, and 4 hours of storage, and was also compared using either 50 or 100 μ L of whole blood for the assay. The results showed that there was

almost no variation between the oxidative burst under any of these conditions (Figure 2.12b).

In order to compare neutrophil function at different time points following infection it was necessary to ensure that results obtained for rhodamine MFI at different time points would be comparable and not influenced by, for example, drift in the intensity of lasers on the flow cytometer. For this reason, before each batch was processed, the FL-1 voltage was adjusted to achieve the same FL-1 MFI using the same batch of fluorescent beads (Spherotech). Furthermore, samples from the same healthy individual were tested 5 times over a one month period to assess the amount of variation that occurred (Figure 2.12c). For stimulated samples there was little variation in rhodamine MFI (mean value 1381, range 1270-1465, s.d. 73.19, coefficient of variation 5.3%), whereas for CD11b fluorescence there was more variation (mean 975.6, range 741-1207, s.d. 217, coefficient of variation 22.25%). When CD11b upregulation was assessed as the fold change in MFI from the corresponding unstimulated sample, there was also substantial variation (mean 5.0 fold increase, range 2.1-8.1, s.d 2.54, coefficient of variation 50.5%).

Figure 2.12. Modification of the oxidative burst and degranulation assay for human subjects.

(a) Gating strategy for selection of human neutrophils. (b) Stability of oxidative burst response was assessed when samples were stored for different lengths of time before the assay, and for different volumes of whole blood used in the assay. (c) Variation in PMA stimulated rhodamine and CD11b MFI was assessed using blood from the same healthy individual tested 5 times over a one month period.



HO-1 Expression (flow cytometry)

Following the methodological problems encountered when optimizing an assay to detect HO-1 expression in mouse leukocytes by flow cytometry, it seemed appropriate to assess the performance of different methods for detection of HO-1 expression in human blood leukocytes. Similar to the approach in the mouse studies, hemin was used to induce HO-1 *in vitro*, so that quantitative changes in expression could be detected. In 24 well plates, aliquots of fresh whole blood from a healthy donor were mixed and incubated with 500 μ L of RPMI containing varying concentrations of hemin for 18 hours at 37°C in the dark. Initially the following methods were assessed: use of direct vs. indirect immunofluorescent staining for HO-1 and fixation/permeabilisation with FixPerm (BD) or formaldehyde/ methanol as described for fixation of mouse cells. After incubation with hemin, 500 μ L aliquots of the blood/RPMI mixture were mixed with 10ml ammonium chloride RBC lysis buffer for 5 minutes at room temperature, then washed with PBS. Cells were then fixed and permeabilised by using warm formaldehyde followed by ice cold methanol and staining in FACS buffer as previously described, or by using 250 μ L FixPerm for 30 minutes at 4°C before washing and staining in PermWash (BD). Cells were stained with anti-CD15 and either no anti-HO-1 antibody, FITC-conjugated anti-HO-1 (HO-1-2, Abcam) or the polyclonal anti-HO-1 antibody (SPA-895, Assay Designs) or polyclonal rabbit serum (Covance) as a control followed by a FITC-conjugated secondary antibody (goat anti-rabbit IgG F(ab')₂), as described earlier in this chapter. Whilst direct staining with the monoclonal antibody produced fluorescence in all leukocytes, which was clearly above that seen in unstained cells, there was no evidence that fluorescence increased following treatment with hemin, indicating that this antibody was not suitable to quantify changes in HO-1 expression (Figure 2.13). In contrast, using the polyclonal anti-HO-1 antibody for indirect labelling of HO-1 showed that induction of HO-1 in response to hemin treatment was detectable, particularly in CD15^{dim} cells (likely to be monocytes) and to a lesser extent in CD15^{bright} cells (neutrophils). Fixation and permeabilisation using FixPerm appeared superior to formaldehyde and methanol as judged by the intensity of fluorescence relative to the control antibody. In order to validate this further, it was confirmed that HO-1 induction was detectable when combined with the

full panel of surface antibodies to be used in the study, and using a different conjugate for the secondary antibody for detection of HO-1 (because some surface markers were only available with certain conjugates, a PE-Cy7-conjugated secondary had to be used for detection of HO-1). Surface staining (with antibodies against CD14, CD16b, CD91 and CD163; see Chapter 6 for further details) was performed prior to fixation and permeabilization with FixPerm. This confirmed that in response to hemin treatment of whole blood, the greatest changes in HO-1 expression were seen in monocytes (Figure 2.14).

Figure 2.13 Optimization of intracellular staining for HO-1 in human blood.

HO-1 expression was compared between healthy donor leukocytes treated with varying doses of hemin for 18 hrs and then either directly stained with a FITC-conjugated monoclonal anti-HO-1 antibody, or indirectly stained with anti-HO-1 polyclonal antibody followed by a FITC-conjugated secondary antibody. For the indirect polyclonal antibody staining, two fixation methods (FixPerm or formaldehyde methanol) were also compared.

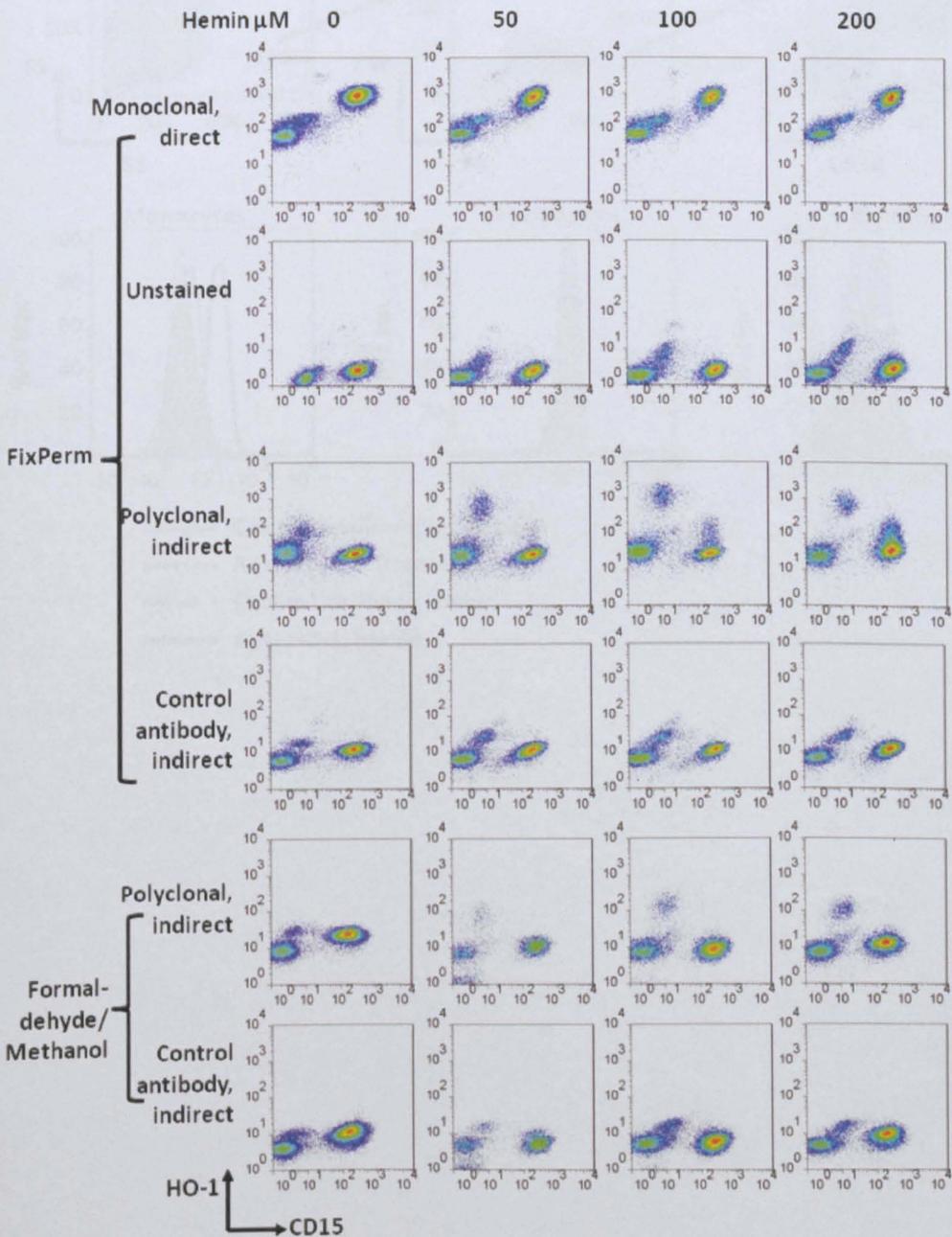
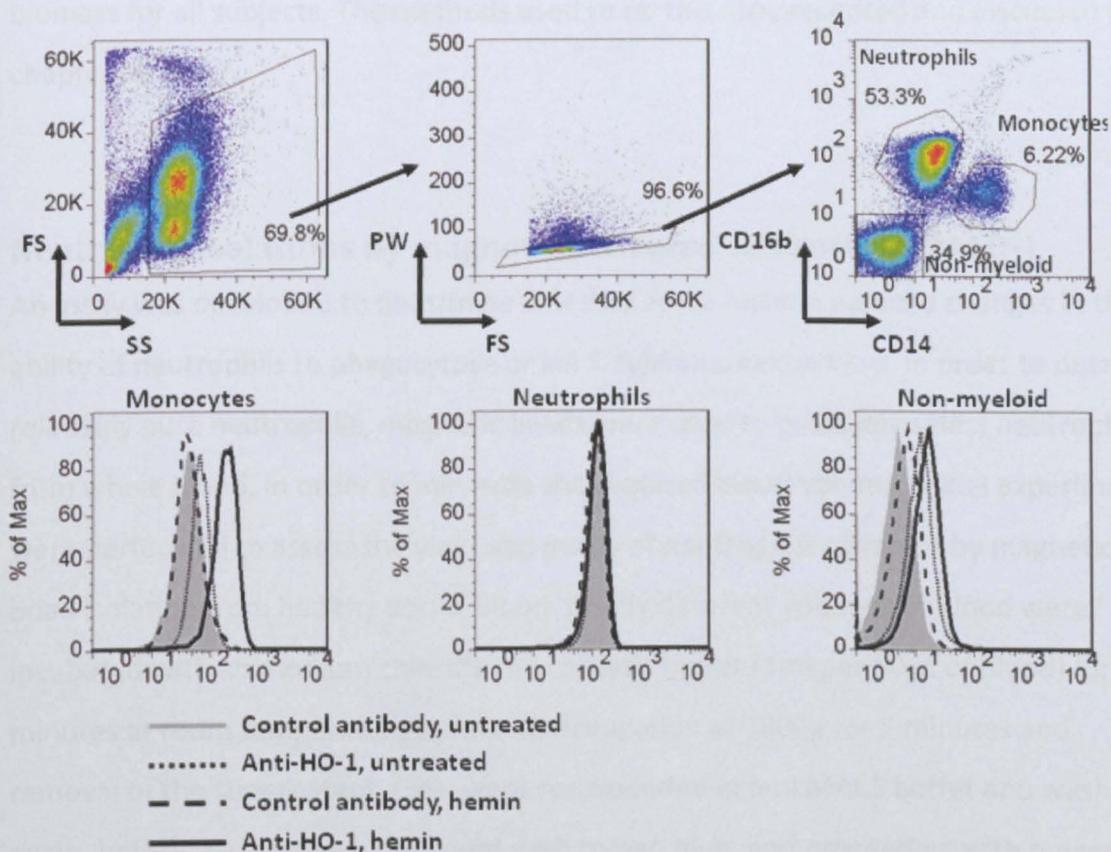


Figure 2.14 HO-1 induction in different cell types in human blood.

Detection of inducible HO-1 expression was confirmed in healthy donor leukocytes after 18 hours incubation with 0 or 100 μ M hemin, when also using a surface antibody panel. Gating strategy is shown in the upper panels and histograms in the lower panels show HO-1 expression in different cell populations.



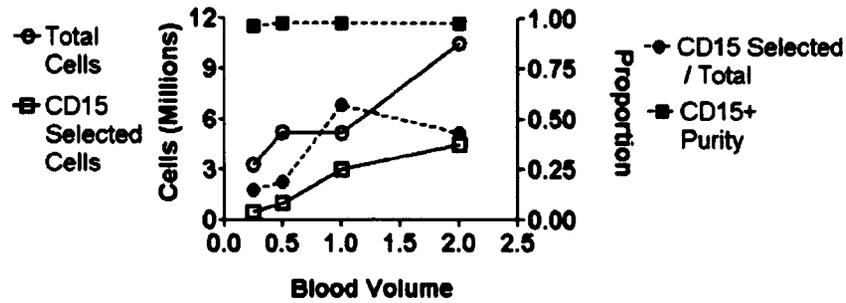
Determination of parasite biomass based on plasma PfHRP-2 concentration

Understanding the relationships between malaria parasite burden, hemolysis, HO-1 induction and neutrophil function was fundamental to this study. In order to avoid underestimation of parasite burden using peripheral blood parasitemia in subjects who may have intravascular sequestration of parasites, we calculated total parasite biomass for all subjects. The methods used to do this are presented and discussed in chapters 6 and 7.

Neutrophil isolations by magnetic activated cell sorting (MACS)

An assay was developed to determine whether acute malaria induced changes in the ability of neutrophils to phagocytose or kill *S. typhimurium in vitro*. In order to obtain relatively pure neutrophils, magnetic beads were used to positively select neutrophils from whole blood. In order to minimize the required blood volume, initial experiments were performed to assess the yield and purity of neutrophils obtained by magnetic bead isolation from healthy donor blood. Briefly different volumes of blood were incubated with ammonium chloride red cell lysis buffer (1ml per 50 μ L of blood) for 10 minutes at room temperature before centrifugation at 1000g for 5 minutes and removal of the supernatant. Cells were resuspended in 5ml MACS buffer and washed again, before performing a cell count with trypan blue, and proceeding with magnetic separation of neutrophils using anti-CD15 beads and MS columns (both Miltenyi Biotec) according to the manufacturer's instructions. Cell counts were repeated after separation of neutrophils and purity was assessed by staining with an anti-CD15 antibody. The purity of CD15 selected cells was high (>97%) and appeared unaffected by the initial blood volume but the absolute number of neutrophils and the yield of cells was dependent on the starting volume (Figure 2.15), and these results confirmed that using at least 1ml of whole blood for neutrophil isolation would probably be optimal.

Figure 2.15 Relationship between blood volume and recovery and purity of cells isolated by MACS.



Salmonella phagocytosis and killing assay

The gentamicin protection assay used to determine phagocytosis and killing of *S. typhimurium* in mice was extremely labour intensive and required access to a plate centrifuge in which infected samples could be centrifuged between wash steps. Unfortunately it was clear that this would not be practical in the study using fresh human blood samples for the following reasons:

1. There was no plate centrifuge available for use with infectious samples.
2. All assays needed to be performed on fresh blood as soon as possible after its arrival in the laboratory, meaning that time available to perform all assays was limited.
3. The study protocol dictated that assays for oxidative burst and HO-1 expression were prioritized, meaning that time available to perform the phagocytosis and killing assays was the most limited.

For these reasons the phagocytosis and killing assay was performed using a modification of the protocol developed by Gondwe *et al.*⁹⁴ The methodology is described in detail in the manuscript presented in Chapter 6. The principle of this assay is rather different to the gentamicin protection assay, in that a high concentration of cells is inoculated with a much lower concentration of opsonized bacteria and constantly mixed to ensure a high collision frequency and therefore phagocytosis of almost all bacteria. Cells can be removed at various time points during the assay and viable bacterial concentration can be determined by cell lysis and quantitative dilution

culture, or GFP⁺ cells can be enumerated directly by flow cytometry. In order to achieve optimal phagocytosis in this assay, Salmonella were opsonized using pooled serum from 10 healthy adult Gambian rural villagers, based on the assumption that within this pool of serum there would likely be antibodies which would opsonize Salmonella, as described by Gondwe *et al.* in pooled Malawian adult serum.⁹⁴ Opsonization with pooled heterologous serum is preferable to autologous serum from children with acute malaria, since child sera might contain highly variable levels of anti-Salmonella antibodies,⁹⁷ and other serum factors induced by acute malaria infection might influence the assay. However, this assay is likely to be a test of the best possible phagocytic and killing function of the neutrophils, and not necessarily the function which would be achieved *in vivo* in each subject.

Chapter 3. Does malaria significantly impair vaccine responses?

Suppression of vaccine responses by malaria. Insignificant or overlooked?

The material presented in this chapter was commissioned as a review article by the Editor of *Expert Review of Vaccines*. It was peer reviewed prior to publication. In the article we discuss the evidence that malaria impairs vaccine responses, the potential public health implications and the underlying biological mechanisms.

Cover sheet for each 'research paper' included in a research thesis

1. For a 'research paper' already published

1.1. Where was the work published? **Expert Reviews of Vaccines**

1.2. When was the work published? **April 2010**

1.2.1. If the work was published prior to registration for your research degree, give a brief rationale for its inclusion

1.3. Was the work subject to academic peer review?

Yes _____

1.4. Have you retained the copyright for the work? No _____

If yes, attach evidence of retention

If no, or if the work is being included in its published format, attach evidence of permission from copyright holder (publisher or other author) to include work

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2. For a 'research paper' prepared for publication but not yet published

2.1. Where is the work intended to be published?

2.2. List the paper's authors in the intended authorship order

2.3. Stage of publication – Not yet submitted/Submitted/Undergoing revision from peer reviewers' comments/In press

3. For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)

I performed the literature review, appraised, synthesised and interpreted the findings of the studies and wrote the manuscript.

Candidate's signature _____

Supervisor or senior author's signature to confirm role as stated in (3)

Suppression of vaccine responses by malaria: insignificant or overlooked?

Expert Rev. Vaccines 9(4), 409–429 (2010)

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Malaria is widely reported to suppress immune responses to heterologous antigens, including vaccines, but the evidence base for this assumption is patchy and confusing. Here we review the evidence for malaria-mediated suppression of responses to vaccination and conclude that: there is evidence of impairment of responses to heterologous polysaccharide antigens in children with clinical malaria or asymptomatic parasitemia; there is little evidence of impairment of responses to routine, protein-based childhood vaccine regimens; and the underlying mechanisms of impaired responsiveness, and especially of impaired responses to T-independent polysaccharide antigens, remain unclear. We suggest that, with the possible exception of vaccines against encapsulated bacteria, the benefits of postponing vaccination until a malaria infection has cleared are probably outweighed by the risk of missing opportunities to vaccinate hard-to-reach populations.

KEYWORDS: antibody • cellular immunity • chemoprophylaxis • immunosuppression • malaria • T-independent • vaccine

Malaria causes a huge global burden of ill health: an estimated 243 million cases of malaria and 863,000 deaths in 2008 [201]. Of the five species known to infect humans (see Box 1), *Plasmodium falciparum* causes the greatest morbidity and mortality, predominantly in young children in sub-Saharan Africa. However, the direct burden of disease may underestimate the overall effect of *P. falciparum* on the health of a population. Epidemiological and ecological studies suggest that infection with *P. falciparum* is a stronger risk factor for death than can be directly attributed to malaria itself [1–3], and it has been estimated that up to half of the variation in child mortality in Africa may be accounted for by parasite prevalence [1]. In other words, there is a major indirect effect of malaria infection on child survival. Consistent with this is the association of clinical malaria episodes with increased risk of bacterial infections [4–6], increased HIV viral load [7], inability to suppress chronic Epstein–Barr virus infection [8,9] and reduced responses to some vaccines [10–16]. These observations have led to the suggestion that malaria is immunosuppressive, although the term immunomodulatory may be more appropriate since it is unclear how

parasite-induced changes in the host immune response influence the clinical manifestations of *P. falciparum* infections.

The populations suffering the greatest burden of ill health from malaria also suffer substantial ill health from vaccine-preventable diseases [17]. The possibility that clinical malaria or asymptomatic parasitemia (see Box 1) might reduce the efficacy of vaccines administered around the time of infection is very worrying. This is not only a potential problem for 'routine' vaccines, but also an obstacle to assessment of new vaccines in malaria-endemic settings. Furthermore, there is a possibility that concurrent *P. falciparum* infection at the time of vaccination may alter the efficacy of an experimental malaria vaccine in an endemic setting. Despite these concerns, the effect of malaria on vaccine responses in humans has been investigated less extensively and less rigorously than might be expected.

In this review we aim to critically re-examine the evidence regarding whether and how *P. falciparum* infection alters either heterologous antigen or malaria vaccine responses in humans and to contrast the effects of *P. falciparum* with the effects of other infections. We concentrate on *P. falciparum*, at the expense of the other *Plasmodium* species that infect humans, because

Box 1. Human malaria.

- Five species of protozoan parasite cause malaria in humans: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae* and *Plasmodium knowlesi*.
- The *Plasmodium* species are transmitted by the bite of female anopheline mosquitoes.
- The first stage of infection involves the rapid transit of injected sporozoites through the skin, into the blood and then to the liver.
- The parasite develops and replicates in liver cells before merozoites burst out to infect red blood cells.
- In red blood cells, parasites undergo further replication and release more merozoites to infect additional red blood cells in a repeating cycle.
- Some parasites differentiate to become gametocytes, which can be taken up by mosquitoes during a blood meal and reinitiate the cycle of infection.
- *Plasmodium* infection may cause no symptoms (asymptomatic parasitemia), mild disease characterized by fever and nonspecific symptoms, or severe disease and death (usually only with *P. falciparum* infection).
- The intensity of malaria infection varies markedly, even within nearby areas of the same country and with changes in rainfall and mosquito numbers.
- Individuals exposed to repeated *P. falciparum* infections acquire immunity to malaria and parasitemia.
- Immunity to severe disease is acquired faster than immunity which reduces parasite load.
- In some settings, nearly everyone will have parasitemia detectable on a blood film, although few of them will have any clinical symptoms.
- The density of parasitemia is related to the likelihood of presenting symptoms.
- Effective immune responses directed against sporozoites could produce sterile immunity (i.e., prevent sporozoites from an infectious mosquito bite from generating merozoites).
- Effective immune responses against blood-stage parasites are more likely to result in lower parasite densities and a reduced likelihood of symptoms.

this parasite has been studied in most detail. Some of the issues we identify may also be relevant to infections with other *Plasmodium* species, particularly *Plasmodium vivax*, which also causes a huge burden of disease [18] but, to date, the evidence is either completely lacking or insufficient to draw any firm conclusions. We find that the strongest evidence is for suppression of responses to heterologous polysaccharide antigens by *P. falciparum* infection, whilst evidence for an effect on heterologous protein antigen responses is less robust. The effect of malaria or asymptomatic parasitemia on responses to experimental malaria vaccines has not been investigated in detail, but there is some preliminary evidence to suggest that immunogenicity is reduced. Paradoxically, nearly all recent studies seeking to explain the reduction of vaccine-induced immune responses in malaria have focused on T-lymphocyte-dependent mechanisms, whereas the most convincing evidence of suppression is for responses to polysaccharide antigens that do not require T-cell help. We propose that practical issues surrounding vaccination may be more important than the immunomodulatory effect of malaria when administering routine childhood vaccines. However, understanding why T-cell-independent responses are most clearly suppressed by malaria may reveal fundamental aspects of the immunological host–pathogen relationship, and may assist development of more effective malaria vaccines. We lay down a challenge for all future vaccine studies conducted in malaria-endemic countries to actively assess the effect of parasitemia on vaccine immunogenicity and protective efficacy.

Does malaria suppress responses to vaccination with heterologous antigens?

Although it is frequently stated that malaria suppresses vaccine responses, to our knowledge the evidence base supporting this statement has never been critically appraised. In fact, it is difficult

to imagine how one may answer this question experimentally in humans. The gold standard would be to randomize volunteers in a blinded fashion to receive infected or uninfected mosquito bites, then to allow time for development of parasitemia before double-blind, randomized allocation to immunization with the vaccine of interest or a control vaccine, followed by assessment of the vaccine-induced immune response and, eventually, of protective efficacy of the vaccine. This type of experiment has been performed in an animal model, for example, the protective efficacy of whole-cell *Bordetella pertussis* vaccine in mice was reduced by blood-stage malaria [19], but never in humans. While challenge experiments are possible in human volunteers, and have played a role in development of malaria vaccines [20], they are costly and, for ethical and safety reasons, parasitemia would not be allowed to proceed for long enough to be comparable with naturally acquired malaria. It is also unlikely that the groups of most interest for vaccination, for example, African infants, would ever be enrolled in such a study. For this reason, most published studies have addressed this question in the context of naturally occurring infections in malaria-endemic areas. Unfortunately, studies undertaken in this way are more subject to bias. For example, comparison of vaccine responses in a group of children with malaria and a group of age- and sex-matched children without malaria may be confounded by a common immunological factor which both increases susceptibility to malaria and reduces responses to vaccination. If the vaccine responses were lower in the children with malaria, the erroneous conclusion may be that malaria reduces vaccine responses. Examples of study designs and potential sources of bias are described in TABLE 1.

A total of 22 studies were identified that either directly or indirectly assessed the effect of *P. falciparum* infection on heterologous vaccine responses (TABLE 2) [10–16,21–35]. Some studies were

Table 1. Limitations and benefits of different study designs to assess the effect of *Plasmodium falciparum* infection on vaccine responses.

| Study design | Example | Sources of bias and confounding | Other advantages/disadvantages |
|---|--|--|---|
| <i>Observational</i> | | | |
| Effect of clinical malaria | Comparison of vaccine responses between children with clinical malaria and healthy control children (may include matching for age, sex and location) | <ul style="list-style-type: none"> • Selection bias • Confounding by uncontrolled factors (e.g., nutritional status, underlying illness, common factor[s] predisposing to malaria and reducing vaccine responses) | <ul style="list-style-type: none"> • Sample size is easy to calculate • A direct method of assessing association between malaria and vaccine response |
| Effect of asymptomatic parasitemia | Retrospective evaluation of vaccine response in children with/without parasites in blood film at the time of mass vaccination | <ul style="list-style-type: none"> • Confounding by uncontrolled factors as above • Submicroscopic parasitemia might reduce the power to detect any difference between groups | <ul style="list-style-type: none"> • Results easily added onto another study • Often the study is not designed to answer this question, so it may be underpowered for this outcome |
| Effect of natural protection from malaria | Vaccine responses in children with HbAA versus HbAS | <ul style="list-style-type: none"> • Confounding by factors as above • Hemoglobinopathy itself may alter vaccine responses | <ul style="list-style-type: none"> • May be difficult to estimate the degree of protection afforded by HbAS |
| <i>Interventional</i> | | | |
| Intervention to create groups which are protected or unprotected from naturally occurring infection | Randomization of children to chemoprophylaxis or placebo | <ul style="list-style-type: none"> • The intervention may itself effect vaccine responses • Selection bias may be present but can be eliminated by randomization • Observer bias may be present which can be eliminated by blinding | <ul style="list-style-type: none"> • The effectiveness of the protective intervention and the rate of natural infections may be hard to predict in advance. These factors will influence the power of the study to detect an effect on vaccine responses |

HbAA: Normal hemoglobin A; HbAS: Sickle cell trait

not primarily designed to assess whether *P. falciparum* infection influenced vaccine-induced immune responses, but rather to test whether the intervention to prevent malaria itself influenced the immune responses to routine childhood vaccines [22,30,31,34]. All studies used immunological outcomes to quantify vaccine responses; no studies have assessed the effect of malaria or asymptomatic parasitemia on vaccine efficacy. Although many different methodologies were used, all had the potential to introduce bias. Individual vaccines, combinations of vaccines and schedules of administration differed between studies, so that direct comparison of results is very difficult. Despite these caveats, there are several consistent themes that suggest that *P. falciparum* does indeed have a detrimental effect on immune response to vaccination with some heterologous antigens.

The antibody response to polysaccharide antigens, especially meningococcal polysaccharides, was most consistently suppressed by *P. falciparum* infection. This effect was reported in observational studies where children with acute malaria or asymptomatic parasitemia had weaker antibody responses to meningococcal polysaccharides than control children without parasitemia [10,11,13]. Children protected from *P. falciparum* infection by chemoprophylaxis or sickle-cell trait had higher antibody responses to meningococcal polysaccharides than unprotected children in areas of high transmission intensity [11–13]. Comparison of the relative effects of clinical malaria and asymptomatic parasitemia

have not been performed within the same study, but children with acute malaria vaccinated with group C meningococcal polysaccharide in one study had lower antibody titers relative to their respective controls than children with asymptomatic parasitemia at the time of vaccination in another study [10,13]. In observational studies, higher levels of symptomatic or asymptomatic parasitemia were associated with lower antibody responses to group C meningococcal polysaccharide, suggesting a 'dose-response' relationship [10,11]. Vaccination of children at different intervals after an episode of acute malaria suggested that the duration of the suppressive effect of acute malaria was at least 28 days for meningococcal C vaccine [10], but this has not been assessed following clearance of asymptomatic parasitemia.

The response to *Salmonella typhi* polysaccharide antigens, as a result of whole-cell vaccination, was also suppressed, but the effect was less pronounced than for meningococcal polysaccharide. This may reflect the difference between administration of a whole organism (which might induce potent cellular immune responses) or just a polysaccharide component (which might not). Antibody responses to the *S. typhi* O antigen, but not to the rather more immunogenic H antigen, were lower in children with acute malaria infection than in controls [15], although the duration of this suppression was shorter than for meningococcal polysaccharide vaccine [10], and chemoprophylaxis was associated with only a small (not statistically significant) increase

Table 2. Studies assessing the effect of *Plasmodium falciparum* infection on heterologous antigen vaccine responses.

| Vaccine(s) | Study (year) and study type ^a | Population | Groups for comparison of vaccine responses | Outcome measures and main findings | Comments/limitations ^b | Ref. |
|--|---|--|--|---|---|------|
| <i>Polysaccharide antigens</i> | | | | | | |
| <i>Salmonella typhi</i> One dose administered at the same time as tetanus toxoid | Greenwood <i>et al.</i> (1972) Observational | 6-month–6-year-old children attending outpatient department of a teaching hospital in northern Nigeria | 51 children with malaria, 31 children with other acute illness (without parasitemia) or 34 healthy siblings | <ul style="list-style-type: none"> Antibody titers and response rates (two or more doubling dilutions) 16 days postvaccination Children with malaria had approximately 2.8-fold lower rise in postvaccination antibody titers to <i>S. typhi</i> O antigen than healthy children. There were fewer 'responders' to <i>S. typhi</i> O antigen (49 vs 85%) compared with healthy children. There were no differences for the H antigen Higher parasite density was associated with lower antibody responses to <i>S. typhi</i> O antigen Other acute illness was not associated with significantly different results to healthy children. 74% of 'sick' controls responded to <i>S. typhi</i> O antigen | <ul style="list-style-type: none"> Few details were provided to compare similarity of groups at baseline Children with malaria received curative treatment on the day of presentation/vaccination All children, regardless of diagnosis, received pyrimethamine 48 h after presentation and 1 week later | [15] |
| <i>S. typhi</i> and group C meningococcal polysaccharide One dose of each | Williamson <i>et al.</i> (1978) Observational | 6-month–6-year-old children attending outpatient department of a teaching hospital in northern Nigeria | 79 children with acute <i>P. falciparum</i> malaria ($\geq 1\%$ parasitemia) compared with 40 age, sex and ethnic group matched healthy siblings. Children with malaria were randomized to receive vaccines on day 1, 7 or 28 | <ul style="list-style-type: none"> Increase in antibody titer and proportion of responders (\geq doubling titer) measured 2 weeks after vaccination Children with malaria had approximately twofold lower increases in antibody titers for <i>S. typhi</i> vaccine given on day 1 Children with malaria had less increase in antibody titers to group C meningococcal polysaccharide at all time points (~fourfold lower on day 1). The proportion of responders was lower on days 1 and 7 There was a strong negative correlation between log(rise in antibody titer) and log(absolute parasite count) | <ul style="list-style-type: none"> All children with malaria received curative treatment on day 1 All children received weekly prophylaxis after enrollment | [10] |

^aSee TABLE 1 for further details of the classification of study types and for generic limitations of each study type.

^bHepatitis B vaccine not strictly equivalent between groups, since RTS,S/AS02D contains hepatitis B surface antigen, but in a different formulation/adjuvant.

BCG: Bacillus Calmette-Guérin; DTP: Diphtheria, tetanus and pertussis; EIA: Enzyme immunoassay; EPI: Expanded Program on Immunization; HbAa: Normal hemoglobin A, HbAs: Sickle-cell trait; IPTi: Intermittent preventive treatment of infants; IPV: Inactivated polio vaccine.

Table 2. Studies assessing the effect of *Plasmodium falciparum* infection on heterologous antigen vaccine responses (cont.).

| Vaccine(s) | Study (year) and study type ¹ | Population | Groups for comparison of vaccine responses | Outcome measures and main findings | Comments/limitations ¹ | Ref. |
|--|---|--|--|--|---|------|
| <i>Polysaccharide antigens (cont.)</i> | | | | | | |
| Group C meningococcal polysaccharide One dose | Greenwood <i>et al.</i> (1980) Observational | 360 children and adults (all ages) in a village in northern Nigeria. 88% of individuals parasitemic at baseline | High vs low level/absent parasitemia HbAA vs HbAS | <ul style="list-style-type: none"> Antibody response (change from pre- to postvaccine titers) 2 weeks after vaccination Antibody response was lower in individuals with high parasitemia within each age group Approximately twofold higher antibody response in individuals with HbAS within each age group | <ul style="list-style-type: none"> Regression analysis was performed to control for effect of age and HbAA/HbAS genotype on the effect of density of parasitemia on vaccine response | [11] |
| Group A and C meningococcal polysaccharide One dose | Blakebrough <i>et al.</i> (1981) Observational | 288 11–20-year-olds attending an urban secondary school in northern Nigeria | Children with HbAA and HbAS | <ul style="list-style-type: none"> No difference in antibody titer 2 weeks after vaccination | <ul style="list-style-type: none"> The intensity of malaria transmission was low (the study was conducted in the dry season) so it was unlikely to find any effect on vaccine responses from the protective effect of HbAS against malaria | [21] |
| Combined group A and C meningococcal polysaccharide One or two doses Initial dose combined with measles and tetanus toxoid | Greenwood <i>et al.</i> (1981) A. Interventional B. Observational | 206 3–17-month-old children in northern Nigeria, parasitemia in 59% of children not receiving prophylaxis at the time of vaccination | <p>A. Children randomized to receive chloroquine doses 1 week before and on the day of vaccination</p> <p>B. Children with parasitemia at the time of vaccination in group not receiving chloroquine</p> | <p>Antibody titers 1 month postvaccination</p> <p>A. Approximately 1.5-fold higher rise in antibody titers against meningococcal A and C polysaccharides in children receiving chloroquine</p> <p>B. Approximately 1.6-fold lower titers of antibodies against meningococcal A and C polysaccharides in children who were parasitemic at the time of vaccination</p> | <ul style="list-style-type: none"> Although subjects were allocated randomly to chloroquine treatment, there was no placebo treatment and no evidence of blinding | [13] |

¹See TABLE 1 for further details of the classification of study types and for generic limitations of each study type.

²Hepatitis B vaccine not strictly equivalent between groups, since RTS,S/AS02D contains hepatitis B surface antigen, but in a different formulation/adjuvant.

BCG: Bacillus Calmette-Guérin; DTP: Diphtheria, tetanus and pertussis; EIA: Enzyme immunoassay; EPI: Expanded Program on Immunization; HbAA: Normal hemoglobin A; HbAS: Sickle-cell trait; IPT: Intermittent preventive treatment of infants; IPV: Inactivated polio vaccine.

Table 2. Studies assessing the effect of *Plasmodium falciparum* infection on heterologous antigen vaccine responses (cont.).

| Vaccine(s) | Study (year) and study type ¹ | Population | Groups for comparison of vaccine responses | Outcome measures and main findings | Comments/limitations ¹ | Ref. |
|--|--|--|---|--|---|------|
| <i>Polysaccharide antigens (cont.)</i> | | | | | | |
| Group A and C meningococcal polysaccharide and <i>S. typhi</i> at 1–2 years of age One dose | Bradley-Moore <i>et al.</i> (1985) Interventional | 1–2-week-old infants in a village in northern Nigeria 41% parasitemia in children not receiving prophylaxis | 198 children allocated to receive chloroquine prophylaxis or 185 receiving placebo for 1–2 years | <ul style="list-style-type: none"> Antibody titers and proportion of children with protective titers one month after vaccination In the chloroquine-treated group, rises in antibody titers were higher against group A (~1.2-fold) and group C (~1.6-fold) meningococcal polysaccharides. There were higher proportions of children with protective titers against group A (72 vs 41%) and group C (44 vs 25%) meningococcal polysaccharides Responses to <i>S. typhi</i> O antigen were not significantly different | <ul style="list-style-type: none"> Allocation was not random and blinding was partial 60% loss to follow-up occurred over the duration of the study. Comparison of age at time of vaccination with meningococcal and <i>S. typhi</i> vaccines was not reported for those remaining under follow-up at this time | [12] |
| <i>Haemophilus influenzae</i> type b capsular polysaccharide conjugated to tetanus protein One dose | Usen <i>et al.</i> (2000) Observational | 12–30-month-old Gambian children attending hospital clinic | 57 children with malaria, 57 with other (aparasitemic) febrile illness, or 60 healthy (aparasitemic) controls | <ul style="list-style-type: none"> Antibody titers 1 month after vaccination and proportions of children with protective antibody titers Children with malaria or other febrile illnesses had lower antibody titers than healthy children (medians of 6.3, 7.5 and 23.0 µg/ml, respectively) More children with malaria failed to achieve protective antibody titers than healthy children (11 vs 0%) | <ul style="list-style-type: none"> The groups were not exactly matched at baseline, children with malaria were slightly older and had slightly lower weight-for-age z scores Results would be affected by a common immunological factor causing both susceptibility to clinical malaria and poorer vaccine responses | [16] |
| <i>S. typhi</i> Ty21a live oral vaccine Three doses First dose coadministered with <i>Vibrio cholerae</i> CVD103–HgR vaccine | Faucher <i>et al.</i> (2002) Interventional | 330 4–16-year-old public school children in Gabon Parasitemia in 33–35% at baseline | Children randomized to receive atovaquone/proguanil chemoprophylaxis or placebo for 3 weeks prior to vaccination, and continuing for 12 weeks | <ul style="list-style-type: none"> Serum IgG and IgA antibody titers against <i>S. typhi</i> lipopolysaccharide, and vibriocidal antibody titers, 4 weeks after vaccination, and proportion of responders (doubling or quadrupling titers, respectively) No significant difference between groups | <ul style="list-style-type: none"> The study was designed to assess whether atovaquone/proguanil suppressed immunogenicity of the live vaccines due to antibacterial effects The power to detect an effect of parasitemia on immunogenicity was limited. All children received curative treatment 7 days before, such that only one child was parasitemic at the start of the chemoprophylaxis or placebo treatment. Only 9% of the placebo group were parasitemic at the time of vaccination | [22] |

¹See TABLE 1 for further details of the classification of study types and for generic limitations of each study type

²Hepatitis B vaccine not strictly equivalent between groups, since RTS,S/AS02D contains hepatitis B surface antigen, but in a different formulation/adjuvant

BCG: Bacillus Calmette-Guérin; DTP: Diphtheria, tetanus and pertussis; EIA: Enzyme immunoassay; EPI: Expanded Program on Immunization; HbAA: Normal hemoglobin A; HbAS: Sickle-cell trait; IPTi: Intermittent preventive treatment of infants; IPV: Inactivated polio vaccine

Table 2. Studies assessing the effect of *Plasmodium falciparum* infection on heterologous antigen vaccine responses (cont.).

| Vaccine(s) | Study (year) and study type ¹ | Population | Groups for comparison of vaccine responses | Outcome measures and main findings | Comments/limitations ¹ | Ref. |
|--|--|------------------------------------|--|--|---|------|
| <i>Polysaccharide antigens (cont.)</i> | | | | | | |
| <i>Haemophilus influenzae</i> type b conjugate vaccine Three doses at 8, 12 and 16 weeks of age coformulated with DTP and coadministered with hepatitis B* or RTS,S/AS02D | Abdulla <i>et al.</i> (2008) Interventional | 340 8-week-old infants in Tanzania | Infants randomized to receive either the experimental malaria vaccine RTS,S/AS02D or hepatitis B vaccine at 8, 12 and 16 weeks of age | <ul style="list-style-type: none"> Seroconversion or seroprotection rates and antibody titers measured 1 month after the third dose of vaccines No significant difference in seroprotection rate between groups Geometric mean antibody titers were lower in the infants receiving RTS,S/AS02D | <ul style="list-style-type: none"> Rates of parasitemia during the course of vaccination are unknown Parasitemia was cleared in all children 2 weeks before the third doses of vaccines 20 of 151 children receiving hepatitis B vaccine, and eight of 146 children receiving RTS,S/AS02D had at least one episode of parasitemia during the 6-month period starting 2 weeks after the third vaccine. This low rate of parasitemia results in low power to detect an effect of the protection from parasitemia on antibody response to vaccination | [34] |
| <i>Protein antigens</i> | | | | | | |
| Tetanus toxoid Two doses, 6 weeks apart | McGregor and Barr (1962) Observational | 3-year-old children in The Gambia | 16 children receiving chloroquine prophylaxis from birth, 14 receiving primaquine prophylaxis from birth or 36 children receiving no prophylaxis | <ul style="list-style-type: none"> Antitoxin titers measured 10–14 days after second dose of tetanus vaccine There were significantly more non-responders in the unprotected group (14/36, 38%) than the group receiving chemoprophylaxis (4/30, 13%) | <ul style="list-style-type: none"> Similarity of groups in terms of potential confounding factors was not assessed Allocation of prophylaxis was not reported to be random Relatively small study size The response rate to tetanus toxoid in both groups is very low compared with the response to 3 doses given in current EPI regimes (close to 100% response rates) | [14] |

¹See **TABLE 1** for further details of the classification of study types and for generic limitations of each study type

*Hepatitis B vaccine not strictly equivalent between groups, since RTS,S/AS02D contains hepatitis B surface antigen, but in a different formulation/adjuvant

BCG: Bacillus Calmette-Guérin, DTP: Diphtheria, tetanus and pertussis; EIA: Enzyme immunoassay, EPI: Expanded Program on Immunization, HbAA: Normal hemoglobin A, HbAS: Sickle-cell trait, IPTi: Intermittent preventive treatment of infants, IPV: Inactivated polio vaccine

Table 2. Studies assessing the effect of *Plasmodium falciparum* infection on heterologous antigen vaccine responses (cont.).

| Vaccine(s) | Study (year) and study type ¹ | Population | Groups for comparison of vaccine responses | Outcome measures and main findings | Comments/limitations ¹ | Ref. |
|---|---|--|--|---|--|------|
| <i>Protein antigens (cont.)</i> | | | | | | |
| Tetanus toxoid One dose administered at the same time as <i>S. typhi</i> | Greenwood <i>et al.</i> (1972) Observational | See Greenwood <i>et al.</i> (1972) in Polysaccharide antigens section [15] | See Greenwood <i>et al.</i> (1972) in Polysaccharide antigens section [15] | <ul style="list-style-type: none"> Antibody titers and response rates 16 days post vaccination There were fewer 'responders' to tetanus (24 vs 50%) compared with healthy children. Higher parasite density was associated with a lower response rate Other acute illness was not associated with significantly different results to healthy children. 42% of 'sick' controls responded to tetanus | <ul style="list-style-type: none"> See Greenwood <i>et al.</i> (1972) in Polysaccharide antigens section [15] The response rate to tetanus toxoid in all groups is very low compared with the response to 3 doses given in current EPI regimes (close to 100% response rates) | [15] |
| Measles (one dose) and tetanus toxoid (one or two doses) Initial dose combined with group A and C meningococcal polysaccharide | Greenwood <i>et al.</i> (1981) | See Greenwood <i>et al.</i> (1981) in Polysaccharide antigens section [13] | See Greenwood <i>et al.</i> (1981) in Polysaccharide antigens section [13] | <ul style="list-style-type: none"> No significant differences in antibody titers to measles and tetanus toxoid | <ul style="list-style-type: none"> Tetanus appeared to be very immunogenic in this combination | [13] |
| Tetanus toxoid one or two doses, 5 weeks apart | A. Monjour <i>et al.</i> (1982) Interventional | 12–36-month-old children in Burkina Faso. Holoendemic | 159 children receiving amodiaquine prophylaxis commencing at the time of vaccination, who were protected (aparasitemic 28 days later) compared with 126 children not given prophylaxis who were parasitaemic at baseline and 28 days later | <ul style="list-style-type: none"> Antibody titers to tetanus toxoid 48–58 days after single vaccination dose, or 86–96 days after first vaccine if two doses were given There was no difference in protective titers between groups. 91.3% achieved protective titres after one dose of tetanus vaccine, and 99.5% after 2 doses No difference in titers 5 years later | <ul style="list-style-type: none"> Unclear whether protected and infected subjects were matched in any way In the majority, prophylaxis was not given prior to the day of vaccination Antibody titers were only available for a small proportion of the total in each group | [23] |
| | B. Monjour <i>et al.</i> (1988) (5-year follow-up) | | | <ul style="list-style-type: none"> No difference in titers 5 years later | <ul style="list-style-type: none"> In the 5-year follow-up, it was unclear how the subjects for follow-up were selected | [35] |

¹See TABLE 1 for further details of the classification of study types and for generic limitations of each study type.

²Hepatitis B vaccine not strictly equivalent between groups, since RTS,S/AS02D contains hepatitis B surface antigen, but in a different formulation/adjuvant.

BCG: Bacillus Calmette-Guérin, DTP: Diphtheria, tetanus and pertussis, EIA: Enzyme immunoassay, EPI: Expanded Program on Immunization, HbAA: Normal hemoglobin A, HbAS: Sickle-cell trait, IPTi: Intermittent preventive treatment of infants, IPV: Inactivated polio vaccine.

Table 2. Studies assessing the effect of *Plasmodium falciparum* infection on heterologous antigen vaccine responses (cont.).

| Vaccine(s) | Study (year) and study type ¹ | Population | Groups for comparison of vaccine responses | Outcome measures and main findings | Comments/limitations ¹ | Ref. |
|--|---|--|--|--|---|------|
| <i>Protein antigens (cont.)</i> | | | | | | |
| Tetanus toxoid Three doses at 28 day intervals | Brabin <i>et al.</i> (1984) Observational | 187 pregnant women after 12 weeks gestation attending rural hospital antenatal clinic in western Kenya | Presence or absence of parasitemia in peripheral blood at the time of vaccination and 7, 28 and 56 days postvaccination | <ul style="list-style-type: none"> Antibody titers measured at days 0, 7 (in primigravidae), 28 or 56 No effect of peripheral blood parasitemia on antibody titers | <ul style="list-style-type: none"> All women received chemoprophylaxis (although its efficacy was uncertain) Substantial loss to follow-up Peripheral blood parasitemia underestimates infection in pregnancy where parasites can sequester in the placenta Primigravidae were divided into multiple groups for comparison based on pattern of parasitemia, which may reduce power to detect any effect | [24] |
| DTP and oral polio at 4, 5 and 6 months Measles at 7 months BCG at 1 or 2 years if Mantoux negative | Bradley-Moore <i>et al.</i> (1985) Interventional | See Bradley-Moore <i>et al.</i> (1985) in Polysaccharide antigens section [12] | See Bradley-Moore <i>et al.</i> (1985) in Polysaccharide antigens section [12] | <ul style="list-style-type: none"> Antibody titers at 1 and 18 months postvaccination. Conversion from negative to positive Mantoux test 5 weeks after BCG In the group receiving chemoprophylaxis there were higher antibody levels to tetanus toxoid at 18 months post vaccine. Other antibody responses to vaccination were not significantly different There was no difference in the rate of conversion to a positive Mantoux test | <ul style="list-style-type: none"> See Bradley-Moore <i>et al.</i> (1985) in Polysaccharide antigens section [12] | [12] |
| Measles One dose | Monjour <i>et al.</i> (1985) Interventional | 1–3-year-old children in Sudan 56–60% parasitemia prior to vaccination | 48 children receiving amodiaquine prophylaxis from 3 days prior to vaccination (‘protected’) vs 65 children without prophylaxis and positive blood films 3 days prior and 28 days after vaccination (‘infected’) | <ul style="list-style-type: none"> Seroconversion 28 days after vaccination Seroconversion rate was not significantly different between groups (89.5% in unprotected versus 92.3% in protected children) | <ul style="list-style-type: none"> Not randomized, blinded or placebo controlled | [25] |

¹See Table 1 for further details of the classification of study types and for generic limitations of each study type.²Hepatitis B vaccine not strictly equivalent between groups, since RTS,S/AS02D contains hepatitis B surface antigen, but in a different formulation/adjuvant. BCG: Bacillus Calmette-Guérin, DTP: Diphtheria, tetanus and pertussis, EIA: Enzyme immunoassay, EPI: Expanded Program on Immunization, HbAA: Normal hemoglobin A, HbAS: Sickle-cell trait, IPTi: Intermittent preventive treatment of infants, IPV: Inactivated polio vaccine.

Table 2. Studies assessing the effect of *Plasmodium falciparum* infection on heterologous antigen vaccine responses (cont.).

| Vaccine(s) | Study (year) and study type ¹ | Population | Groups for comparison of vaccine responses | Outcome measures and main findings | Comments/limitations ¹ | Ref. |
|--|--|--|--|--|--|------|
| <i>Protein antigens (cont.)</i> | | | | | | |
| Measles One dose | Cenac <i>et al.</i> (1988) Interventional | 580 9–48-month-old children in Niger | Children randomized to parasite clearance with chloroquine on the day of vaccination or no treatment | <ul style="list-style-type: none"> Seroconversion 28 days after vaccination The seroconversion was not significantly different between groups (75.5% in the chloroquine group, 81.7% in the untreated group) | <ul style="list-style-type: none"> Randomized but not placebo controlled or blinded | [26] |
| Tetanus toxoid (booster) Single dose | Corrigan (1988) Observational | 197 school children (mean age 9 years) in Papua New Guinea 68.9% of children parasitemic on day of vaccination | Presence or absence of parasitemia in peripheral blood at the time of vaccination | <ul style="list-style-type: none"> Antibody titers measured 28 days after vaccination No effect of peripheral blood parasitemia on antibody titers | <ul style="list-style-type: none"> Few details were provided to compare similarity of groups at baseline | [27] |
| Acellular pertussis Administered with diphtheria, tetanus and IPV (three doses), and one dose of BCG Three different schedules | Simondon <i>et al.</i> (1999) Observational | 390 1–2-month-old infants in rural Senegal | Children with or without parasitemia 5–8 months after vaccination | <ul style="list-style-type: none"> Antibody responses to pertussis toxoid and filamentous hemagglutinin 1 month after the third dose of vaccine Reduced pertussis toxoid titer (geometric mean titer: 81.1 vs 97.3) by EIA 1 month after third dose of DTP vaccine | <ul style="list-style-type: none"> Assessment of the effect of parasitemia was a tertiary objective of the trial Significant effect of parasitemia on pertussis response was only present in a pooled analysis for one of three assays of the antibody response to pertussis vaccine | [28] |
| Measles One or two doses | Spindel <i>et al.</i> (2001) Observational | 65 20–75-month-old children from two indigenous tribes in Brazil Serological evidence of exposure to malaria in 73–100% of children | Seroconversion rates for these populations compared with expected rates for other populations | <ul style="list-style-type: none"> Seroconversion rates (89–95%) measured at variable times after one or two doses were as expected for populations not exposed to malaria | <ul style="list-style-type: none"> No within-study comparison group | [29] |

¹See TABLE 1 for further details of the classification of study types and for generic limitations of each study type

²Hepatitis B vaccine not strictly equivalent between groups, since RTS,S/AS02D contains hepatitis B surface antigen, but in a different formulation/adjuvant

BCG: Bacillus Calmette-Guérin, DTP: Diphtheria, tetanus and pertussis; EIA: Enzyme immunoassay; EPI: Expanded Program on Immunization; HbAA: Normal hemoglobin A, HbAS: Sickle-cell trait; IPTi: Intermittent preventive treatment of infants; IPV: Inactivated polio vaccine.

Table 2. Studies assessing the effect of *Plasmodium falciparum* infection on heterologous antigen vaccine responses (cont.).

| Vaccine(s) | Study (year) and study type ¹ | Population | Groups for comparison of vaccine responses | Outcome measures and main findings | Comments/limitations ¹ | Ref. |
|--|---|---|---|--|--|------|
| <i>Protein antigens (cont.)</i> | | | | | | |
| DTP and oral polio vaccines (three doses) and measles (one dose) | Schellenberg <i>et al.</i> (2001) Interventional | 701 2-month old infants in semirural southern Tanzania | Infants receiving placebo or sulfadoxine-pyrimethamine intermittent (at 2, 3 and 7 months of age) preventive treatment at the same time as second and third doses of DTP and measles vaccines | <ul style="list-style-type: none"> Seroconversion rates for tetanus and diphtheria at 9 months of age, and protective antibody titers against measles at 12 months of age No difference between groups | <ul style="list-style-type: none"> The effect of IPTi on vaccine responses was a subsidiary analysis, designed to assess whether sulfadoxine-pyrimethamine prophylaxis impaired vaccine responses Power to detect an effect of malaria on vaccine responses was limited because IPTi commenced with the second dose of vaccines, and the absolute difference in the proportion of children between the groups free from clinical malaria was only approximately 21%. Seroconversion rates were high in both groups | [30] |
| DTP and oral polio vaccines (three doses) | Massaga <i>et al.</i> (2003) Interventional | 291 12–16-week-old infants in rural north-eastern Tanzania 24–36% parasitemia at baseline | Infants receiving placebo or intermittent (every 2 months) preventive treatment with amodiaquine commencing at the time of their third set of routine DTP vaccine | <ul style="list-style-type: none"> Antibody titers to tetanus (IgG and IgM), poliovirus (IgG and IgM) and diphtheria (IgG) 60 days after their third vaccination No difference between groups | <ul style="list-style-type: none"> The effect of IPTi on vaccine responses was a subsidiary analysis, designed to assess whether amodiaquine prophylaxis impaired vaccine responses Power to detect an effect of malaria on vaccine responses was limited because IPTi only commenced with the third dose of vaccines, and the absolute difference in the proportion of children between the groups free from malaria was only approximately 30% | [31] |
| Measles (one dose) or DTP (2 doses, 1 month apart) | Rosen <i>et al.</i> (2005) Interventional | 996 4-month- to 6-year-old children in six villages in rural Burkina Faso 52% parasitemia prior to study | Children receiving amodiaquine prophylaxis for 5 months prior to vaccination or no prophylaxis | <ul style="list-style-type: none"> Antibody responses 2 months after first vaccination Antibody responses were not significantly different between groups for any vaccines | <ul style="list-style-type: none"> Cluster allocation was not random, not controlled and not blinded There was nearly 50% loss to follow-up Overall response to tetanus was 91% in those without prophylaxis | [32] |

¹See TABLE 1 for further details of the classification of study types and for generic limitations of each study type.

²Hepatitis B vaccine not strictly equivalent between groups, since RTS,S/AS02D contains hepatitis B surface antigen, but in a different formulation/adjuvant.

BCG: Bacillus Calmette-Guérin, DTP: Diphtheria, tetanus and pertussis, EIA: Enzyme immunoassay, EPI: Expanded Program on Immunization, HbAA: Normal hemoglobin A, HbAS: Sickle-cell trait, IPTi: Intermittent preventive treatment of infants, IPV: Inactivated polio vaccine.

Table 2. Studies assessing the effect of *Plasmodium falciparum* infection on heterologous antigen vaccine responses (cont.).

| Vaccine(s) | Study (year) and study type ¹ | Population | Groups for comparison of vaccine responses | Outcome measures and main findings | Comments/limitations ² | Ref |
|---|--|---|---|--|--|------|
| Protein antigens (cont.) | | | | | | |
| Tetanus (booster) One dose | Van Riet <i>et al.</i> (2008) Observational | 53 7–12-year-old children in Gabon who had previously received a tetanus-containing vaccine | Children with parasitemia at the time of vaccination or during the following 14 days compared with children without parasitemia during the same time period | <ul style="list-style-type: none"> Antibody titers and IgG₁ avidity to tetanus toxoid 28 days after vaccination There was no difference between groups in antibody titers or IgG₁ avidity There was no difference between groups in the cellular immune response to tetanus toxoid measured by cytokine production from a whole-blood assay | <ul style="list-style-type: none"> Small study, with secondary analysis of the effect of malaria on vaccine responses Only 11 of 53 subjects had parasitemia (nine <i>P. falciparum</i>). Five were symptomatic and given treatment during the study Subjects were from different locations and had different rates of other parasitic infections | [33] |
| DTP 1 dose, Co-formulated with <i>Haemophilus influenzae</i> type b, hepatitis B ³ or RTS,S/AS02D Three doses | Abdulla <i>et al.</i> (2008) in Polysaccharide antigens section [34] | See Abdulla <i>et al.</i> (2008) in Polysaccharide antigens section [34] | See Abdulla <i>et al.</i> (2008) in Polysaccharide antigens section [34] | <ul style="list-style-type: none"> Seroconversion or seroprotection rates and antibody titers measured one month after the third dose of vaccines No significant difference in seroprotection rate between groups for DTP. Geometric mean antibody titers against DTP, were lower in the infants receiving RTS,S/AS02D Hepatitis B antibody titers and seroprotection rates were higher in RTS,S/AS02D recipients | <ul style="list-style-type: none"> See Abdulla <i>et al.</i> (2008) in Polysaccharide antigens section [34] | [34] |

¹See Table 1 for further details of the classification of study types and for generic limitations of each study type
²Hepatitis B vaccine not strictly equivalent between groups, since RTS,S/AS02D contains hepatitis B surface antigen, but in a different formulation/adjuvant
 BCG: Bacillus Calmette-Guérin; DTP: Diphtheria, tetanus and pertussis; EIA: Enzyme immunoassay; EPI: Expanded Program on Immunization; HBAA: Normal hemoglobin A; HBAS: Sickle-cell trait; IPT: Intermittent preventive treatment of infants; IPV: Inactivated polio vaccine

in the vaccine response to the O antigen [12]. There was also evidence that acute malaria suppressed the antibody response to *Haemophilus influenzae* type b (Hib) capsular polysaccharide antigen (polyribosylribitol phosphate [PRP]) even when this was conjugated to tetanus protein [16]. However, the anti-PRP titers were almost as low in aparasitemic children with other febrile illnesses, suggesting this effect may not be specific to malaria. An important potential confounding factor may have been the administration of paracetamol to febrile children, which was not reported in this study, but has recently been shown to reduce anti-PRP titers in response to vaccination [36]. Unfortunately, there have been no direct comparisons of the relative effects of parasitemia on plain polysaccharide and the relevant conjugate vaccine responses, although this information would be very important if parasitemia eroded the benefit of conjugate vaccines in young children.

In studies designed to assess whether chemoprophylaxis with atovaquone/proguanil or the experimental malaria vaccine RTS,S/AS02D themselves adversely influenced antibody responses to *S. typhi* Ty21a and *Vibrio cholerae* CVD103-HgR oral vaccines, or a Hib-containing vaccine, respectively, there was no evidence for an effect of parasitemia on responses to the polysaccharide antigens [22,34]. However, neither study was designed to assess the effect of parasitemia on vaccine-induced antibody responses and as such both studies lacked sufficient power for this outcome to be meaningfully assessed.

In a single study of the antibody response to tetanus toxoid administered at the time of presentation to hospital with acute malaria, children with malaria had lower antibody titers and response rates than healthy control children or children with other illnesses [15]. However, three observational studies of the effect of asymptomatic parasitemia on antibody responses to tetanus toxoid (one in pregnant women and two investigating booster vaccination in older children) found no significant effect

of malaria infection on anti-tetanus antibody responses [24,27,33], although all studies had methodological limitations (TABLE 2). We identified eight studies that investigated the effect of chemoprophylaxis or parasite clearance on the response to tetanus toxoid [12–14,23,30–32,35]. In one small study, the antibody response was significantly higher among 3-year-old Gambian children who had been protected from malaria from birth than in those who had not received prophylaxis [14], and in another study where chemoprophylaxis was also given for a prolonged period, children on prophylaxis showed slower declines in anti-tetanus antibodies following vaccination [12]. By contrast, the response to two or three doses of combined diphtheria, tetanus and pertussis vaccine in younger infants was not improved by prolonged antimalarial prophylaxis [12,32] either because any suppressive effect was overcome by the high immunogenicity of this vaccination regime or because the power of the study was limited by low susceptibility to malaria infection of small infants [37]. In addition, the response to tetanus vaccine was not improved by 1 week of chemoprophylaxis when tetanus was combined with group A and C meningococcal and measles vaccines in 3–17-month-old children [13], by chemoprophylaxis or parasite clearance given at the time of vaccination [23,30,31] or by the apparent protective effect of an experimental malaria vaccine [34]. As shown in TABLE 2, there are many methodological limitations of these studies which might reduce their power to detect any effect of malaria infection on antibody responses to tetanus toxoid. Overall, the effect of asymptomatic parasitemia on the tetanus vaccine response is uncertain, and may be negligible, while one observational study supports an association between acute malaria and reduced response to tetanus vaccine given in isolation, but does not prove causality. Studies in mice indicate that whole-cell pertussis vaccine can act as an adjuvant for coadministered antigens thereby overcoming malaria-induced suppression of the tetanus vaccine response [38], and so it is likely that the immunogenicity of tetanus toxoid is enhanced in the human triple vaccine. Differences in the vaccination and chemotherapeutic regimes used between studies may be sufficient to account for the discrepant findings regarding responses to tetanus toxoid in appropriately powered studies.

We found no evidence that antibody responses to measles, diphtheria or polio were impaired by *P. falciparum* infection. The isolated finding of a reduced response to pertussis in one study must be interpreted with caution since this was only found in a subsidiary analysis of pooled data and is only significant in one of the three assays used [28]. The antibody responses to modern vaccines given in their usual Expanded Program on Immunization (EPI) combinations and timings were universally high in children in malaria-endemic settings, and by inference appear to be relatively unaffected by malaria or parasitemia [30,31,34].

The effect of *P. falciparum* infection on heterologous vaccine efficacy, that is whether vaccination protects against disease, has not been assessed. This issue is of far more clinical and public health significance than the antibody responses to vaccination, since the quality (affinity and subclass) and longevity of antibodies and memory cells generated by vaccination may be just as important for protection as the absolute quantity of antibody

detected shortly after vaccination [39]. In resource-poor, malaria-endemic settings there is little capacity for high-quality surveillance for clinical vaccine failures, and hence data on vaccine effectiveness are scarce.

In summary, therefore, the evidence that *P. falciparum* impairs antibody responses to polysaccharide antigens is quite robust and this is of public health significance since effective vaccination against encapsulated bacteria such as Hib, *Streptococcus pneumoniae* and *Neisseria meningitidis* could produce huge health benefits in poorer countries [40]. Polysaccharide antigens are well-known to be poorly immunogenic in young children and so vaccines have been developed with polysaccharide antigens conjugated to immunogenic proteins, to convert a T-lymphocyte-independent immune response to one with T-lymphocyte help, and greater immunogenicity [41]. It is therefore worrying that even Hib-conjugate vaccine responses were suppressed by acute malaria [16], raising important and, as yet, unanswered questions as to the effect of asymptomatic parasitemia on Hib vaccination. Of concern, in an efficacy trial of a nine-valent pneumococcal conjugate vaccine in The Gambia, vaccine efficacy was lowest in children immunized during the malaria season, although the study was not designed to specifically test the effect of *P. falciparum* on vaccine efficacy and effect of season was not statistically significant [42]. These findings suggest that the effect of *P. falciparum* on new generation vaccines against encapsulated bacteria should be evaluated in any trials taking place in malaria-endemic regions.

Is the effect of malaria any different to other infections?

Immunosuppression by infection is not a unique feature of malaria. Measles suppresses both cell-mediated and humoral immunity during acute infection, and possibly for some time after [43,44]. Similar to malaria, measles causes both direct mortality during the acute illness and late mortality, which has been attributed to its immunosuppressive effect [45–47]. The effect of measles on vaccine responses was assessed by Whittle *et al.* [43] in a study of similar design to an earlier trial on acute malaria [15]. Measles infection reduced antibody responses to *S. typhi* O and H antigens and tetanus toxoid (the latter not statistically significant) when children 7–72 months of age were immunized within 4 days of appearance of their rash. Helminth infections such as onchocerciasis [48] and lymphatic filariasis [49] have also been shown to reduce responses to vaccination with tetanus toxoid and a large study is currently underway to evaluate whether intestinal helminths have a similar effect [50]. These helminth infections perhaps have more in common with *P. falciparum* because significant proportions of the population can be asymptotically infected for long periods of time.

We identified only two studies that compared responses to vaccination in children presenting to hospital with malaria, with responses in children with other acute illnesses and with healthy controls. In Nigeria, Greenwood *et al.* found that whilst malaria was associated with lower vaccine responses, other ill children did not have significantly lower responses to tetanus and *S. typhi* O antigen than healthy control children [15]. On the other hand,

in The Gambia, Usen *et al.* found that malaria and other febrile illnesses suppressed the response to Hib-conjugate vaccine to a similar extent, although the proportion of children with protective titers was lowest in those with malaria [16]. Aside from the different vaccines used, the selection of the nonmalarial ill children differed slightly with the later study, only including those with a fever, which may partially explain the discrepancy in the effect on vaccine responses. In other words, it is possible that vaccine responses are reduced after vaccination during a febrile illness but not during a nonfebrile illness. More studies are required to resolve this question.

Should malarious children be vaccinated?

In resource-rich countries such as the UK and USA, vaccination is usually deferred in unwell febrile children [202,203], although the rationale for this is not that the febrile illness may diminish vaccine responses, but that any potential adverse vaccine reaction may be indistinguishable from the existing febrile illness and may thus cause diagnostic uncertainty. Furthermore, administration of a potentially reactogenic vaccine to a clinically unstable patient might precipitate a severe adverse event. Several large studies have shown that mild intercurrent illness [51,52], or even more severe febrile illness [53], have little effect on vaccine responses, although many of these relate to measles vaccine, which seems to be the most impervious to any effect of malaria. Studies on other vaccines would be helpful to clarify whether vaccine immunogenicity and efficacy are influenced by vaccination during intercurrent illness.

The limited evidence available suggests that impairment of vaccine responses by malaria is qualitatively and quantitatively similar to measles, and perhaps to other febrile illnesses of equal severity. However, in resource-poor countries, where opportunities to vaccinate children may be limited, vaccination often proceeds regardless of whether a child has an intercurrent illness [16]. Even if some febrile children have a slightly poorer response to vaccination, this is unlikely to be of great public health significance if the proportion of children who are febrile at any time is low and the duration of the effect is short. Asymptomatic parasitemia potentially poses a greater problem because the proportion of affected children at any time may be high and the duration of infection may be prolonged. Thus, even if the effect of asymptomatic parasitemia is not very different from other illnesses, its prevalence and duration might create a much greater public health problem. At the moment, there is insufficient evidence regarding the effects of asymptomatic parasitemia – particularly on responses to vaccines to encapsulated bacteria – to make any policy recommendations in this area.

Does *P. falciparum* infection reduce the protective response to experimental malaria vaccines or natural infection?

There are currently no licensed vaccines for the prevention of malaria, but there are many candidates in development, with the RTS,S vaccine (recombinant circumsporozoite protein antigen fused to hepatitis B surface antigen in a novel particle) being in the most advanced clinical trials [54]. The development of

malaria vaccines has seen many candidate vaccines that have appeared immunogenic in animal models and in Phase I trials in nonendemic populations, but fail because of negligible efficacy in field trials. There remains a fundamental problem that the nature of naturally acquired immunity to malaria is poorly understood [55]. Consistent with this, there has never been a clear correlation between humoral or cellular immune responses generated by candidate malaria vaccines and their protective efficacy. These observations make it very difficult to determine whether *P. falciparum* infection does suppress relevant immune responses to experimental malaria vaccines, or indeed the response to itself in human subjects. Furthermore, to our knowledge, this issue has never been addressed as the primary goal of a clinical trial in humans. Many studies have used a simplified system, examining the effect of *P. falciparum* infection on the responses of human peripheral blood mononuclear cells (PBMCs) to malaria antigens *in vitro*, and depressed responses to malaria antigens have usually been found in subjects with current infection [56–58]. However, these studies made the assumption that the proportion of antigen specific cells circulating in peripheral blood at the time of infection is the same as in convalescence or healthy controls, and this is unlikely to be true [59]. There is also the possibility that naturally occurring *P. falciparum* infection at the right time interval after vaccination might enhance the response to a malaria vaccine by acting as a 'natural booster'.

Evidence from experimental malaria vaccine efficacy trials

To date, only two experimental malaria vaccines have claimed efficacy against naturally occurring infection in endemic settings: SPf66 and RTS,S. The SPf66 vaccine (a synthetic polymer composed of blood-stage, protein-derived, amino acid sequences, linked by a circumsporozoite repeat sequence) is now considered to have had negligible efficacy after a series of clinical trials that began with adult subjects under low transmission intensity and progressed to increasingly rigorous trials in infants and children under higher transmission intensity, with ever decreasing estimates of protective efficacy [60–62]. The effect of *P. falciparum* infection at the time of SPf66 vaccination on vaccine efficacy was considered in a secondary analysis within a trial in Brazil [63]. Although, overall, the vaccine did not show significant efficacy, the estimate of efficacy was even lower in the subgroup with intercurrent parasitemia during vaccination than in those free from parasitemia. As exposure was not assessed it is not possible to exclude that this was confounded by differences in exposure, but randomization should have reduced the likelihood of this. In a trial in 1–5-year-old Tanzanian children with over 90% parasite prevalence prior to vaccination, under conditions of intense perennial transmission, sulphadoxine-pyrimethamine (S-P) was administered 2 weeks prior to each of three doses of vaccine, ensuring that children were parasite free at the time of vaccination. This was the only trial of SPf66 in African children, which demonstrated significant efficacy against the first or only episode of clinical malaria (35% efficacy; 95% CI: 0–52) [64]. A subsequent study in infants in the

same setting found efficacy of only 2% (95% CI: -16–16); this time parasites were not cleared prior to vaccination but it is not clear whether this, the age of the vaccinees or chance explains the discrepancy between the two trials [62]. However, no significant beneficial effect of the vaccine was seen in Gambian infants, in a highly seasonal malaria transmission setting, given three doses of SPf66 and S–P treatment 1–2 weeks before the first and third doses (vaccine efficacy 8% [95% CI: -18–29] [65], suggesting that age at vaccination may indeed be important. This study was marred by a coding error resulting in incorrect vaccine administration in almost a quarter of children, and it is unclear whether S–P treatment was re-administered prior to revaccination with the correct vaccine. Finally, in 2–15-year-old children under lower intensity transmission in Thailand, parasitemia was only treated at the time of vaccination and the protective vaccine efficacy of SPf66 was found to be -9% (95% CI: -33–14) [61]. Whilst results of all four of these trials would be consistent with a true vaccine efficacy of 0–14%, it is also possible that the differences in treatment/clearance of *P. falciparum* during the course of vaccination might have contributed to differences in observed vaccine efficacy. Even differences as subtle as parasite clearance 1 versus 2 weeks before vaccination might be important if parasitemia has a prolonged suppressive effect, as observed for meningococcal C vaccine [60], although given the peptide nature of the Spf66 vaccine, a major effect of malaria is perhaps hard to argue.

Clinical trials of the RTS,S vaccine in Africa have shown 45–66% efficacy against the first episode of parasitemia [66,67], and 30–53% efficacy against the first episode of clinical malaria [66,68]. These trials have varied in size and geographical location, and have included iterative optimizations of both vaccination schedule and adjuvant [54]. None of the trials included deliberate clearance of parasitemia or chemoprophylaxis prior to vaccination; however, all trials using the first episode of parasitemia as an end point required a curative treatment to be given prior to the third (final) dose of vaccine so that any parasitemia observed during follow-up might be classified as a new infection. Parasite clearance was not undertaken in either of the large studies using first episode of clinical malaria as an end point [66,68]. From the available evidence it is not possible to determine whether *P. falciparum* infection prior to vaccination has any detrimental effect on the efficacy of RTS,S, although clearly this issue is of great importance as RTS,S is a strong candidate to become the first licensed malaria vaccine.

Other malaria vaccines have reached clinical trials in endemic countries, but none have demonstrated clinical efficacy. Only one assessed the effect of parasite clearance with S–P prior to vaccination (with Combination B: merozoite surface proteins [MSP] 1 and 2, and ring-infected erythrocyte surface antigen) and, using parasite density during the follow-up period as a measure of vaccine efficacy, the authors suggested that parasite clearance might actually be associated with a rebound increase in parasite density and a consequent reduction in vaccine efficacy [69]. This study was small and randomization may have been inadequate, so this finding should be viewed with caution.

Evidence from experimental malaria vaccine immunogenicity trials

Far more trials of experimental malaria vaccine immunogenicity have been conducted than for vaccine efficacy, but again few have assessed the effect of parasitemia on response to vaccination. In the small study of the Combination B vaccine mentioned above, antibody titers to the component antigens were not enhanced by S–P treatment 1 week prior to vaccination but cellular responses to one of three antigens (MSP-1) appeared lower in the vaccinated subjects who had received S–P [70]. In a larger study, Bejon *et al.* examined the effect of naturally occurring *P. falciparum* infection on the development and longevity of T-cell responses to an experimental vaccine (multiple-epitope thrombospondin-related adhesion protein [ME-TRAP]) designed to stimulate strong cellular immunity [71]. The ME-TRAP vaccine was administered by a sequential vector regime (attenuated fowl pox followed by modified vaccinia virus Ankara) and although it did not protect against febrile malaria in the primary study [72] it was immunogenic. Asymptomatic parasitemia at the start of vaccination, or prior to the peak transmission season in control subjects, was associated with a smaller IFN- γ recall response to vaccine antigens at the end of the vaccination schedule or transmission season, respectively. While the results of these studies are interesting, their relevance is uncertain given the inefficacious immune response to the vaccine.

Evidence from studies of naturally acquired infection

Naturally acquired protective immunity to *P. falciparum* develops slowly, incompletely and can wane in the absence of ongoing challenge [55,73]. Epidemiological studies suggest that there are at least two components: immunity to clinical disease and immunity to parasitemia. The development of clinical immunity is relatively fast, is hastened under conditions of more intense transmission and is lost relatively quickly in the absence of reinfection. By contrast, antiparasite immunity appears to develop more gradually with age and, once established, can be maintained with infrequent boosting [73,74]. The two components are likely to be related, since antiparasite immunity may reduce parasitemia below a threshold necessary to cause symptoms or severe disease. It is possible that either of these components of naturally acquired immunity are subverted or subdued by *P. falciparum* infection. Unfortunately, the exact nature of the antibody and cellular immune responses necessary for protection against malaria remain unknown. Antibody levels correlate well with exposure to *P. falciparum* [75], exposure correlates strongly with the acquisition of protective immunity and protective antiparasitic immunity reduces the amount of *P. falciparum* antigen available to restimulate immune responses. Thus, it is very difficult to assess whether naturally occurring *P. falciparum* infection actually suppresses the immune response to subsequent *P. falciparum* infection or whether any change is actually due to protective immunity efficiently limiting infection and hence antigen exposure.

One way to circumvent this problem would be to assess the effect of intensity of exposure on the development of immune responses to *P. falciparum* antigens before antiparasitic immunity has developed. This has been attempted in a small but detailed

longitudinal study of Kenyan infants in the first 2 years of life under different transmission intensities, with frequent assessment of parasitemia and entomological inoculation rate to determine specific antibody responses [76]. Levels of IgG to MSP-1_{19-kDa} (a blood-stage antigen) and circumsporozoite protein (CSP; a pre-erythrocytic antigen) varied dramatically over time and with episodes of infection. Higher levels of IgG to MSP-1_{19-kDa}, but not CSP, were found in children who were subjected to lower transmission intensity and had fewer infections during the first 2 years of life than in those subjected to a very high intensity of infection. Another approach to the problem is to assess immune responses in children who are exposed to malaria but protected from blood-stage infection by chemoprophylaxis. In 5-year-old Gambian children who had received 3 years of intermittent chemoprophylaxis, cellular responses to blood-stage antigens were higher than in controls who had received placebo, but antibody responses to a crude schizont extract were lower [77]. Furthermore, a small study comparing exposed individuals with and without natural protection against the blood stage of *P. vivax*, due to absence or presence of the Duffy antigen on erythrocytes, respectively, demonstrated strong cellular responses to sporozoite antigens but absent responses to blood-stage antigens in Duffy antigen-negative individuals, and the opposite trend in Duffy antigen-positive individuals [78]. These findings provide some preliminary evidence that *Plasmodium* species may suppress cellular and antibody responses to some of their own antigens *in vivo*, although the relationship may be complex, with different thresholds for generation and suppression of protective responses.

Overall, there is scarce evidence that *P. falciparum* infection suppresses protective responses to malaria vaccines or to naturally acquired malaria infection, but this is because protective immune responses remain poorly defined, rather than because there is strong evidence to refute the hypothesis. Some observations are compatible with suppression of specific responses to malaria antigens by *P. falciparum*, but these fall far short of proving causality, or that there is an effect of clinical or public health relevance. Alternative explanations for the slow development of antiparasite immunity, such as age-dependent maturation of the immune system [74], and parasite antigenic variation [79] have more compelling evidence to support them.

Mechanisms of immunomodulation by malaria

The evidence for modulation of vaccine responses by *P. falciparum* is limited by the methods available for study of human immune responses, but experimental infections in animals have allowed much more detailed exploration of immunomodulatory effects and the potential mechanisms. In mice, humoral and cellular responses to vaccination with a wide variety of heterologous antigens can be shown to be suppressed by experimental malaria, recapitulating and often exceeding the effects observed in humans [80–82]. Interestingly, this effect is particularly prominent for splenic antibody responses rather than those mounted in lymph nodes [83]. There is conflicting evidence regarding whether blood-stage infection in mice can suppress immunity to liver-stage antigens [84,85], which would be a problem for vaccines such as RTS,S

if it were also true in humans. However, experimental infections in mice do not necessarily closely replicate naturally occurring, repeated episodes of *P. falciparum* infection in humans, so here we will focus on only the most pertinent mechanistic studies.

The most convincing effect of *P. falciparum* is on responses to heterologous vaccine polysaccharide antigens. These antigens, which contain repetitive sequences, elicit T-lymphocyte-independent type-2 immune (TI-2) responses, through a mechanism which involves cross-linking of multiple B-cell receptors, allowing signal transduction in the absence of MHC-II restricted T-cell co-stimulatory signals [86]. Conventionally, CD4⁺ T lymphocytes have been thought to recognize only protein antigen presented via MHC-II and hence are excluded from the response to polysaccharide antigens. Although it is worth noting that the quality of the response to T-independent (TI) antigens is affected by T-lymphocytes [86] and that some polysaccharide antigens can be presented on MHC-II and recognized by the $\alpha\beta$ T-cell receptor [87], if the primary defect in the vaccine response was at the level of CD4⁺ $\alpha\beta$ T cells we would expect to see widespread effects on responses to protein vaccines, and this is not the case.

Splenic marginal zone B cells are particularly adapted for responding to TI-2 antigens [88,89]. The marginal zone allows slow passage and trapping of blood-borne antigen, carried – at least in mice – on circulating CD11c^{lo}Mac-1^{hi} ‘dendritic cells’ (DCs) [89,90], which also provide survival signals for plasmablasts, ensuring maximum exposure of marginal zone B cells to antigen. TI-2 responses are weak in young children, for reasons which remain uncertain but may reflect a need for maturation of the marginal zone B-cell population in early childhood [88]. Most of the evidence for suppression of polysaccharide vaccine responses by *P. falciparum* was generated in young children, so it is perhaps not surprising that the most weakly immunogenic types of antigen in this age group are seen to be most affected by malaria, and this may involve a completely nonspecific mechanism. However, TI-2 responses are an essential component of the host response to encapsulated bacteria in the bloodstream and individuals with impaired TI-2 responses due to malaria would be expected to be susceptible to bacterial coinfections. This has been repeatedly observed in numerous settings where malaria is endemic [4–6]. If a specific mechanism is involved, modulation of either B-cell or DC function, rather than T-lymphocyte function, are the most likely explanations. Early experiments in mice suggested that B-cell dysfunction *per se* was an unlikely explanation for hyporesponsiveness to TI-2 antigens since, although splenic B cells responded poorly in malaria-infected mice, these same B cells were able to respond after transfer into irradiated uninfected mice [81]. It was also realized early on that certain antigens did not localize normally in the spleen during malaria infection [91], suggesting that changes in splenic architecture or antigen-presenting cell function may be the underlying problem. DC function has been extensively studied during malaria infection and although the findings may appear superficially contradictory they can, on the whole, be reconciled by considering a natural progression from activation of DCs early in infection to homeostatic inhibition later in infection [92]. However, attention

has focused on the interaction of DCs with T lymphocytes and subsequent T-effector and T-dependent antibody responses with little regard for TI responses. Whether the role of DCs in localizing antigen for the TI response, or in promoting survival of marginal zone B cells is defective, is unknown at present, and an explanation as simple as continuous depletion of circulating DCs by persistent parasitemia and high levels of malaria antigen exposure, may be all that is necessary. Indeed, a recent study has shown that a CD11c^{hi}Mac-1^{hi} 'monocyte' population, with a very similar immunophenotype to the previously described circulating DCs, selectively transports *Plasmodium chabaudi* to the spleen, accumulating there during blood-stage infection with simultaneous depletion from the bone marrow [93]. This may be compounded by widespread activation of marginal zone B cells during malaria infection by highly repetitive malaria antigens acting as TI antigens [94–96], or by complement-binding IgM immune complexes [97–99], leaving them with little capacity to respond to other TI antigens that are quite simply crowded out. Crucially, obliteration of the normal splenic architecture during both *P. falciparum* and rodent malaras, including progressive dissolution of the splenic marginal zone and follicular structures [100,101], probably precludes optimal interactions between marginal zone B cells and antigen-presenting DCs. Intriguingly, the time taken for splenic marginal zones to regain their normal conformation (up to 30 days after clearance of infection in mice [100]) is similar to the duration of the adverse response to polysaccharide vaccination.

A possible scenario by which malaria infection leads to suppression of heterologous polysaccharide vaccine responses might thus be as follows. Malaria antigens in the blood stream are trafficked to the spleen by circulating DCs. If the antigen load is sufficient this may deplete DCs from the blood or overwhelm their capacity to traffic other antigens. Through TI-2 repetitive sequence proteins and TI-1 polyclonal B-cell-activating antigens, and through immune complex-mediated stimulation of marginal zone B cells, proliferation and migration of marginal zone B cells leads to dissolution of the normal marginal zone structure. Heterologous polysaccharide antigens or bacteria entering the circulation at this stage of the infection may not be trafficked to the splenic marginal zone due to a lack of transporting DCs or because the marginal zone has disintegrated. Even if the marginal zone lymphocytes are encountered elsewhere in the spleen, they may be unable to respond normally to TI antigens, and a relative defect may persist until the marginal zone architecture returns to normal.

Implications & challenges for the future

The evidence that *P. falciparum* infection reduces vaccine responses is less robust than is often implied. In part this reflects the difficulty of studying immune responses in humans in the context of a complex parasitic disease, which most severely affects young children in some of the world's most deprived countries. It also reflects the fact that the potential impact of concurrent malaria infection has been largely ignored in vaccine trials in endemic countries. Based on the available evidence, there is no reason to suppose that asymptomatic *P. falciparum* infection significantly reduces the immunogenicity

of routine EPI vaccines when given in their usual formulation and schedule. Whether clinical malaria would influence these vaccine responses is unclear, and the decision whether or not to vaccinate in the context of an acute febrile illness that may be malaria has to be a pragmatic one – in an ideal situation vaccination might be deferred until the illness has been treated, but if the opportunity for vaccination may not recur, then vaccination would be better than not. In the case of very high fever, severe illness or where there is diagnostic uncertainty, vaccination may risk causing harm and we would recommend deferring vaccination.

For vaccination with polysaccharide antigens, consideration should be given to curative treatment prior to vaccination in areas where parasite prevalence is high, particularly in younger children who already respond suboptimally to these vaccines. The length of time between parasite clearance and restitution of normal vaccine responsiveness may vary from vaccine to vaccine, but a good rule of thumb appears to be 4 weeks and practical considerations such as accessibility of the population, the effective half-life of the antimalarial drug and likelihood of reinfection will be at least as important as the immunological recovery. Even for newer conjugate vaccines, responses may be impaired by acute malaria or other febrile illness, but the same considerations apply as for polysaccharide vaccines, when deferring vaccination may result in a missed opportunity to vaccinate. Clinical trials of new conjugate vaccines undertaken in malaria-endemic areas should aim to assess the effect of parasitemia on vaccine efficacy as well as immunogenicity. In the context of a well-conducted, randomized control trial this should not be a great additional burden on resources.

Determining whether *P. falciparum* modulates the immune responses to itself or to antigens in malaria vaccines seems to be a high priority for understanding protective immune responses against malaria and for the development of an effective malaria vaccine. One way to assess this would be a combined trial in a high-transmission setting of a malaria vaccine with another intervention, such as intermittent preventive treatment of infants (IPTi) [102]. If IPTi was given prior to the malaria vaccine, and a 2 × 2 factorial design (IPTi + malaria vaccine vs placebo + malaria vaccine vs IPTi + control vaccine vs placebo + control vaccine) was employed, this could be very informative, and would be a realistic assessment of the value of a malaria vaccine, which is unlikely to ever be used in isolation, to prevent malaria in an endemic setting. The major drawbacks of this approach would be the very large sample size required and the resulting cost. However, even the systematic collection and analysis of observational data in ongoing and future trials, monitoring vaccine efficacy in children with or without parasitemia before and during vaccination may provide important information to address this issue. Subsequent mathematical modeling of vaccine efficacy and cost-effectiveness under different transmission intensities or in combination with other control measures could assist the most rational implementation of the vaccine.

The potential significance of the relative suppression of heterologous TI-2 antibody responses by *P. falciparum* has been overlooked to date. We speculate that this may be directly related to the alterations in splenic architecture and marginal zone B-cell activation and distribution that occur in malaria. Investigation of the underlying

mechanisms of suppression of vaccine responses by malaria might reveal novel insights into the capabilities and limitations of human immunity, particularly in infants, and might enhance development of vaccines for malaria and other infectious diseases.

Expert commentary

We are entering the second wave of vaccination to reduce burden of childhood disease in resource-poor countries. The initial package of vaccines introduced by the WHO EPI in the 1970s and 1980s is now being supplemented by the *H. influenzae* type b conjugate, pneumococcal conjugate and rotavirus vaccines, and potentially affordable malaria, meningococcal conjugate and typhoid vaccines suitable for Africa are in advanced trials [103]. However, routine vaccine coverage remains incomplete and the determinants of vaccine effectiveness for preventing disease in routine use remain largely unknown.

For malaria, a challenge of global eradication has been set and many innovative strategies besides vaccines are being assessed. There has also been a resurgence of interest in the immunomodulatory effects of malaria. Unfortunately, the extraordinary opportunities afforded by malaria intervention and eradication studies have not yet been fully exploited to assess the immunomodulatory effects of *P. falciparum* infection in humans. If *P. falciparum* infection influences the effectiveness of new vaccines, it may have major implications for their cost-effectiveness and their mode of delivery. Understanding the effect of *P. falciparum* on vaccine effectiveness is therefore crucial.

It is sad to have to restate year on year that the nature of the protective immune response to malaria remains poorly understood. By better understanding the nature of the suppressive effect of *P. falciparum* on heterologous vaccine responses we may better understand whether immune responses to any parasite antigens are also likely to be suppressed. If so, such antigens may prove to be the 'Achilles heel' of the parasite and be targets for development of more effective vaccines.

Five-year view

In the next 5 years, malaria control and eradication efforts will make progress in reducing the global burden of malaria. This may produce unanticipated benefits in terms of other vaccine-preventable diseases, especially meningococcal and pneumococcal disease.

Results of the Phase III RTS,S malaria vaccine trials will reveal whether RTS,S is a good enough vaccine to enter routine use. Other new vaccines will also become available and decisions will have to be made as to which are most effective and which are affordable. None of these decisions can be made on a sound basis for malaria-endemic countries without considering the possible impact of malaria on the effectiveness of these vaccines and we hope that studies will be undertaken to answer this question definitively.

Acknowledgements

The authors would like to thank Brian Greenwood for his helpful comments on the initial manuscript.

Financial & competing interests disclosure

Aubrey J Cunningham holds a Medical Research Council clinical research training fellowship (G0701427). The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Key issues

- *Plasmodium falciparum* infection suppresses antibody responses to polysaccharide vaccine antigens
- Responses to protein antigen vaccines and some *P. falciparum* antigens may be suppressed, but the evidence is weaker
- There is no evidence that antibody responses to highly immunogenic combinations of routine vaccines are significantly suppressed by *P. falciparum*.
- The effect of *P. falciparum* on vaccine efficacy has never been formally assessed, even though this is more important than assessment of the antibody response. There is an urgent need to incorporate this assessment into efficacy trials of new vaccines in malaria-endemic countries
- The mechanisms of the protective immune response against malaria and the suppression of vaccine responses remain poorly explained but attention should perhaps be focused on cellular interactions in the marginal zone of the spleen.
- Better understanding of the effect of *P. falciparum* on vaccine responses may be crucial to develop and implement new vaccines for malaria-endemic countries

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Chapter 4. What are the consequences of malaria and HIV co-infection?

HIV and malaria co-infection

The material presented in this chapter was commissioned by Dr Tracey Lamb as a contribution for the textbook (in Press) *Immunity to Parasitic Infection* (Wiley-Blackwell). In this review we consider the scale of the problem of malaria and HIV co-infection, the many different facets of the interactions between the two pathogens with a particular emphasis on the immunological mechanisms involved in the pathogenesis of HIV which may be important in the reciprocal interactions with malaria, and finally, we describe the consequences of co-infection. The material presented here has been edited by Tracey Lamb, in her role as editor of the book, to ensure consistency and avoid duplication within the book, but no substantive changes in content have been made.

HIV and Malaria Co-infection

19

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19.1 The endemicity of HIV and malaria

The Human Immunodeficiency Virus (HIV) has spread as a global pandemic, affecting all continents. Malaria, caused by the five *Plasmodium* species known to infect man (*P. falciparum*, *vivax*, *ovale*, *malariae* and *knowlesi*) (see Chapter 3), has a more limited geographical distribution, defined by the availability of suitable mosquito vectors. The greatest burden of disease due to both HIV and malaria (predominantly *P. falciparum*) occurs in sub-Saharan Africa, although the geographical overlap of the highest risk areas for both infections is limited to central and southern Africa (Figure 19.1). Nevertheless, HIV infection persists lifelong, and *P. falciparum* can be both a chronic and a frequently recurring cause of infection, increasing the likelihood of HIV and malaria co-infections in the same individual.

Understanding the potential consequences of interaction between the two infections – including understanding their reciprocal effects on host immune responses to HIV and malaria, their combined effect on host responses to other infections, and the implications of HIV-malaria co-infection on transmission, diagnosis, treatment and prevention – is therefore of considerable importance

19.2 HIV infection

19.2.1 A short history of HIV infection

HIV is the cause of the acquired immunodeficiency syndrome (AIDS), a progressive loss of immune competence which results in susceptibility to opportunistic infection and cancer. HIV is caused by two related retroviruses – HIV-1 and HIV-2 – both of which evolved from viruses of non-human primates. It is likely that the ancestral HIV-1 virus entered the human population in the early

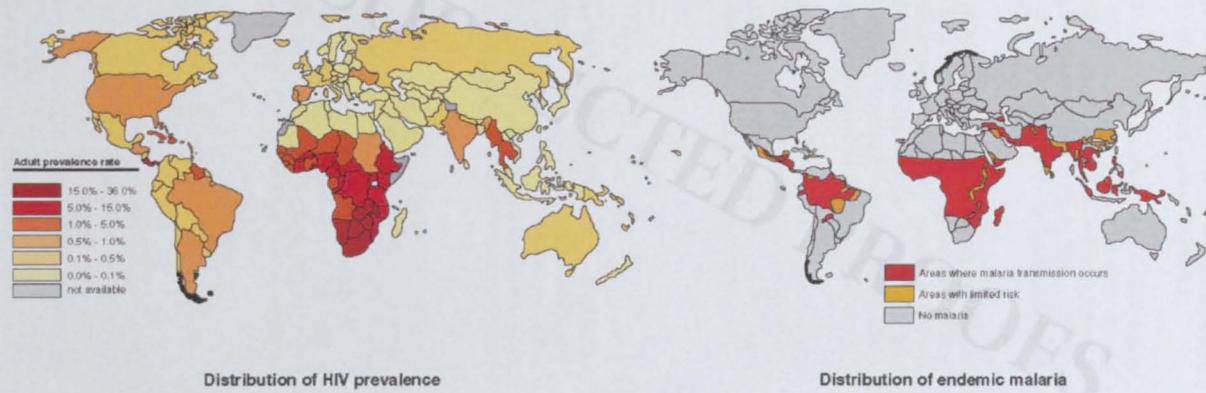


Figure 19.1 HIV/AIDS and malaria are highly endemic and there is wide geographical overlap in sub-Saharan Africa. Among the most severely affected countries are Cameroon, Central African Republic, Malawi, Mozambique and Zambia, where more than 90 per cent of the population is exposed to malaria, and HIV prevalence (among adults 15–49 years of age) is above ten per cent. Outside Africa, the two diseases overlap in certain at-risk groups in southeast Asia and South America, and in several Indian cities such as Mumbai. Reproduced by kind permission of The World Health Organization.

20th century in the Democratic Republic of Congo, but AIDS was not formally recognized until its description in 1981 in the United States of America.

HIV-1 is widespread, while the HIV-2 epidemic is focused around West Africa and its migrant populations; HIV-2 infection progresses to AIDS more slowly than HIV-1. Globally, more than 30 million people are estimated to be infected with HIV, two-thirds of these in the sub-Saharan African region. This compares with 85 per cent of 240 million global malaria cases, and nearly 90 per cent of the 860,000 malaria deaths occur in the African region.

Despite this broad overlap, the burden of co-infection is unevenly distributed within the African region, and even within individual countries, due to local variation in malaria transmission and the nature of the HIV epidemic. For example, malaria is often more common in rural areas, whereas HIV is often more prevalent around urban centres. Also, immunity to malaria is acquired in childhood, before the peak of HIV acquisition in adolescence and early adulthood.

The countries most severely affected by malaria-HIV co-infection include Malawi, Mozambique, the Central African Republic, Zambia and Zimbabwe. In this chapter, we limit discussion to interactions between HIV-1 and *P. falciparum*, because there is less evidence available for interactions involving HIV-2 or the other *Plasmodium* species.

19.2.2 The HIV virus

Before considering the interactions between HIV and malaria, it is useful to consider the challenges that each infection poses on its own for host immunity, and to take note of some similarities between the two infections in the ways that they have co-evolved with the host's immune system (Table 19.1). The immunology and pathology of malaria infections has been described in Chapter 3.

HIV is a retrovirus; each enveloped viral particle (virion) contains two copies of the viral RNA genome as well as the enzymes essential for the reverse transcription of this RNA into cDNA and its integration into the host genome. The surface of the HIV virion expresses glycoproteins, which mediate binding to, and fusion with, host target cells. The most important of these are gp120, which binds to CD4, as well as the co-receptors CCR5 and CXCR4 on the host cell membrane, and gp41 which is necessary for fusion of the membranes and viral entry.

19.2.3 Cellular sources of HIV virus

Activated CD4+ T cells, which express CCR5, are the principal target for HIV invasion and the main site of viral replication, producing the majority of HIV which is detectable in the plasma (the viral load). However, HIV is also able to infect other cell types, including naïve and memory CD4 T cells, monocytes, macrophages and dendritic cells. Infection of these other cell types makes relatively little contribution to plasma viral load, but it creates a latent (i.e.

Table 19.1 Some similarities between *Plasmodium* and HIV of relevance to host immunity.

| <i>Plasmodium</i> species | HIV |
|---|---|
| Evolutionary origin Zoonotic transmission from non-human primates. Host adaptation to invade red blood cells using specific sialic acid residues. | Zoonotic transmission from non-human primates. Adaptation to human host, e.g. HIV-1 Vpu protein antagonizes the innate defence protein tetherin (which would prevent release of viral progeny from cells). |
| Immune evasion at infection Rapid transit of sporozoites to the liver limits stimulation of humoral responses. | Rapid invasion of CD4+CCR5+ cells for active replication. Dendritic cells and B cells carry virus to activated CD4 T cells. Early establishment of latently infected cells. |
| Immune activation Repeated exposure/chronic persistent parasitaemia Immune activation causes increased endothelial adhesion molecule expression, sequestration of <i>P. falciparum</i> parasitized RBCs, protection from splenic clearance and enhanced replication. Immune activation may cause severe disease manifestations such as cerebral malaria. | Persists lifelong after infection. Translocation of microbial products across intestinal mucosa causes immune activation. Immune activation increases viral replication. Immune activation hastens progression to AIDS. |
| Evasion of the humoral immune response Antigenic variation. Mutation. Cryptic B cell epitopes. Alternative invasion pathways, e.g. Sialic acid dependent and independent invasion of RBC. Intracellular replication cycles. Polyclonal B cell activation diverts from specific response. (Hypergammaglobulinaemia). Aberrant memory B cell development. Latent infection (trophozoite) in the liver in (<i>P. vivax</i> and <i>P. ovale</i>) | Antigenic variation. Mutation. Cryptic B cell epitopes. Alternative invasion pathways e.g. CCR5 and CXCR4. Intracellular replication cycle. Polyclonal B cell activation diverts from specific response. (Hypergammaglobulinaemia). Aberrant memory B cell development. Latent intracellular infection of macrophages and resting T cells. |
| Evasion of the cell mediated immune response Sporozoites down-regulate MHC I on Kupffer cells in the liver. Altered peptide ligands interfere with T cell receptor interactions and activation. Intraerythrocytic replication cycle – RBCs lack MHC expression. Cryptic T cell epitopes. | Viral Nef (negative regulation factor) protein inhibits MHC I expression and presentation of peptides on MHC II. Mutation of T cell epitopes interferes with T cell response to wild type immunodominant epitopes. Viral reservoirs in privileged sites, e.g. central nervous system. |

non-replicating) virus reservoir, relatively protected from the immune response and from antiretroviral drugs.

Latent virus can begin to replicate following immunological activation of the host cell; for example, there are NF- κ B responsive elements in the viral long terminal repeat (LTR) region, which result in transcriptional activation of the

virus by NF- κ B. HIV replication in activated CD4+ T cells has a direct cytopathic effect, whereas latently infected cells may have a prolonged lifespan.

19.2.4 Transmission of HIV

HIV is transmitted between humans as cell-free or cell-associated virus in bodily fluids, principally semen, vaginal secretions and blood. It can also be transmitted from mother to child across the placenta, in the birth canal or in breast milk. The risk of transmission is closely related to the viral load in the blood and the integrity and state of inflammation of the mucosal (or placental) barriers. HIV can cross the mucosal barriers through interaction with dendritic cells or CCR5-expressing epithelial cells, with subsequent infection of CD4 T cells in the submucosa or lymphoid tissues. Individuals with mutations that limit CCR5 expression are resistant to HIV infection.

19.2.5 The immune response against HIV

When HIV is transmitted into a new host, it establishes infection in CD4+ T cells and there is a phase of rapid viral replication, high viral load, and depletion of CCR5+CD4+ T cells from the gut and, to a lesser extent, from the peripheral blood (Figure 19.2). This acute phase manifests as an influenza-like illness in 50–80 per cent of cases. An innate immune response is initiated by binding of uridine-rich HIV RNA to Toll-like receptors 7 and 8 and triggering of

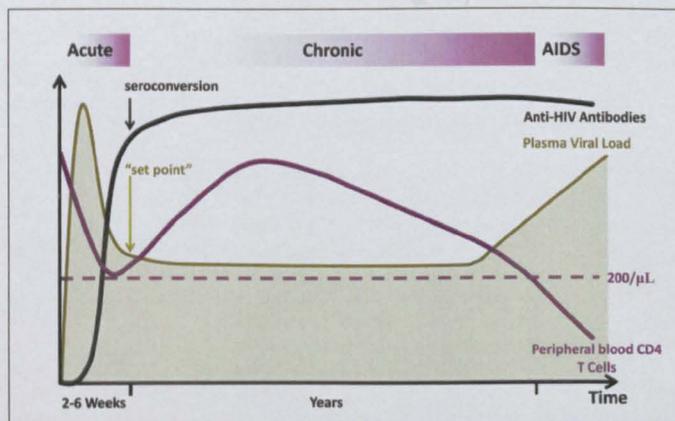


Figure 19.2 Three phases of HIV infection. Acute HIV infection is characterized by a high viral load and depletion of CD4+ T cells. Plasma viral load drops to a relatively constant 'set-point' as host immunity establishes imperfect control of viral replication, antibody seroconversion occurs, and there is recovery of CD4+ T cell numbers in the peripheral blood. In the chronic phase, there is gradual loss of functional immunity, most commonly measured by the depletion of CD4+ T-lymphocytes, and eventually a loss of control of viral replication. When the CD4+ T cell count falls below the threshold of 200 cells/ μ L, there is severe immunocompromise: AIDS.

interferon- α production by gp120 in monocytes and dendritic cells. It is accompanied by CD8+ T cell activation and production of antibodies to viral proteins (seroconversion). This cytotoxic CD8+ T cell response to acute infection limits, but does not eradicate, infection. Antibodies appear to be ineffective, because the humoral response is too slow to keep up with the rapid rate of mutation of HIV epitopes, such that neutralization of intact virions is poor *in vivo*.

After 2-6 weeks, viral load falls to a 'set-point' and peripheral blood CD4+ T cell numbers rebound. In the absence of treatment, a chronic phase of infection begins; there is now a gradual decline in CD4+ T cell numbers and a gradual increase in HIV viral load, with eventual progression to AIDS. In reality, there is great variation in the time taken to progress from acute infection to AIDS, with a median of 8-10 years (although in children, progression is often faster). Some individuals progress very rapidly, while others appear not to progress (long-term non-progressors). Long-term non-progression is strongly associated with genetic variants affecting the peptide binding groove of HLA class 1, indicating that interaction of HLA class 1 with viral peptide and the quality of its presentation to CD8+ T cells is a major determinant of the effectiveness of the host response.

The acute phase of infection (seroconversion illness) is rarely identified clinically, unless there is a particular reason to expect an individual is at risk of HIV infection. The beginning of the chronic phase is asymptomatic. This means that individuals may be infected with HIV for several years without knowing that they have the virus, during which time they may transmit the virus to others. Progression to advanced stages of HIV and AIDS is defined by the onset of recurrent, severe or opportunistic infections, malignancies (cancers) or pathological effects of HIV itself (wasting syndrome or encephalopathy), or by a fall in the CD4+ T cell count below a threshold value (<200 cells/ μ l for adults).

19.2.6 Drug therapy against HIV infection

Highly active anti-retroviral therapy (HAART) targets the replicating virus by inhibiting the reverse transcriptase and protease enzymes necessary for the production of infective virions, but it does not affect the virus in latently infected cells, making eradication of the virus (i.e. a cure) impossible. HAART usually comprises a combination of three drugs, two nucleoside reverse transcriptase inhibitors (NRTIs) and either a non-nucleoside reverse transcriptase inhibitor (NNRTI) or a protease inhibitor (PI). HAART can effectively suppress viral replication to undetectable levels, reverse immune activation and slowly allow recovery of the immune system.

In the chronic phase, treatment of HIV with HAART can prevent the progression to AIDS by suppressing viral replication and delaying the decline in peripheral blood CD4 count. Treatment of individuals after they have an AIDS defining-illness can also suppress viral load and restore peripheral blood CD4+ T cell counts over time, reversing the immunodeficiency.

Some consequences of HIV/AIDS, such as lung, kidney and neurological damage, are not reversible with restoration of the CD4 count and, unfortunately,

not all immunological dysfunction appears reversible. Notably, memory B cell numbers and function do not recover well after initiation of HAART, and this results in persisting defects in humoral immunity, which might only be avoided by initiation of HAART very early in the course of infection, before irreversible damage is done. Restoration of immune competency by HAART carries risk in those who have been very immunosuppressed, because the resurgent immune system may mount a vigorous and damaging response to covert pathogens, causing immune reconstitution inflammatory syndrome (IRIS).

Anti-retroviral drugs may also be used to prevent mother-to-child transmission (MTCT) of HIV and to prevent infection immediately following exposure to HIV (post-exposure prophylaxis). Individuals with low CD4+ T cell counts are often also given prophylactic treatment with trimethoprim-sulfamethoxazole, antibiotics which help to prevent bacterial and *Pneumocystis jirovecii* infection.

19.3 Immunopathogenesis of HIV

19.3.1 Immune activation in acute HIV infection

Although it is now accepted that HIV is the cause of AIDS, the mechanisms leading to immunosuppression remain the subject of some debate. Immunosuppression due to HIV is not a state of immunological quiescence, but quite the opposite. HIV promotes immune activation, and immune activation strongly predicts progression to AIDS (Figure 19.3).

Immune activation is identified by increased circulating pro-inflammatory mediators (chemokines and cytokines), polyclonal B cell activation, increased T cell proliferation and activated T cell phenotypes. Although only a small proportion (<1 per cent) of all CD4+ T cells are infected by HIV, the increased activation and turnover of T cells not only creates new target cells (expressing CCR5) for further viral replication, but may also ultimately exhaust the proliferative capacity of the T and B memory cell pools.

Activation and infection of important functional subsets of T cells, such as central memory CD4+ T cells, may be particularly damaging. Central memory T cells constitute a pool of precursors for effector memory T cells, and their depletion is strongly associated with development of AIDS. Similarly, depletion of polyfunctional T cells (able to secrete high levels of several cytokines) is correlated with increased viral load and progression to AIDS. Polyfunctional cytotoxic (CD8+) T cells are believed to be important in defence against HIV itself, but the capacity of these effector cells to limit HIV replication is impaired by chronic immune activation, which drives terminal differentiation towards exhausted cells, secreting lower levels of a more limited repertoire of cytokines.

Similar phenomena occur in B cells during HIV infection, with chronic stimulation leading to exhausted cell phenotypes and a reduction in their capacity to mount antibody responses to vaccination and infection. One mechanism by which chronic immune activation impairs T cell function is increased expression of the surface receptor Programmed Death-1 (PD-1) on T cells, and

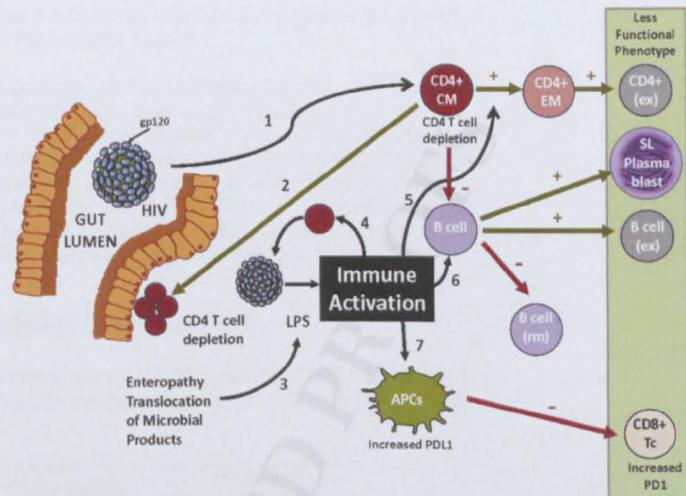


Figure 19.3 Immune activation is central to the pathogenesis of AIDS. 1. HIV preferentially infects activated CD4+ T cells expressing CCR5. 2. HIV is directly cytopathic to activated CD4+ T cells, resulting in acute depletion of CD4+ T cells in peripheral blood, particularly in the gut. This causes damage to the defensive mucosal barrier of the gut, allowing translocation of bacteria and their products into the circulation. 3. Bacterial products such as lipopolysaccharide (LPS) and HIV synergize to cause chronic immune activation. 4. Consequent activation of CD4+ T cells allows increased viral replication. It promotes depletion of central memory (CM) cells and differentiation of effector memory (EM) cells to less functional terminal effector and exhausted (ex) phenotypes. 5. Depletion of CD4+ T cells limits helper function for B-lymphocytes, and immune activation causes polyclonal stimulation of mature naive B-lymphocytes, enhanced differentiation to short-lived (SL) plasmablasts and exhausted (ex) phenotypes, while irreversibly depleting resting memory (rm) B cell pools. 6. Chronic immune activation enhances the expression of Programmed Death-1 (PD-1) on CD8+ T cells and of its ligand PD-L1 on antigen presenting cells (APCs), which reduces the proliferation and enhances apoptosis of HIV-specific CD8+ T cells.

of its ligand, PD-L1, on antigen presenting cells. Ligation of PD-1 to PD-L1 reduces survival, proliferation and cytokine production of CD8+ and CD4+ T cells. Chronic immune activation also disrupts the architecture of lymphoid tissues (e.g. thymus and lymph nodes), preventing their orchestration of normal immune responses.

19.3.2 Chronic immune activation in HIV infection

The chronic immune activation that occurs during HIV infection is not attributable exclusively to the virus. It is currently believed that immune activation is largely driven by a loss of functional integrity of the gastrointestinal mucosal barrier, which allows translocation of microbial products such as lipopolysaccharide (LPS) from the gut lumen into the circulation. These microbial products are able to stimulate the innate immune response through

Toll-like receptor signalling. Furthermore, HIV infection increases the sensitivity of macrophages to Toll-like receptor ligands.

HIV infection causes inflammation of the gastrointestinal tract, with destruction of the epithelial surface and death of enterocytes. The majority of lymphocytes in the body are located in the gastrointestinal tract, and there is a dramatic depletion of CD4+ T cells from this site during acute HIV infection. This continues in the chronic phase of infection, and it is of much greater magnitude than the CD4+ T cell depletion from peripheral blood. The occurrence of opportunistic infections in HIV-infected individuals provides another stimulus to immune activation, viral replication and disease progression.

19.4 Interactions between malaria and HIV

There are many possible interactions between malaria and HIV (summarized in Figure 19.4), but good quality evidence is available to support or refute only

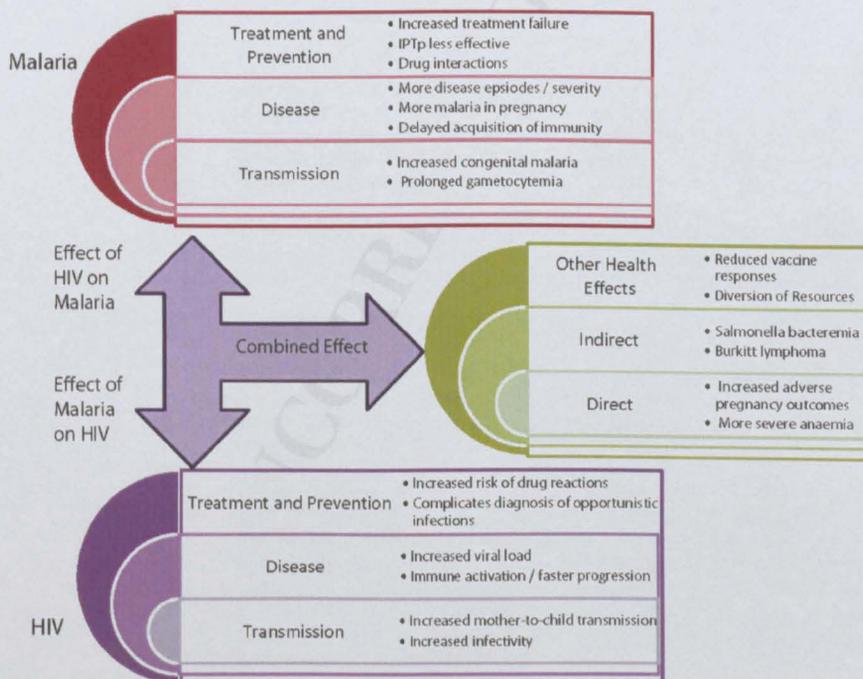


Figure 19.4 HIV and malaria have reciprocal effects on transmission, disease, treatment and prevention. The combination of HIV and malaria co-infection may have additional implications for susceptibility to other infections and poor health. Of the many potential consequences shown here, as yet only a few (shown in bold type) are supported by reliable immunological or epidemiological data.

some of these interactions. In this chapter, we will consider the likely immunological explanations for the best-established interactions between malaria and HIV, and indicate areas of potential concern that warrant further research.

19.4.1 Effect of HIV infection on the incidence and severity of clinical malaria

Assessment of the effect of HIV/AIDS on the incidence of clinical malaria, and the likelihood of severe disease, can only be meaningfully interpreted when the intensity of malaria transmission and the prior acquisition of immunity to severe disease are taken into account. As the exact nature of 'immunity' to malaria remains poorly defined, quantifying any effect immunologically is almost impossible, and thus we have to rely on inference from epidemiological studies.

Even this, however, is not straightforward. Even within a defined geographical area, individuals may be exposed to different intensities of transmission of malaria and have different levels of anti-malarial immunity, regardless of their HIV status. Those who go on to acquire HIV may not necessarily be similar (genetically, immunologically, or in terms of malaria exposure) to those who remain HIV-negative. In addition, the diagnosis of clinical malaria can be difficult; the presence of parasitaemia and compatible clinical symptoms does not necessarily mean that malaria is the cause of the illness. Asymptomatic parasitaemia is common in clinically-immune individuals, and individuals with HIV are more likely to have other infections, which may be misdiagnosed as malaria if parasitaemia is present.

Thus, although many studies have claimed that HIV-infected individuals are more susceptible to clinical malaria and to severe malaria, establishing a link between HIV/AIDS and incidence and severity of malaria is problematic.

To date there are no robust data to indicate whether or not acquisition of clinical (anti-disease) or anti-parasitic immunity to malaria are impaired, differentially affected or unaffected by HIV infection. This is mainly because the very high mortality of children with vertically acquired HIV, in settings where there is also high-intensity transmission of malaria, has prevented longitudinal studies of the effect of HIV infection on acquired immunity to malaria.

However, several studies in adults who have acquired immunity to malaria prior to becoming HIV infected suggest that horizontally-acquired HIV appears to have a relatively modest effect on pre-existing anti-malarial immunity. Although the risk of clinical malaria increases several-fold, and is inversely related to the CD4+ count, the susceptibility to malaria is much less dramatic than susceptibility to bacterial infections. The risk of severe malaria does not appear to be significantly increased, indicating that HIV has relatively little effect on established anti-malarial immunity.

Importantly, however, this is not the case for adults with HIV who do not have pre-existing immunity to malaria. In settings where malaria occurs sporadically or in epidemics, the risk of severe malaria – including coma, acidosis and severe

anaemia – is significantly higher in HIV-infected adults than in those without HIV. Some severe manifestations of malaria, such as cerebral malaria, are frequently considered to be due to immunopathology, so it is an interesting observation that HIV-related immunosuppression exacerbates, rather than prevents, severe malaria. This may be reconciled by remembering that immune activation and dysregulation – rather than silencing of the immune system – is central to the pathogenesis of AIDS, and perhaps supports the concept that dysregulation is also important in the pathogenesis of severe malaria.

There has been very little research on whether HIV may increase malaria transmission, but it is conceivable that clearance of gametocytes from the blood is impaired in HIV-infected individuals, resulting in a prolonged 'carrier' state. This may be of particular relevance as a factor hindering global efforts to eliminate and eradicate malaria.

19.4.2 Effect of malaria on HIV viral load and progression

In contrast to the paucity of robust information on the effects of HIV on malaria incidence and severity, there are numerous studies demonstrating an effect of malaria on HIV infection and progression. Longitudinal studies of intercurrent malaria infections in HIV-infected individuals indicate that acute clinical malaria increases plasma viral load. This is presumed to result from immune activation by malaria, which would increase viral replication. The increase in viral load is relatively modest (less than a tenfold increase), and it resolves with anti-malarial treatment.

This is not unique to malaria, since other pathogens have also been reported to increase viral load in a similar manner (e.g. tuberculosis, herpes simplex, schistosomiasis). However, it is the possibility of frequent episodes of malaria, and of persistent asymptomatic parasitaemia affecting a large proportion of the population, that distinguish malaria as, potentially, an important cause of elevation of HIV viral load and, thus, progression to AIDS. Frequent episodes of malaria could thus hasten progression to AIDS and increase mortality. In addition, HIV plasma viral load is a major determinant of the risk of HIV transmission between individuals, and so transient increases in viral load might increase the spread of HIV.

Unfortunately, there are currently insufficient data from longitudinal studies to know if malaria is really a major force driving HIV transmission and morbidity in sub-Saharan Africa, and studies are urgently needed to address this issue. Although the immunological arguments are compelling, there are many reasons why these may not have the predicted effects. For example, adults with symptomatic malaria may be less likely to engage in sexual activity and would therefore be less likely to transmit HIV during acute malaria episodes.

19.4.3 Interactions between malaria and HIV in pregnant women

In settings with stable, high-level malaria transmission, adults are generally immune to clinical malaria and have lower levels of parasitaemia when they

become infected. This immunity is strikingly impaired by pregnancy, with a susceptibility to clinical malaria and dense infection of the placenta with *P. falciparum* being a feature of (particularly first) pregnancies. Pregnancy-associated malaria (PAM) is associated with poor outcomes for the mother (severe anaemia) and the foetus (reduced birth weight, increased neonatal mortality).

In subsequent pregnancies, the risk of PAM and adverse maternal and neonatal outcomes decreases. The most compelling explanation for this phenomenon is that PAM is caused by a subset of *P. falciparum* parasites which express variant surface antigens (VSA) on the surface of infected erythrocytes, enabling their binding to chondroitin sulphate A (CSA) on placental trophoblast and, thus, sequestration of infected erythrocytes in the placenta.

In non-pregnant individuals, acquisition of protective humoral immunity to malaria is likely to be due to acquisition of specific IgG against the predominantly expressed VSAs. CSA is rarely used as a receptor for *P. falciparum* adhesion in non-pregnant individuals, so there is no stimulus for an antibody response against the CSA-binding VSAs. In pregnancy, there is an opportunity for selection of parasite clones able to adhere to CSA, which is highly expressed in the placental intervillous space. Since there is no pre-existing immunity to CSA-binding VSAs, these clones can adhere, replicate and cause symptomatic infection. Pregnant women acquire increasing levels of antibodies to these clones during sequential pregnancies, which correlates with the acquisition of immunity to PAM.

HIV exacerbates the effects of malaria in pregnancy. Pregnant HIV-infected women suffer more frequent and more severe attacks of malaria, develop more severe anaemia and have worse neonatal outcomes than HIV-negative women. HIV is, in itself, a cause of adverse pregnancy outcomes; for example, pregnant women with HIV are particularly vulnerable to opportunistic infections. Furthermore, the remarkable perturbation of B cell function caused by HIV impairs the acquisition of protective antibodies against the pregnancy associated VSAs, and thus reduces the protective immunity that is acquired during sequential pregnancies. This means that the vulnerability to pregnancy associated malaria seen in first pregnancies also persists in subsequent pregnancies in HIV-infected women.

Maternal HIV infection also increases the risk of congenital malaria infection of the newborn, i.e. blood-stage *P. falciparum* transmitted directly across the placenta from mother to foetus. The increased risk of congenital malaria is likely a direct consequence of the increased risk of placental infection in women with HIV.

In the absence of any intervention, MTCT occurs in 35 per cent of cases. Approximately 20 per cent of these infections occur *in utero*, ~40 per cent occur during childbirth and the remainder occur during breast-feeding. There are several reasons to think that malaria might increase MTCT of HIV. First, malaria increases HIV plasma viral load and plasma viral load is an independent predictor of MTCT. Second, placental malaria causes inflammation in the placenta, which may locally increase HIV replication and facilitate passage of

HIV across the placental barrier, increasing *in utero* transmission. It will be important for future studies to resolve the relationship between pregnancy-associated malaria, placental malaria and MTCT of HIV.

19.5 Effect of co-infection on treatment of HIV and malaria infections

There is considerable potential for co-infection to influence the treatment of malaria and HIV, both through reciprocal effects on the effectiveness of treatment and also the interaction of drugs used to treat each infection. Drug interactions can be extremely complex and difficult to predict, influencing pharmacokinetics (liberation, absorption, distribution, metabolism and elimination of the drug), pharmacodynamics (effects of the drugs on the body, HIV or the malaria parasites) and, ultimately, the effectiveness of drug treatment and emergence of resistance. Whether clinical malaria or asymptomatic parasitaemia impairs the treatment of HIV is currently unknown, but it is conceivable that, during episodes of severe malaria, there may be reduced compliance with antiretroviral drugs and changes in host factors which determine pharmacokinetics and pharmacodynamics.

The effectiveness of drug treatment of malaria is determined in part by host immunity. Adults who have acquired protective immunity, through repeated exposure to malaria, have a lower risk of treatment failure than do children in the same transmission setting, and children with higher concentrations of IgG to some parasite antigens have better treatment responses than those with lower levels. In addition, it is generally true that treatment of any infection is more likely to fail in an immunocompromised host.

If the major effect of HIV is to impair the acquisition of immunity to malaria, then it would be predicted that this would also be associated with increased rates of treatment failure in older children and adults. There is some evidence from clinical studies that HIV-infected individuals are less likely to clear their malaria infections completely after treatment, leading to recrudescence of infection after treatment and being more prone to rapid reinfection. There is also evidence that HIV infection may diminish the effectiveness of intermittent preventive treatment of malaria in pregnancy (IPTp), a strategy of providing intermittent treatment doses of sulfadoxine-pyrimethamine to pregnant women in order to eliminate subclinical malaria infections and protect against PAM.

Interactions between anti-malarial and anti-retroviral drugs have been predicted on theoretical grounds and from *in vitro* studies, but convincing evidence of clinical relevance from studies in humans is lacking. Interestingly, some of the most widely used HIV PIs have been described to have anti-malarial activity, suggesting that they may prevent or reduce the severity of malaria in HIV patients receiving PI-containing HAART. Similarly, *in vitro* studies indicate that anti-malarial drugs such as mefloquine synergize with PIs to enhance their antiretroviral activity. While such interactions might be beneficial, both anti-malarial drugs and anti-retroviral drugs have undesirable side effects, and thus further study is needed to establish that these strategies would be safe.

19.6 Combined effects of HIV and malaria on susceptibility to other diseases

Co-infection with HIV/AIDS and malaria may have cumulative effects, resulting in increased susceptibility to other diseases. Notable examples are susceptibility to non-typhoid *Salmonella* (NTS) bacteraemia, B cell lymphoma and vaccine-preventable diseases.

19.6.1 *Salmonella* bacteraemia

Malaria and HIV are independently associated with an increased risk of invasive infection with NTS, which is itself one of the most common invasive bacterial infections in children in sub-Saharan Africa. Antibodies are an important component of protection against NTS bacteraemia. Young children often lack the capacity to make antibodies against encapsulated organisms such as NTS, and dysregulated humoral immunity as a result of HIV infection or malaria infection further contributes to susceptibility. If different mechanisms are involved in the susceptibility caused by malaria and HIV respectively, then their combined effect on susceptibility to NTS may be dramatic.

19.6.2 Burkitt's B cell lymphoma

Malaria and HIV are independently associated with an increased risk of the B cell malignancy, Burkitt lymphoma. Endemic Burkitt lymphoma is a childhood cancer that occurs in malarious regions of sub-Saharan Africa, and its aetiology appears dependent on the co-occurrence of repeated exposure to malaria and infection with the B-lymphotropic Epstein-Barr virus (EBV). Children repeatedly exposed to malaria have reduced control, and thus greater replication, of EBV, presumably as a consequence of polyclonal B cell stimulation by malaria antigens and suppression of T cell-mediated immunity to EBV.

EBV is an oncogenic virus which can trigger the translocation of the proto-oncogene MYC into a IgG locus within infected B cells, resulting in over-expression of the transcription factor c-myc, enhanced cellular proliferation and reduced apoptosis. HIV-related Burkitt lymphoma is less often associated with EBV (only about 40 per cent of cases), but in those EBV-positive cases it is believed that impairment of cell mediated immunity by HIV allows reactivation of EBV. While HIV and malaria are both important risk factors for Burkitt lymphoma, whether HIV increases susceptibility to endemic Burkitt lymphoma, or malaria increases susceptibility to HIV-related Burkitt lymphoma, remains to be established.

19.6.3 Vaccination

Vaccination against infectious diseases has been one of the most successful ways to reduce their global burden. Childhood vaccines recommended universally by the World Health Organization are BCG (except in those with

confirmed HIV infection), hepatitis B, polio, diphtheria, tetanus and pertussis, *Haemophilus Influenzae* type B, pneumococcal conjugate, rotavirus, measles and humanpapilloma virus.

Vaccines protect from infectious diseases both at an individual and a population level. If the number of people susceptible to an infectious agent falls below a critical threshold, then the agent will not be able to spread within the population and can potentially be eradicated. Individuals who are unvaccinated, or who did not achieve a protective response to vaccination, are protected by 'herd immunity'; when the majority of individuals in the population have been vaccinated, the chance of a susceptible individual coming into contact with someone who has not been vaccinated is very small. So long as herd immunity is maintained, immunocompromized individuals are afforded some protection against the vaccine-preventable diseases.

However, herd immunity can be eroded rapidly by disruption of vaccine distribution and uptake, or by increased rates of vaccine failure. Dysregulation of B-lymphocyte function and CD4+ T-lymphocyte help, and functional impairment of CD8+ T cells in HIV-infected individuals, impairs responses to most of the vaccines studied (including polysaccharide, protein subunit and live attenuated vaccines) and leads to loss of pre-existing vaccine mediated immunity, which cannot be restored by HAART alone. Although revaccination after starting HAART generally allows effective immune responses to be mounted, these may wane more rapidly than in non-HIV infected individuals.

Children with vertically-acquired HIV may only achieve the capacity to mount normal vaccine responses if they are identified and commenced on HAART early in life, allowing preservation of their memory B cell pool. Delayed anti-retroviral therapy may cause long-lasting impairment of their ability to respond to vaccination, even after commencing HAART. In areas where the HIV prevalence is high, it is likely that a large proportion of the population is susceptible to vaccine preventable diseases, and conditions are created where outbreaks may occur.

Malaria has also been reported to impair vaccine responses, most notably for T cell-independent polysaccharide antigens and possibly polysaccharide-protein conjugate vaccines administered during episodes of clinical malaria. The effect of malaria on T-dependent vaccine responses, those to protein vaccines like tetanus, and to live vaccines like measles, appears to be minimal. The mechanisms by which malaria impairs vaccine responses are uncertain, but they may include polyclonal B cell activation and disruption of normal splenic function. Although it has never been formally evaluated, the potential for erosion of the population benefits of vaccination in areas of co-existing high malaria transmission and high HIV prevalence is very worrying.

19.7 Malaria and HIV vaccines

Strenuous efforts are being made to develop vaccines against malaria and HIV, but vaccines which provide complete protection from infection remain a distant prospect. If realized, these vaccines would be of huge public health

significance, but even partially effective vaccines may have a valuable role in reducing malaria mortality and stemming the HIV epidemic, when implemented alongside other preventive measures.

Imperfect vaccines, which might reduce the likelihood of clinical and severe malaria without providing sterilizing immunity, or reduce the rate of progression of HIV without preventing infection, are in development, and these may be the first generations of vaccines against these diseases to reach widespread use. However, any host factors which erode their effectiveness when used operationally (i.e. outside of clinical trials) may drastically reduce their cost-effectiveness. In other words, if HIV substantially impairs the effectiveness of a malaria vaccine, then the money spent on the vaccine might be better spent on other measures to protect from malaria in populations with a high prevalence of HIV.

19.7.1 Malaria vaccine candidate RTS,S

The RTS,S vaccine is the malaria vaccine candidate closest to achieving approval for widespread operational use (see Chapter 25). The efficacy of RTS,S in HIV-infected individuals is unknown, but there are reasons to be pessimistic: the immunogenicity of the hepatitis B surface antigen (HBsAg, which is a component of the vaccine) is diminished by HIV; the generation and longevity of antibody responses to the circumsporozoite protein are likely to be impaired; and chronic immune activation is likely to diminish the quality, quantity and durability of the polyfunctional T cell response. In order to maximize any benefit from RTS,S it may need to be employed in conjunction with measures to reduce MTCT of HIV and treat infected adults.

19.7.2 Vaccination against HIV infection

An effective vaccine to prevent HIV infection is not yet available, but much research effort has justifiably been directed into trying to identify one. HIV vaccine development has been hampered by failure of killed virus or protein subunits to elicit broadly neutralizing antibodies – which appear to be necessary to prevent infection – or to generate effective CD8+ T lymphocyte responses, which might help to prevent infection and limit viral replication. This is a consequence of the rapid mutation of key viral molecules such as gp120, coupled with conformational protection of conserved regions until the moment of ligation with target cells.

Mutation of HIV CD8+ T cell epitopes also occurs rapidly, which necessitates that vaccine strategies seeking to protect through cell-mediated immunity should elicit a broad range of CD8+ T cell responses in order to be protective. However, cell-mediated responses would probably not prevent transmission of HIV and establishment of infection, even if they could attenuate viral replication and progression to AIDS. Thus, vaccines aiming to elicit a cell-mediated immune response might reduce the 'set point' of plasma viral load (essentially the equilibrium between replication and control) and preserve CD4 cell counts, and these are being actively pursued.

The most successful clinical trial to date—using a six-dose prime-boost strategy based on gp120—only showed a slight (less than one-third) reduction in risk of infection with HIV, and no effect on viral load or CD4 count in infected subjects. Given the inherent difficulties in the ability of the human immune system to mount an effective response to protect against or control HIV, it is likely that anything that perturbs the ability to mount an optimal immune response will threaten the success of such vaccination.

There is, as yet, little evidence on which to evaluate the effect of malaria on novel vaccine technologies, so we cannot predict for certain whether malaria will hamper efforts to control HIV by vaccination, but the co-incidence of the two infections is likely to be another hurdle to be overcome. Since individuals with acute febrile illness, such as malaria, are usually not vaccinated until recovery, and there might be major operational difficulties in administering a complicated vaccine schedule in an area where there is both high malaria transmission and a high incidence of clinical malaria.

19.8 Summary

The combination of HIV and malaria has the potential to be very detrimental to health, but there is surprisingly little evidence that this is, in fact, the case. Although there are broad geographical overlaps between the greatest burdens of *P. falciparum* infection and HIV infection, the overlap at an individual level may be less dramatic. One situation where there is clear evidence that HIV and malaria have a detrimental interaction is in pregnancy, and there are obvious virological, parasitological and immunological reasons why this should be the case. Formulating safe and effective strategies to limit HIV-malaria co-infections in pregnant women is, thus, a priority.

HIV and malaria have some common effects on the host immune response, and some similar mechanisms of immune evasion, probably explaining why they represent two of the most intractable human pathogens. Lessons learned from one infection may be instructive in the future to guide strategies for tackling the other, and there is a clear pressing need for more research on the adverse effects and best management of co-infection.

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Chapter 5. Does heme oxygenase-1 play a role in the increased susceptibility to Salmonella of malaria infected mice?

Malaria impairs resistance to Salmonella through heme- and heme oxygenase-dependent dysfunctional granulocyte mobilization.

The material presented in this chapter is an uncommissioned, fully peer-reviewed, published article, in which we describe the mechanisms accounting for increased bacterial replication and accelerated mortality in mice co-infected with the rodent malaria parasite *P. yoelii* 17X Non-lethal, and *S. typhimurium*. Although I performed most of the laboratory work myself, some of the experimental work presented in Figures 3h, 4b, and 5, was undertaken with assistance from Dr J. Brian de Souza.

Cover sheet for each 'research paper' included in a research thesis

1. For a 'research paper' already published
 - 1.1. Where was the work published? Nature Medicine
 - 1.2. When was the work published? December 2011
 - 1.2.1. If the work was published prior to registration for your research degree, give a brief rationale for its inclusion

 - 1.3. Was the work subject to academic peer review?
_____ Yes _____
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 - 2.1. Where is the work intended to be published?

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 - 2.3. Stage of publication – Not yet submitted/Submitted/Undergoing revision from peer reviewers' comments/In press

3. For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)

I had the idea for the research, planned the project, conducted the laboratory work, analysed and interpreted the results and wrote the manuscript.

Candidate's signature _____

Supervisor or senior author's signature to confirm role as stated in (3)

Malaria impairs resistance to *Salmonella* through heme- and heme oxygenase-dependent dysfunctional granulocyte mobilization

Aubrey J Cunningham¹, J Brian de Souza^{1,2}, Michael Walther^{3,4} & Eleanor M Riley¹

In sub-Saharan Africa, invasive nontyphoid *Salmonella* (NTS) infection is a common and often fatal complication of *Plasmodium falciparum* infection. Induction of heme oxygenase-1 (HO-1) mediates tolerance to the cytotoxic effects of heme during malarial hemolysis but might impair resistance to NTS by limiting production of bactericidal reactive oxygen species. We show that co-infection of mice with *Plasmodium yoelii* 17XNL (Py17XNL) and *Salmonella enterica* serovar Typhimurium 12023 (*Salmonella typhimurium*) causes acute, fatal bacteremia with high bacterial load, features reproduced by phenylhydrazine-induced hemolysis or hemin administration. *S. typhimurium* localized predominantly in granulocytes. Py17XNL, phenylhydrazine and hemin caused premature mobilization of granulocytes from bone marrow with a quantitative defect in the oxidative burst. Inhibition of HO by tin protoporphyrin abrogated the impairment of resistance to *S. typhimurium* by hemolysis. Thus, a mechanism of tolerance to one infection, malaria, impairs resistance to another, NTS. Furthermore, HO inhibitors may be useful adjunctive therapy for NTS infection in the context of hemolysis.

NTS bacteremia is the most common cause of community-acquired bacteremia in many parts of sub-Saharan Africa¹, and NTS co-infection has been associated with high malaria mortality². The association of NTS infection with hemolysis is well established in humans with malaria (especially severe malarial anemia)^{3,4} and sickle cell disease⁵ and in mice with hemolysis resulting from rodent malaria infection^{6–8}, treatment with phenylhydrazine or erythrocyte-targeting antibodies, or red blood cell enzyme defects^{9–11}. It has been assumed that hemolysis-induced macrophage dysfunction is responsible for this phenomenon, although there is no direct evidence that macrophages are the primary refuge of NTS *in vivo* in the context of hemolysis^{7,8,10}.

Hemolysis results in liberation of heme, leading to expression of the inducible isoform of HO (HO-1)¹², which degrades heme to biliverdin, carbon monoxide and iron¹³. Heme is pro-oxidant, induces neutrophil migration and activates the neutrophil oxidative burst^{14–16}, but HO-1 (and its products) are essential in cytoprotection (reviewed in ref. 17), as evidenced by the severe susceptibility to oxidative stress of mice and humans with HO-1 deficiency^{18–20}. HO-1 induction has been shown to protect against infectious, inflammatory and hypoxic-ischemic insults in mice (reviewed in ref. 21) and has been linked to modulation of malarial pathogenesis²² and sickle cell disease²³. Recently, in mice, induction of HO-1 has been proposed as a tolerance mechanism in severe malaria^{24–26} and polymicrobial sepsis²⁷: HO-1 lessens heme-mediated tissue damage and enhances survival

without reducing pathogen load. A key cytoprotective effect of HO-1, and thus a likely explanation for its ability to confer tolerance, is its ability to limit the production of damaging reactive oxygen species (ROS, reviewed in ref. 17). However, ROS are crucial for resistance to certain pathogens, including *Salmonella* species²⁸, and this raises the possibility that tolerance of one pathogen may sometimes come at the price of loss of resistance to another. We hypothesized that liberation of heme by intravascular hemolysis may lead to HO-1 induction and impairment of resistance to NTS, with increased bacterial replication and mortality.

RESULTS

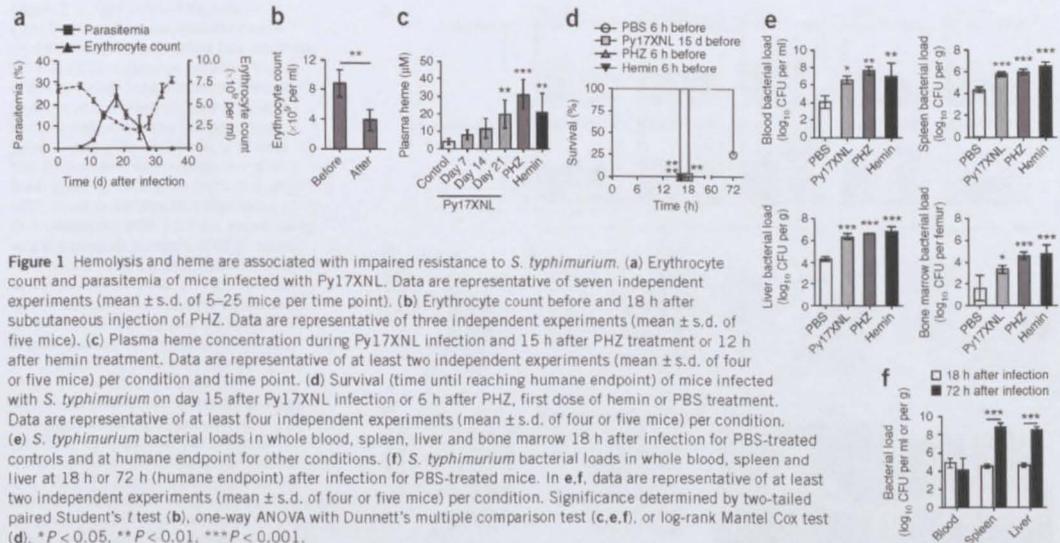
Hemolysis and heme impair resistance to *S. typhimurium* bacteremia

To determine whether heme liberated by hemolysis impairs resistance to NTS infection, we compared survival and bacterial loads after intraperitoneal infection of C57BL/6 mice with GFP-expressing *S. typhimurium* with or without preceding Py17XNL co-infection, phenylhydrazine (PHZ) or hemin treatment. Py17XNL infection of C57BL/6 mice caused a self-resolving infection; parasitemia peaked at 20–30% and was accompanied by progressive hemolytic anemia (Fig. 1a). By contrast, PHZ treatment caused acute hemolysis (Fig. 1b). In both cases, plasma heme concentrations were markedly increased and similar to concentrations achieved 12 h after injection of hemin (Fig. 1c), but without depletion of haptoglobin or hemopexin

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Received 23 March 2010; accepted 8 November 2011; published online 18 December 2011; doi:10.1038/nm.2601





(Supplementary Fig. 1a,b). Survival of Salmonella-infected mice was dramatically shortened by prior Py17XNL infection, PHZ or hemin treatment (Fig. 1d) and was significantly shorter in PHZ- and hemin-treated mice (16 h) than in Py17XNL co-infected mice (18 h) (*P* < 0.01, log-rank Mantel Cox test). PHZ, hemin and Py17XNL did not cause any mortality in the absence of *S. typhimurium* infection (data not shown).

Decreased survival of malaria-infected, PHZ-treated or hemin-treated mice after *S. typhimurium* infection was accompanied by higher bacterial loads in whole blood, spleen, liver and bone marrow (Fig. 1e), and bacteremia was much more pronounced; immediately before death (that is, 16–18 h after infection in Py17XNL-infected, PHZ- or hemin-treated mice and 72 h after infection in control mice), bacterial loads in the blood of infected or treated mice were proportionately higher, and bacterial loads in liver and spleen proportionately lower (Fig. 1e), than in control mice (Fig. 1f).

S. typhimurium localize in granulocytes following hemolysis

By flow cytometry, we identified GFP⁺ (*S. typhimurium*-containing) cells in blood, spleen and bone marrow. In the blood of Py17XNL-infected mice, and of PHZ- or hemin-treated mice, just before death, we found salmonellae predominantly in Gr-1^{hi} cells (Fig. 2a), and they were enriched in this cell population compared to the bacteria in control (PBS-treated) mice (Fig. 2b). The proportion of all GFP⁺Gr-1^{hi} cells in blood, spleen and bone marrow (Fig. 2c) correlated with the bacterial load determined by culture (Fig. 1e,f). We identified the Gr-1^{hi} cells as granulocytes (Ly6G⁺F4/80⁺CD115⁻; Supplementary Fig. 2a,b). Almost all GFP⁺ cells were CD115⁻ (Fig. 2d); moreover, *S. typhimurium* infection caused a higher proportion of Gr-1^{hi}CD115⁻ blood leukocytes (Fig. 2e), suggesting that immature granulocytes are mobilized from the bone marrow to the peripheral blood during infection²⁹. In support of this, blood films from Py17XNL-infected and PHZ- or hemin-treated mice 18 h after *S. typhimurium* infection showed numerous neutrophils containing *S. typhimurium*, and many of these neutrophils had immature nuclear morphology (Fig. 2f).

In contrast, neutrophils from PBS-treated mice showed mature nuclear morphology and did not contain *S. typhimurium*.

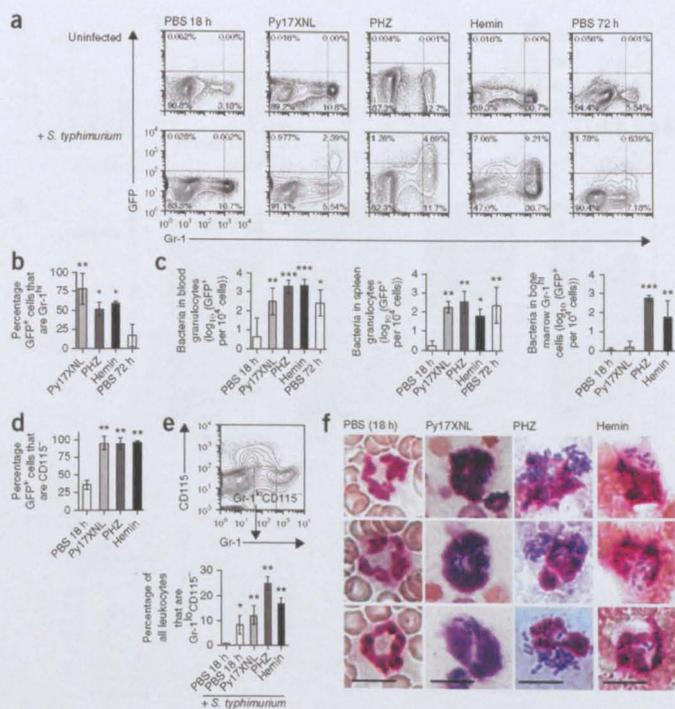
The accumulation of GFP⁺ bacteria in granulocytes was not simply due to failure of bacterial uptake by monocytes and macrophages, as the proportion of GFP⁺ cells in the spleen that were either monocytes or macrophages (F4/80^{hi}CD11b^{hi} and F4/80^{hi}CD11b^{lo}, respectively) did not differ between Py17XNL-infected or PHZ- or hemin-treated mice and those treated with PBS alone (Supplementary Fig. 2c).

Py17XNL inhibits granulocyte oxidative burst and bacterial killing

As there is no obvious defect in uptake of *S. typhimurium* by macrophages and monocytes after hemolysis or hemin treatment, accumulation of *S. typhimurium* in blood granulocytes may result from impaired bacterial killing or a more permissive intracellular environment for bacterial replication. To investigate these possibilities, we isolated CD11b⁺ cells from blood of Py17XNL-infected and uninfected mice and compared their ability to phagocytose and kill *S. typhimurium*. Neither flow cytometric analysis of GFP⁺ cells nor quantitative culture (in a gentamicin protection assay) revealed any differences in the rates of phagocytosis of *S. typhimurium* between neutrophils or monocytes or between cells from malaria-infected or uninfected mice (Fig. 3a,b); we confirmed the intracellular location of GFP⁺ bacteria by confocal microscopy (Supplementary Fig. 3a,b). However, when we lysed cells after 2 h in the gentamicin protection assay and enumerated live bacteria by culture, the live bacterial recovery from cells from Py17XNL-infected mice was significantly higher than from control mice (Fig. 3b), indicating considerable impairment of intracellular killing of *S. typhimurium* by cells from Py17XNL-infected mice.

As HO-1 reduces the production of ROS^{30–33}, and as phagocyte NADPH oxidase is essential for resistance to *S. typhimurium* early in infection²⁸, we investigated whether Py17XNL infection impairs the granulocyte oxidative burst. Using oxidation of dihydrorhodamine to its fluorescent derivative rhodamine as a readout for oxidative burst³⁴, we observed progressive suppression of the phorbol myristate acetate (PMA)-induced oxidative burst of blood granulocytes during

Figure 2 *S. typhimurium* localize in granulocytes after hemolysis and hemin treatment. **(a)** Representative flow cytometry plots of blood leukocytes collected from GFP-expressing *S. typhimurium*-infected (bottom) and uninfected (top) mice at the humane endpoint or the indicated time point. Data are representative of at least four independent experiments ($n = 4$ or 5 mice) per condition. **(b)** Proportion of all GFP⁺ blood leukocytes with high levels of Gr-1 expression (PBS 18 h not shown owing very low absolute numbers of GFP⁺ cells). **(c)** Infected mature granulocytes (GFP⁺Gr-1^{hi}) as a proportion of all Gr-1^{hi} cells in blood, spleen and bone marrow. **(d)** Proportion of all GFP⁺ cells in blood that are CD115⁻. In **b-d**, data are representative of at least two independent experiments (mean \pm s.d. of four or five mice) per condition. **(e)** Representative flow cytometry analysis defining immature granulocytes as Gr-1^{lo}CD115⁻ (top) and quantification of the proportion of blood leukocytes that are Gr-1^{lo}CD115⁻ (bottom). Data are combined from two independent experiments (mean \pm s.d. of four to nine mice) per condition. **(f)** Light microscope images of neutrophils from May-Grünwald Giemsa-stained thin blood films of *S. typhimurium*-infected mice, 18 h after infection in PBS-treated mice or at the humane endpoint in Py17XNL-infected and PHZ- or hemin-treated mice. Images are representative of neutrophils containing bacteria, except for PBS-treated mice where no bacteria were seen, from two independent experiments with three to five mice per condition. Scale bars, 10 μ m. Significance determined by one-way analysis of variance (ANOVA) with Dunnett's multiple comparison test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.



infection (Fig. 3c). Granulocytes were not simply refractory to PMA stimulation, as PMA-induced degranulation (assessed by surface CD11b expression³⁵) was actually enhanced 14 and 21 d after malaria infection (Fig. 3d). In contrast, blood granulocytes isolated 24 h after PHZ or hemin treatment did not differ from those of control mice in oxidative burst capacity, degranulation, *ex vivo* phagocytosis or killing of *S. typhimurium* (Fig. 3c,d and Supplementary Fig. 3c,d).

Hemolysis induces dysfunctional granulocyte mobilization

Accumulation of heme after PHZ-mediated hemolysis or hemin administration is faster than during Py17XNL infection. Heme directly induces neutrophil migration and ROS production¹⁶, whereas the subsequent HO-1 induction in myeloid cells can suppress maturation and oxidative burst^{30,33}. As HO-1 is induced in bone marrow by hemolysis²⁶, we wondered whether the chronic hemolysis associated with Py17XNL infection might induce HO-1 expression in immature bone marrow myeloid cells, suppress their oxidative burst capacity as they mature and allow gradual accumulation of dysfunctional cells in the circulation, as opposed to acute hemolysis (induced by PHZ), which may both activate the oxidative burst of circulating granulocytes and mobilize functionally immature bone marrow granulocytes, resulting in heterogeneous oxidative burst activity of blood granulocytes (as suggested by Fig. 3c).

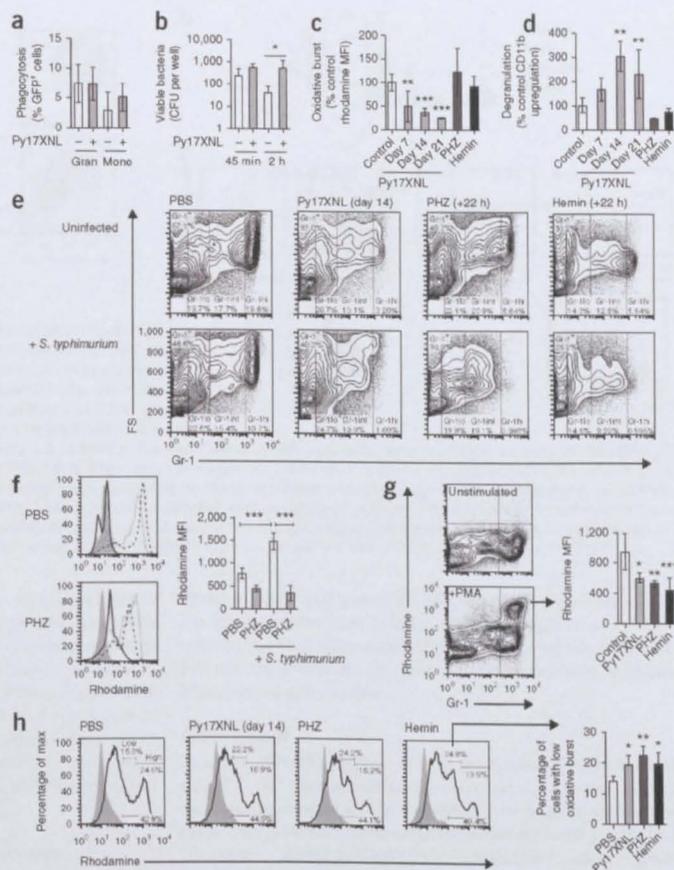
In mice, granulocyte maturation in bone marrow is characterized by increasing expression of Gr-1 (ref. 29). Gr-1^{hi} cells are mature neutrophils, and Gr-1^{lo} cells are immature granulocytes and granulocyte

progenitors; the Gr-1^{int} (intermediate) compartment contains a mixture of cell types. Generation of an oxidative burst is restricted to a functionally mature subpopulation of cells³⁶ in the Gr-1^{hi}, and to a lesser extent the Gr-1^{int}, compartments (Supplementary Fig. 4).

Treatment with hemin or PHZ and Py17XNL infection all caused marked depletion of Gr-1^{hi} cells from bone marrow (Fig. 3e and Supplementary Fig. 5). For PHZ and hemin treatment, loss of Gr-1^{hi} cells from bone marrow was accompanied by an increase in granulocytes in peripheral blood (Supplementary Fig. 5), confirming the effect of free heme in mobilization of granulocytes from bone marrow to the periphery. Although the proportion of circulating granulocytes did not increase during Py17XNL infection, granulocyte mobilization might have been obscured by an overall increase in leukocyte count or granulocyte redistribution (for example, from blood to the spleen)³⁷. *S. typhimurium* infection caused granulocyte mobilization in PBS-treated mice and markedly exacerbated the granulocyte mobilization in Py17XNL-infected and PHZ- or hemin-treated mice (Fig. 3e and Supplementary Fig. 5), consistent with the presence of immature granulocytes in blood (Fig. 2e). To confirm that hemolysis and bacterial challenge did indeed result in granulocytes with reduced oxidative burst activity entering the circulation, we assessed the oxidative burst of circulating granulocytes. Eight hours after *S. typhimurium* infection, the oxidative burst response to PMA was enhanced in PBS-treated mice (presumably due to priming³⁸), but the oxidative burst capacity was markedly lower in PHZ-treated mice (Fig. 3f).

Figure 3 Hemolysis and heme cause dysfunctional granulocyte mobilization. *In vitro* phagocytosis (a,b) and killing (b) of *S. typhimurium* by CD11b⁺ cells from blood of control and Py17XNL-infected (day 15) mice. (a) Phagocytosis: percentage of granulocytes (Gran) and monocytes (Mono) that were GFP⁺. (b) Phagocytosis (45 min) and killing (2 h) of *S. typhimurium* assessed by quantitative culture. Data are representative of two independent experiments (mean \pm s.d. of three to five mice per condition). (c,d) PMA-stimulated oxidative burst (c) and degranulation (d) of granulocytes in whole blood from control, Py17XNL-infected or PHZ- or hemin-treated (24 h after first treatment) mice, relative to mean of PBS controls. Data are representative of at least two independent experiments (mean \pm s.d. of three to five mice) per condition and time point. MFI, median fluorescence intensity. (e) Flow cytometry analysis of Gr-1 expression by bone marrow cells in uninfected mice (top) or 16 h after *S. typhimurium* infection (bottom). Representative of at least four independent experiments per condition.

(f) Left, PMA-stimulated oxidative burst of whole-blood granulocytes 14 h after PHZ or PBS treatment (8 h after *S. typhimurium* infection). Rhodamine fluorescence for unstimulated-uninfected (gray filled), unstimulated-infected (solid lines), PMA-stimulated, uninfected (dotted lines) and PMA-stimulated, infected (dashed lines), PBS- and PHZ-treated mice. Right, quantitative data for PMA-stimulated blood. Representative of two independent experiments (mean \pm s.d. of four mice per condition). (g) PMA-stimulated oxidative burst of Gr-1^{hi} bone marrow granulocytes from control, day 14 Py17XNL and hemin and PHZ treated mice. Representative of at least three independent experiments (mean \pm s.d. of three to five mice) per condition. (h) Proportion of Gr-1^{hi} bone marrow cells with a low-level oxidative burst response to PMA. Total, low- and high-level oxidative burst populations (left; unstimulated control in gray) and quantitative data (right). Data representative of at least three independent experiments (mean \pm s.d. of three to five mice) per condition. Significance determined by two-tailed Student's *t* test (a,b,f) or one-way ANOVA with *post hoc* comparison with control using Dunnett's multiple comparison test (c,d,f,h). **P* < 0.05, ***P* < 0.01, ****P* < 0.001.



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Finally, we investigated whether maturation of the oxidative burst in bone marrow granulocytes was also impaired. On day 14 of Py17XNL infection, and 18 h after PHZ or hemin treatment, there was a clear quantitative defect in the PMA-induced oxidative burst of Gr-1^{hi} cells (Fig. 3g), evident as an increase in the proportion of cells with low oxidative burst capacity and a decrease in the proportion of cells with high burst capacity (Fig. 3h), compared to the PBS control.

Together, these data indicate that intravascular heme (released during hemolysis) mobilizes granulocytes from bone marrow and simultaneously impairs development of their oxidative burst. Thus, granulocytes entering the circulation in response to subsequent infection are able to phagocytose *S. typhimurium* but, owing to their reduced oxidative burst capacity, fail to kill them, providing instead a niche for bacterial replication and dissemination.

HO-1 is induced in immature bone marrow myeloid cells

Given that the cytoprotective effects of HO-1, and of the heme degradation product, carbon monoxide, have been attributed to inhibition

of ROS production^{17,39}, we wondered whether suppression of the granulocyte oxidative burst correlated with HO-1 induction during granulopoiesis. As expected^{25,40}, PHZ treatment and Py17XNL infection led to systemic induction of HO-1 (Supplementary Fig. 6a–c). HO-1 was consistently induced in peripheral blood monocytes by Py17XNL, PHZ and hemin, but only (to a modest extent) by hemin in circulating granulocytes and by Py17XNL in the nonmyeloid population (Supplementary Fig. 6d).

In bone marrow of untreated, uninfected mice, HO-1 is expressed mainly in F4/80⁺ cells (Fig. 4a), presumably macrophages and monocytes. However, in hemin-treated and PHZ-treated mice, there was a significant increase in the proportion of HO-1⁺ bone marrow cells, especially in the Gr-1^{lo}-F4/80⁺ compartment (Fig. 4a,b). There was a small but significant increase in the proportion of HO-1⁺ Gr-1^{lo}-F4/80⁺ cells in Py17XNL-infected mice (Fig. 4b) but no overall increase in bone marrow HO-1⁺ cells, probably owing to mobilization of F4/80⁺ cells from bone marrow to blood and spleen^{37,41} (Supplementary Fig. 7). The Gr-1^{lo}-F4/80⁺ compartment contains

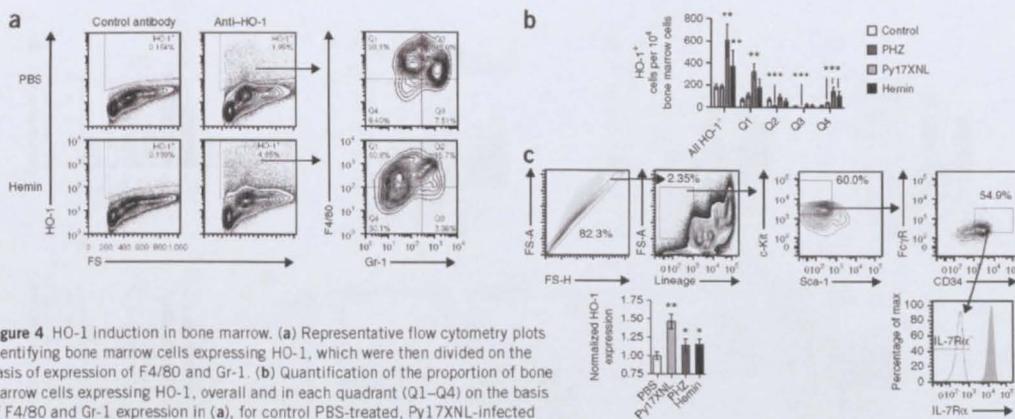


Figure 4 HO-1 induction in bone marrow. **(a)** Representative flow cytometry plots identifying bone marrow cells expressing HO-1, which were then divided on the basis of expression of F4/80 and Gr-1. **(b)** Quantification of the proportion of bone marrow cells expressing HO-1, overall and in each quadrant (Q1–Q4) on the basis of F4/80 and Gr-1 expression in **(a)**, for control PBS-treated, Py17XNL-infected (day 14), PHZ-treated and hemin-treated mice (18 h after treatment). Data are representative of two independent experiments (mean \pm s.d. of three to five mice) per condition. **(c)** Flow cytometry analysis showing identification of granulocyte GMP cells as lineage⁻ c-Kit⁺ Sca-1⁻ Fc γ R⁺ CD34⁺ IL-7R α ⁻, and normalized HO-1 expression in GMP cells determined by the ratio of the MFI of the fluorescence for the antibody against HO-1 to control antibody staining for the same sample, normalized against the average expression in PBS-treated mice in the same experiment (bottom left). The IL-7R α ⁺ population (shaded, bottom right plot) is drawn from the lineage^{hi} population in the top, second left plot. Data are from two independent experiments (mean \pm s.d.) involving four to eight mice per condition. Significance was determined by one-way ANOVA with *post hoc* comparison with control condition using Dunnett's multiple comparison test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

myeloid progenitors and immature myeloid cells. As surface markers that positively identify mouse myeloblasts and promyelocytes have not yet been defined, we assessed HO-1 expression in the granulocyte macrophage progenitor (GMP) population, which is proximal to the myeloblast in the myeloid differentiation pathway⁴². Py17XNL, PHZ and hemin all caused significant induction of HO-1 even at this very early stage of development (Fig. 4c). Thus, malaria infection, hemolysis and hemin treatment all induce HO-1 expression in the earliest stages of granulocyte development and thereby impair subsequent functional maturation of these cells.

HO inhibition restores resistance to *S. typhimurium*

To test the hypothesis that HO-1 impairs resistance of Py17XNL-infected mice to *S. typhimurium* bacteremia, we pretreated mice with the competitive HO inhibitor tin protoporphyrin IX (SnPP). Treatment of Py17XNL-infected mice with SnPP for 48 h before *S. typhimurium* infection reduced bacterial loads in blood, spleen and liver to levels not significantly different from those in PBS treated mice (Fig. 5a). SnPP treatment had no effect on bacterial load in mice without Py17XNL infection (Fig. 5a) or on parasitemia in Py17XNL-infected mice (Fig. 5b) but very effectively prevented accumulation of GFP⁺ *S. typhimurium* within granulocytes in Py17XNL-infected mice (Fig. 5c,d). SnPP also partially restored resistance to *S. typhimurium* when administered 2 h before PHZ treatment (Fig. 5a,c,d) and prolonged the survival of Py17XNL-infected or PHZ-treated mice after *S. typhimurium* infection (Fig. 5e). Cobalt protoporphyrin (CoPP), which induces HO-1 in the absence of hemolysis or free heme⁴³, did not impair resistance to *S. typhimurium* 16 h after infection (Fig. 5a). Thus, both heme and HO-1 are necessary for impairment of resistance to *S. typhimurium* caused by Py17XNL or PHZ hemolysis, and inhibition of HO abrogates this effect. Inhibition of HO by SnPP did not restore the oxidative burst of Gr-1^{hi} bone marrow granulocytes in Py17XNL-infected or PHZ-treated mice (Fig. 5f), presumably owing to enhanced mobilization of mature Gr-1^{hi} cells (Fig. 5g) as a result of greater heme accumulation (Fig. 3e and Supplementary Fig. 5).

However, SnPP did reverse the accumulation of granulocytes with low-level oxidative burst activity in the bone marrow of Py17XNL-infected and PHZ-treated mice (Fig. 5h), indicating that inhibition of HO restores normal development of the oxidative burst in maturing bone marrow granulocytes.

DISCUSSION

Understanding the etiology of NTS septicemia in individuals with malaria and other hemolytic disorders may lead to new strategies for reducing morbidity and mortality. To reflect the clinical association between NTS septicemia and severe malarial anemia^{3,4}, we have used a model in which malaria infection causes progressive hemolysis, eventually resulting in severe (but not lethal) anemia, to assess the impact of *S. typhimurium* co-infection on disease. We have shown that loss of resistance to *S. typhimurium* requires hemolytic release of cell-free heme and subsequent induction of HO-1 and that inhibition of HO-1 reverses this susceptibility to NTS. Thus, although HO-1 is essential for tolerance to the cytotoxic effects of free heme, reducing disease severity without altering pathogen load^{24–27}, HO-1-mediated tolerance to malaria simultaneously impairs resistance to *S. typhimurium*.

We propose (Fig. 6) that during acute hemolysis, heme triggers immediate mobilization of granulocytes from bone marrow to blood and generation of ROS¹⁶, while simultaneously inducing HO-1 in immature myeloid cells and thereby reducing their subsequent oxidative burst capacity^{30,33}, perhaps by limiting the availability of heme for incorporation into NADPH oxidase⁴⁴. This results in mobilization of a heterogeneous population of granulocytes with varying levels of oxidative burst capacity. During malaria infection, however, progressive hemolysis leads to sustained release of free heme, which both impairs maturation of the oxidative burst capacity of granulocytes in the bone marrow and mobilizes functionally immature granulocytes from bone marrow into the peripheral circulation. Accumulation in peripheral blood of functionally impaired granulocytes, which phagocytose but are unable to kill bacteria, provides a new niche for bacterial

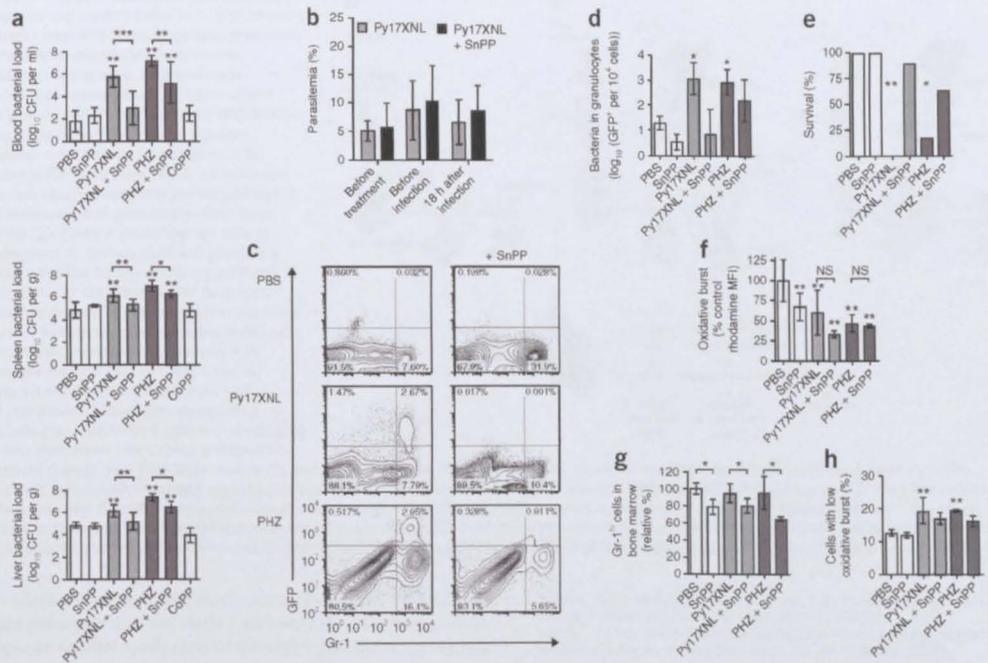


Figure 5 Impaired resistance to *S. typhimurium* is abrogated by inhibition of heme oxygenase. **(a)** *S. typhimurium* bacterial loads in blood, spleen and liver obtained from mice treated as in **Figure 1e**, with additional groups receiving SnPP or CoPP treatment. **(b)** Parasitemia of SnPP-treated and untreated Py17XNL-infected mice immediately before treatment with SnPP, immediately before infection with *S. typhimurium* and 18 h after infection. **(c)** Representative flow cytometry analysis of blood leukocytes from *S. typhimurium*-infected mice treated as in **a**. **(d)** Quantification of GFP⁺ granulocytes as a proportion of all granulocytes in blood. **(e)** Survival (to the humane endpoint) 18 h or 16 h after *S. typhimurium* infection in Py17XNL-infected and PHZ-treated mice, respectively, with or without SnPP treatment. In **a–e**, data represent pooled results (mean \pm s.d.) of two independent experiments (six to nine mice per condition). **(f)** The oxidative burst of Gr-1^{hi} bone marrow granulocytes (as in **Fig. 3g**). **(g)** Proportion of bone marrow cells that are Gr-1^{hi} after SnPP treatment, expressed as a percentage of the average proportion of Gr-1^{hi} cells in the equivalent treatment condition without SnPP. **(h)** Proportion of Gr-1^{hi} cells responding to PMA with a low-level oxidative burst (as in **Fig. 3h**). Data are representative of two independent experiments (mean \pm s.d. of three to five mice) per condition. Significance determined by one-way ANOVA with comparisons using Dunnett's multiple comparison test or Bonferroni's multiple comparison (bracketed groups) **(a,b,d,f,h)**, Student's *t* test **(g)** and Fisher's exact test **(e)**. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

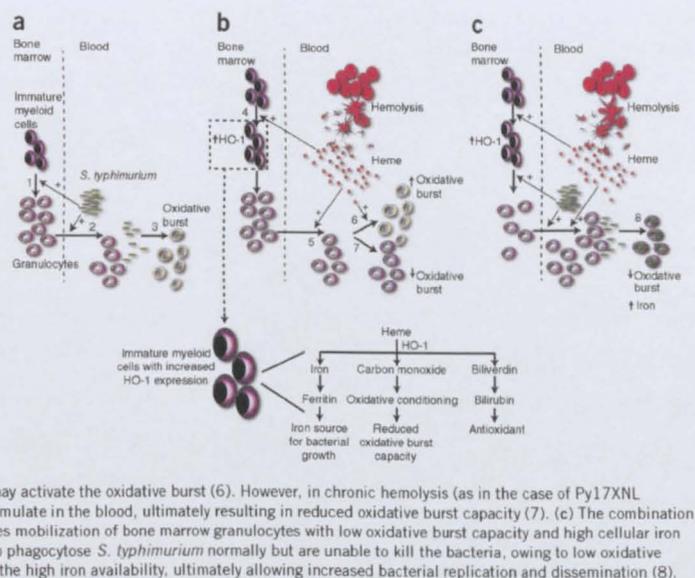
replication and dissemination. In this scenario, HO-1 contributes to impaired resistance to NTS, but heme also has a direct role—either in granulocyte mobilization¹⁶ or as a substrate for HO-1. The heme degradation products carbon monoxide, biliverdin and iron may further impair resistance to NTS by reducing production of ROS²¹ or facilitating bacterial replication⁴⁵. In contrast, nonheme induction of HO-1 (for example, by CoPP) may limit available iron for bacterial replication and protect phagocytic cells from apoptosis^{45,46}.

Our observation that hemolysis specifically suppresses the oxidative burst capacity of neutrophils offers a plausible explanation for the particular susceptibility to NTS bacteremia in individuals with hemolysis. *Salmonella* species have evolved to survive and replicate inside mononuclear phagocytes⁴⁷; hemolysis provides an additional niche for sustained bacterial replication in circulating neutrophils. Our results are also consistent with studies of the cytoprotective role of HO-1 in mice; indeed, limitation of the granulocyte oxidative burst could be a key adaptive mechanism to reduce self-damage by ROS during hemolysis and to prevent tissue injury associated with release of heme.

Very few mechanisms have been clearly identified that confer tolerance to the harm caused by infectious organisms⁴⁸, despite recent interest in the therapeutic potential of augmenting host tolerance⁴⁹. In mice, HO-1 confers tolerance to blood-stage malaria^{24,25} but simultaneously diminishes resistance to malaria parasites developing in the liver⁵⁰, whereas in *Drosophila*, infection-induced anorexia increases tolerance against *S. typhimurium* but reduces resistance against *Listeria monocytogenes*⁵¹, indicating that resistance and tolerance mechanisms can be highly pathogen specific and that a mechanism of tolerance to one pathogen can diminish resistance to another. Although it is well recognized that co-infection with different pathogens can enhance disease severity, and in some cases molecular mechanisms have been elucidated⁵², to our knowledge, this study provides the first direct evidence in a mammalian model of tolerance to one pathogen impairing resistance to another.

To conclude, our findings have a number of key implications. First, they provide an explanation for the susceptibility to NTS bacteremia in individuals with malaria and sickle cell disease. Second, they imply

Figure 6 Proposed mechanism to explain how hemolysis impairs resistance to *S. typhimurium* through heme- and heme oxygenase-dependent dysfunctional granulocyte mobilization. (a–c) Proposed scheme for granulocyte mobilization in response to *S. typhimurium* infection (a) and the dysfunctional mobilization induced by hemolysis (b), which causes impaired resistance to *S. typhimurium* (c). In normal C57BL/6 mice (a), *S. typhimurium* infection causes emergency granulopoiesis (1) and mobilization of granulocytes from bone marrow (2). These granulocytes are able to phagocytose *S. typhimurium* and generate a normal oxidative burst, controlling bacterial replication (3). (b) Intravascular hemolysis caused by malaria or phenylhydrazine treatment liberates hemoglobin and hemoglobin-derived heme. Heme induces HO-1 expression in immature myeloid cells (4), and heme is degraded to biliverdin, carbon monoxide and iron (inset). These heme degradation products may modify the function of developing granulocytes. Heme also causes mobilization of mature granulocytes from bone marrow (5) and may activate the oxidative burst (6). However, in chronic hemolysis (as in the case of Py17XNL infection), functionally immature granulocytes accumulate in the blood, ultimately resulting in reduced oxidative burst capacity (7). (c) The combination of hemolysis and *S. typhimurium* challenge increases mobilization of bone marrow granulocytes with low oxidative burst capacity and high cellular iron stores into the blood. These granulocytes are able to phagocytose *S. typhimurium* normally but are unable to kill the bacteria, owing to the high iron availability, and support bacterial growth due to the high iron availability, ultimately allowing increased bacterial replication and dissemination (8).



that tolerance and resistance mechanisms identified from studies of single pathogens may not easily translate to the 'real world', where people are simultaneously exposed to multiple pathogens. Specifically, the concept that the cytoprotective effects of HO-1 may be harnessed by administering its products therapeutically in humans without adversely affecting host defense against infection^{53,54} may not be valid. Third, we have identified a potential adjunct therapy (SnPP) that might enhance resistance to NTS in patients with hemolytic diseases. SnPP has been used experimentally to prevent severe jaundice⁵⁵, but optimization of treatment would be crucial to avoid impairment of tolerance to heme. The experimental system described here may be a good starting point to assess and optimize such treatments.

METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/naturemedicine/>.

Note: Supplementary information is available on the Nature Medicine website.

ACKNOWLEDGMENTS

This work was supported by a UK Medical Research Council Clinical Research Training Fellowship (G0701427) and a small grant award from the European Society for Pediatric Infectious Diseases awarded to A.J.C. We wish to thank D. Holden (Imperial College, London) for providing GFP-expressing *S. typhimurium* and R. Motterlini, S. Baines, H. Kaur, L. King, C. Stanley, R. Gregory, L. McCarthy, K. Couper, J. Hafalla, E. Findlay and D. Blount for technical advice and assistance.

AUTHOR CONTRIBUTIONS

A.J.C. and J.B.S. conducted the experiments. A.J.C. and E.M.R. wrote the manuscript. All authors contributed to the conception and planning of the experiments, and to critical revision of the manuscript. M.W. and E.M.R. supervised the project.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Bacteria. We stored aliquots from a single broth culture of *S. enterica* serovar Typhimurium 12023 (*S. typhimurium*), constitutively expressing GFP in 10% glycerol at -80°C . For *in vitro* experiments we opsonized *S. typhimurium* in 20% normal mouse serum at 37°C for 30 min before inoculation. For quantification of bacterial loads, we plated tenfold dilutions of lysed organ homogenates and whole blood onto LB agar and incubated for 18 h before counting the number of colony-forming units (CFUs).

Mice. Mouse experimentation conformed with UK Home Office Regulations and was approved by the London School of Hygiene and Tropical Medicine animal procedures and ethics committee. We obtained female, 6- to 10-week-old C57BL/6 mice from Harlan and Charles River (UK) and infected them with *P. yoelii* 17X nonlethal (Py17XNL) by intraperitoneal (i.p.) injection of 1×10^5 parasitized red blood cells. We determined parasitemia by examination of Giemsa-stained thin blood smears. We determined erythrocyte counts using a Z2 Coulter particle counter. We induced acute hemolysis by subcutaneous injection of PHZ (Sigma) ($125 \mu\text{g}$ per kg body weight). We administered hemin (Frontier Scientific) by i.p. injection ($50 \mu\text{mol}$ per kg body weight per dose) in two doses 12 h apart. We initiated *S. typhimurium* infections by i.p. inoculation of 1×10^5 CFU of *S. typhimurium* on day 15 of Py17XNL infection or 6 h after PHZ or first dose of hemin treatment. We administered SnPP (Frontier Scientific) ($40 \mu\text{mol}$ per kg body weight per dose) by i.p. injection 48, 24 and 8 h before *S. typhimurium* infection of Py17XNL-infected mice, 2 h before PHZ treatment or 8 h before *S. typhimurium* alone. We administered CoPP (Frontier Scientific) (10mg per kg body weight) i.p. 6 h before *S. typhimurium* infection. We monitored *S. typhimurium*-infected mice every 6 h until they showed signs of illness (clinical stage 2, see **Supplementary Methods**) and then every 1–2 h until they reached the humane endpoint (clinical stage 4), at which point they were killed. The time of death was used for survival analysis.

Flow cytometry. Antibodies used are described in the **Supplementary Methods**. We performed intracellular staining for HO-1 on the basis of a previously described method⁵⁶.

Microscopy. Chamber slides were protected from light and air-dried for 2 h before nuclear staining and mounting with DAPI dissolved in confocal matrix. To determine whether bacteria were intracellular or adherent to the cell surface, we assessed cells with overlapping GFP⁺ *S. typhimurium* by $0.5\text{-}\mu\text{m}$ interval Z-stack imaging. For light microscopy, we fixed thin blood films with methanol and air dried before staining with May-Grünwald Giemsa stain.

Salmonella phagocytosis and killing assays. We assessed *ex vivo* phagocytosis and killing of *S. typhimurium* in a gentamicin protection assay and quantified by flow cytometry (staining with allophycocyanin-conjugated antibody against Gr-1 (eBioscience) and phycoerythrin-Cy7-conjugated antibody against CD11b (eBioscience)), confocal microscopy and bacterial culture. We seeded isolated CD11b⁺ cells at 1×10^5 per well in flat-bottomed 96-well plates or at 2×10^5 per well in eight-well chamber slides and incubated at 37°C and 5% CO_2 for 20 min

before addition of *S. typhimurium* at a multiplicity of infection (MOI) of ten *S. typhimurium* to one CD11b⁺ cell. We determined phagocytosis at 45 min after infection and killing at 2 h after infection.

Oxidative burst and degranulation assay. We assessed neutrophil oxidative burst using a modification of a previously described flow cytometric assay⁵⁴ in which dihydrorhodamine 123 is converted to rhodamine. We measured neutrophil degranulation by the percentage increase in the median fluorescence intensity of surface CD11b expression in stimulated versus unstimulated samples.

Measurement of heme and hemoglobin. We determined concentrations of heme and hemoglobin using Quantichrom Heme and Hemoglobin assay kits (Bio Assay Systems). We quantified protein-bound plasma heme using a previously described spectrophotometric method⁵⁷ and the concentration of plasma hemoglobin using a previously published method⁵⁸.

Hmox1 expression and HO activity assays. We determined *Hmox1* mRNA expression in liver by quantitative RT-PCR. We standardized cDNA expression for each sample using the housekeeping genes *Gapdh* and *Tbp* and calculated expression as relative fold change compared to healthy control samples. We measured HO activity in whole-liver homogenates after red blood cell lysis using a previously described method⁵⁹. We measured plasma HO-1 by ELISA using an HO-1 Immunoset (Enzo Life Sciences).

Statistical analyses. We performed statistical analysis using Graph Pad Prism 5 software. We used an alpha value of 0.05 for all preplanned statistical analyses. We used the log-rank Mantel Cox test for survival analysis. We analyzed continuous data that were approximately normally distributed using two-sided unpaired or paired Student's *t* test for pairwise comparisons or one-way ANOVA with *post hoc* testing using Dunnett's multiple comparison test for comparison with the control group, Tukey's multiple comparison test for comparison between multiple groups and Bonferroni's multiple comparison test for comparison between two or more selected pairs. We log₁₀-transformed all data relating to bacterial loads before analysis. We compared proportions between groups using Fisher's exact test.

Additional methods. Detailed methodology is described in the **Supplementary Methods**.

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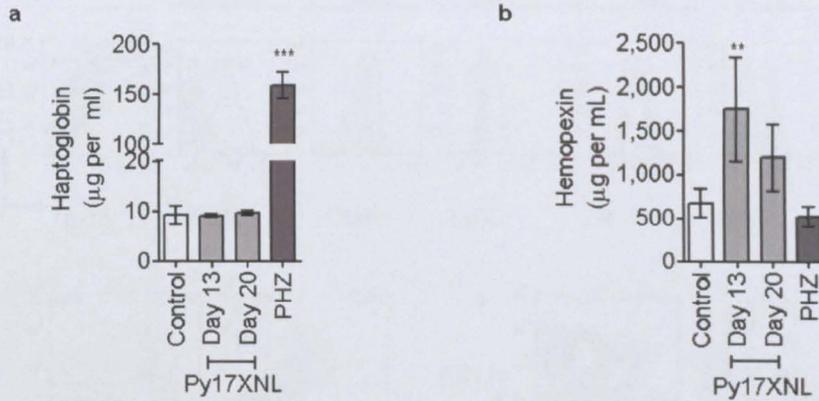
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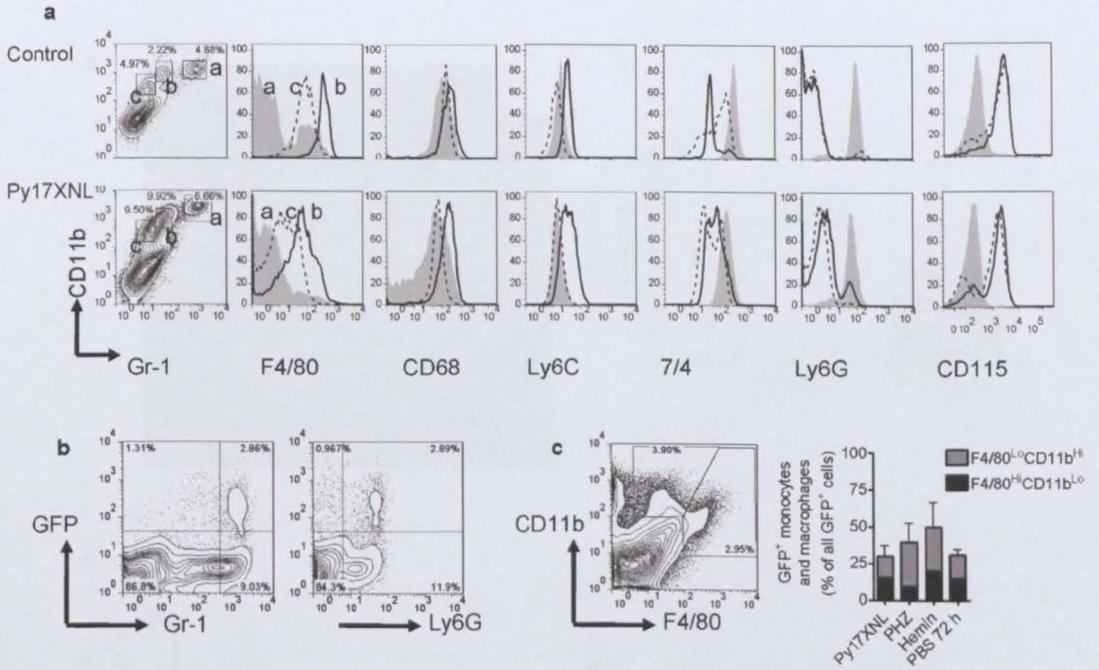
Malaria impairs resistance to Salmonella through heme- and heme oxygenase-dependent dysfunctional granulocyte mobilization.
A. J. Cunnington, J.B. de Souza, R-M. Walther, E. M. Riley

Supplementary Figure 1



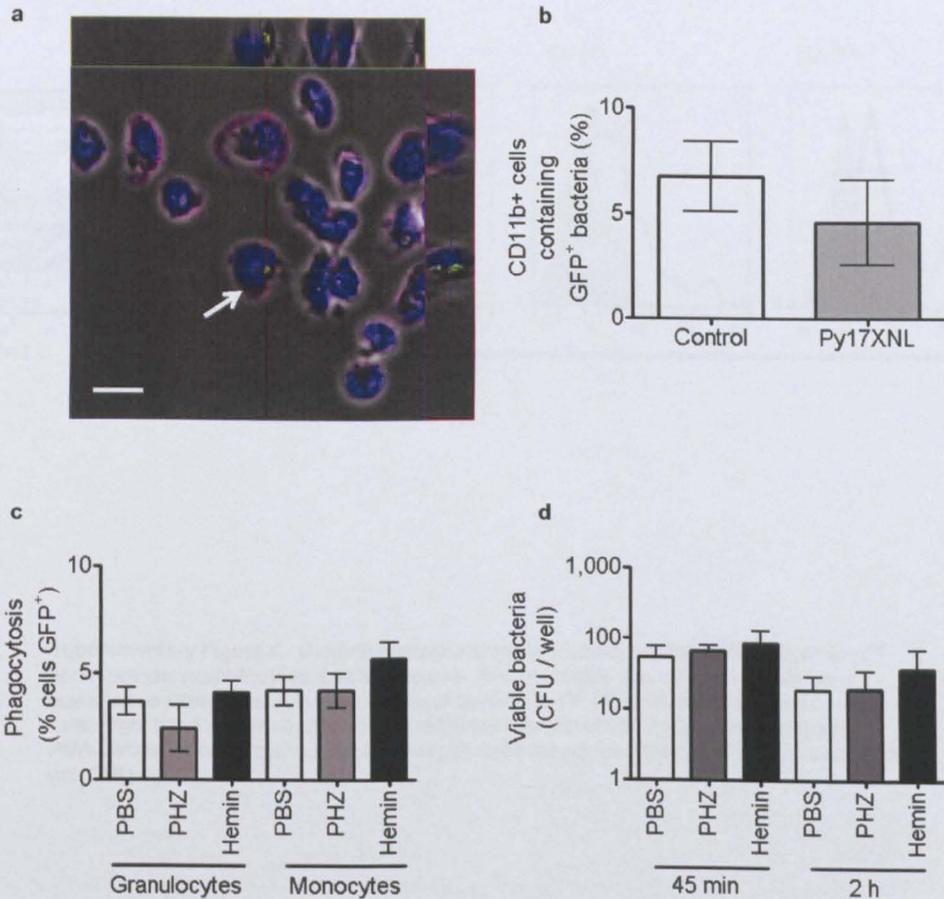
Supplementary Figure 1. Plasma haptoglobin and hemopexin levels are not depleted by Py17XNL or phenylhydrazine hemolysis. (a,b) Plasma haptoglobin (a) and hemopexin (b) were measured by ELISA during Py17XNL infection and 18 h after phenylhydrazine (PHZ) administration. Data are representative of 2 independent experiments (mean \pm s.d. of 3–4 mice) per condition. Significance was determined by one-way ANOVA with post-hoc comparison with control condition using Dunnett's multiple comparison test. ** $P < 0.01$, *** $P < 0.001$.

Supplementary Figure 2



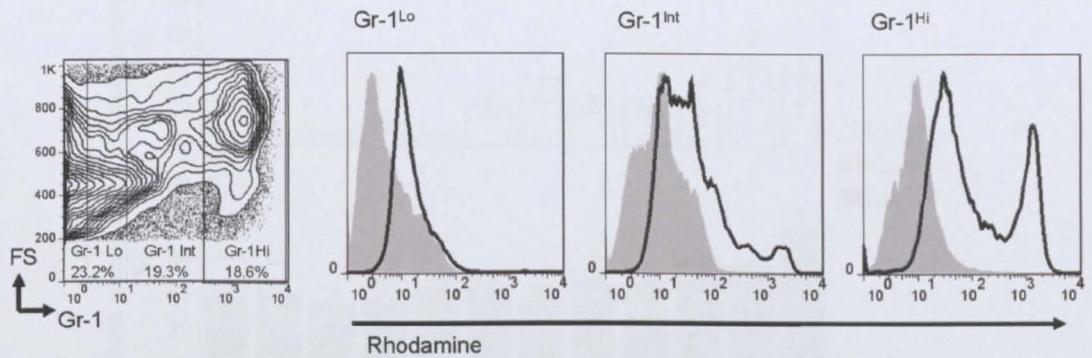
Supplementary Figure 2. Definition of myeloid cell populations by flow cytometry. (a) Myeloid cell populations in blood were defined by differential expression of CD11b and Gr-1 in both uninfected and Py17XNL-infected mice (14 d post infection), and their identity confirmed by expression of other surface markers (histograms). Regions a, b, c (and filled, solid line and dashed line histograms) correspond to granulocyte, inflammatory monocyte and resident monocyte populations respectively. (b) Representative flow cytometric analysis of blood 18 h after infection with GFP-expressing *S. typhimurium* on day 15 of Py17XNL infection. Granulocytes were identified by Gr-1^{Hi} expression (left hand panel) and Ly6G expression (right hand panel). (c) Splenic monocyte and macrophage populations were defined by flow cytometry as F4/80^{Lo}CD11b^{Hi} and F4/80^{Hi}CD11b^{Lo} respectively (left hand panel) and the proportion of all GFP⁺ positive cells in each of these compartments was quantified in Py17XNL-infected, PHZ- and hemin-treated mice at the humane endpoint (right hand panel). GFP⁺ cells are shown 72 h after infection of PBS-treated control mice, but due to very low infection levels in PBS-treated mice 18 h after infection, resulting in no GFP⁺ cells being detected in most mice, this condition is not shown. Data are representative of 2 independent experiments with 3–5 mice per condition. Significance was determined by one-way ANOVA with post-hoc comparison with control condition (PBS) using Dunnett's multiple comparison test.

Supplementary Figure 3.



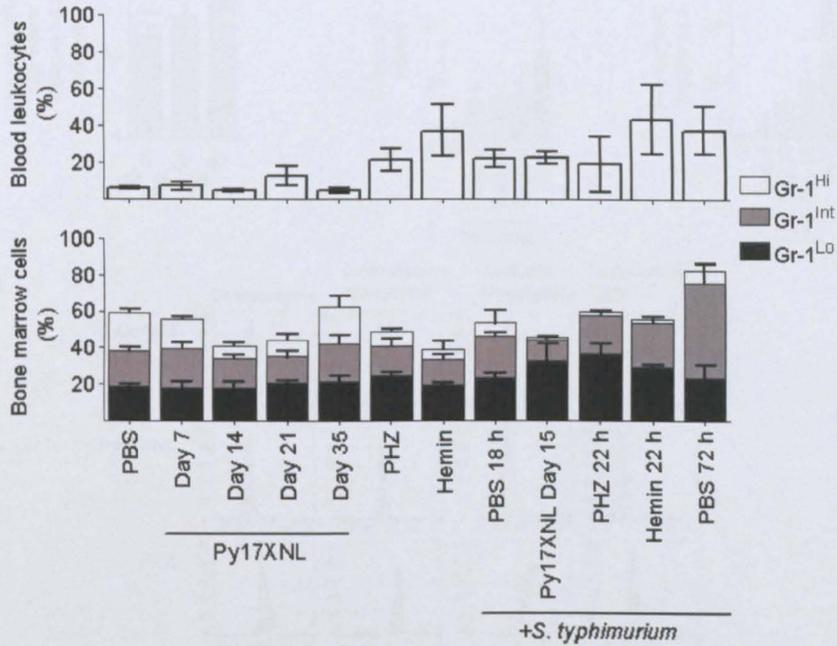
Supplementary Figure 3. Phagocytosis of *S. typhimurium* by monocytes and neutrophils is not impaired by Py17XNL, phenylhydrazine or hemin and *S. typhimurium* killing is not impaired by phenylhydrazine or hemin. (a,b) Intracellular bacteria were quantified by confocal microscopy following incubation of CD11b⁺ cells (isolated from blood of Py17XNL-infected (day 14) and uninfected control mice) with *S. typhimurium* for 45 min in a gentamicin protection assay. (a) Representative orthogonal reconstruction from 17 stacked images at 0.5 μm depth intervals, showing a CD11b⁺ cell (from Py17XNL infected mouse, arrow) containing GFP⁺ *S. typhimurium*. Blue nuclear staining, DAPI; Green, GFP; scale bar 10 μm. (b) Summary data for analysis of confocal images. Data are representative of 2 independent experiments (mean ± s.d. of 3 mice) per condition. Phagocytosis (c,d) and killing (d) of *S. typhimurium* by CD11b⁺ cells isolated from whole blood of control mice or test mice 24 h after PHZ or hemin treatment, and incubated with *S. typhimurium* *in vitro* in a gentamicin protection assay. Phagocytosis was assessed by the percentage of granulocytes and monocytes which were GFP⁺ by flow cytometry (c) and by culture of cell lysates collected after 45 min incubation with bacteria (d). Bacterial killing was assessed by culture of cell lysates collected after 2 h incubation with bacteria (d). Data are representative of 2 independent experiments (mean ± s.d. of 3–6 mice) per condition. Significance was determined by two-tailed Student's *t*-test (b) or one-way ANOVA with post-hoc comparison with control condition using Dunnett's multiple comparison test (c,d).

Supplementary Figure 4



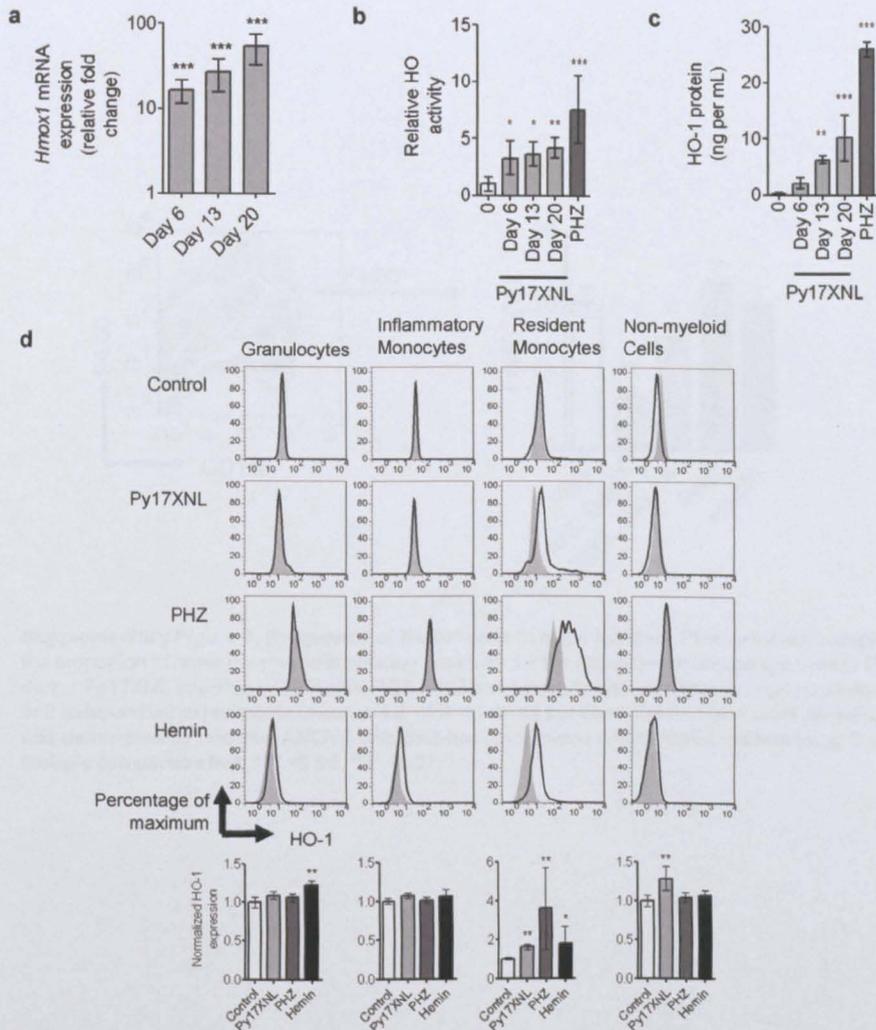
Supplementary Figure 4. Oxidative burst activity is predominantly a property of Gr-1^{Hi} bone marrow granulocytes. Left hand panel: Representative flow cytometric analysis of control bone marrow cells showing gating of Gr-1^{Lo}, Gr-1^{Int} (Gr-1 intermediate) and Gr-1^{Hi} cells. Right hand panels: rhodamine fluorescence intensity of unstimulated (shaded) and PMA-stimulated cells (unshaded, black line). Results are representative of 6 experiments each with 3–5 mice.

Supplementary Figure 5



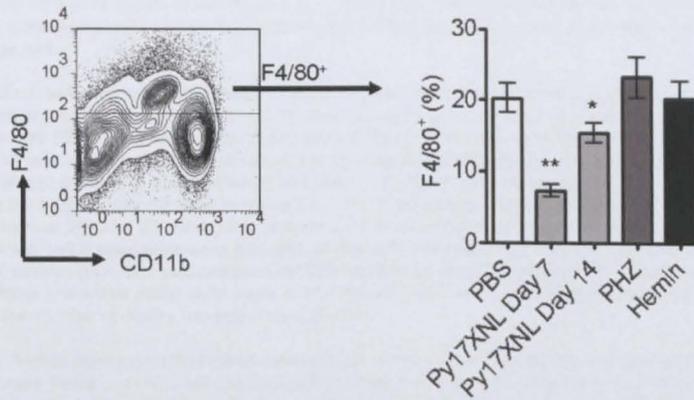
Supplementary Figure 5. Frequency and phenotype of bone marrow and peripheral blood granulocytes. Flow cytometric analysis of blood (upper panel) and bone marrow (lower panel) granulocytes assessed by expression of Gr-1 and divided as low, intermediate and high expression (Gr-1^{Lo}, Gr-1^{Int}, Gr-1^{Hi} respectively), 22 h after mice were treated with PBS, PHZ or hemin treatments, or at different time points during Py17XNL infection, or with additional *S. typhimurium* infection 16 h (PHZ and hemin), 18 h (Py17XNL and PBS) or 72 h (PBS) before harvest. Granulocyte frequencies are shown as a percentage of all leukocytes in blood, and of all bone marrow cells, respectively. Data are representative of at least 3 independent experiments (mean \pm 95% confidence interval of 3–5 mice) per condition and time point.

Supplementary Figure 6



Supplementary Figure 6. Hemolysis and hemin cause systemic and cell-type specific induction of HO-1. Heme oxygenase-1 induction assessed by *Hmox1* mRNA expression in liver (a), HO bio-activity in liver (b) and HO-1 protein concentration in plasma (c). (a) *Hmox1* expression in liver was determined by rt-PCR expressed as fold change relative to the control gene *Gapdh*, relative to the difference between *Hmox1* and *Gapdh* expression in control liver. Data are representative of 2 independent experiments (mean \pm s.d. of 4–5 mice per time point). Similar results were obtained using *Tbp* as the control gene. (b) HO enzyme activity in liver homogenates from mice infected with Py17XNL or 18 h after PHZ treatment, was determined by conversion of hemin to bilirubin, standardized for protein content and expressed relative to controls at each time point. Data are representative of 2 independent experiments (mean \pm s.d. of 3–11 mice per condition and time point). (c) Plasma HO-1 protein concentration was determined by ELISA. Data are representative of 2 independent experiments (mean \pm s.d. of 4 mice per condition or time point). (d) HO-1 expression in myeloid and non-myeloid cells in blood was determined by flow cytometry, using the cell population definitions shown in **Supplementary Fig 2a**. HO-1 expression was quantified (lower panels) by the ratio of anti-HO-1 fluorescence (unfilled histograms) relative to control antibody (filled histograms) (upper panels) and normalized to the average value for control animals in each experiment. Data are representative of at least 2 independent experiments with 3–9 mice per condition. Significance was determined by one-way ANOVA with post-hoc comparison with control condition using Dunnett's multiple comparison test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Supplementary Figure 7



Supplementary Figure 7. Frequency of F4/80⁺ cells in bone marrow. Flow cytometric analysis of the proportion of bone marrow cells staining positively for the monocyte/ macrophage marker F4/80, during Py17XNL infection or 22 h after PBS, PHZ and hemin treatment. Data are representative of 1 or 2 independent experiments (mean \pm s.d. of 3–11 mice) per condition and time point. Significance was determined by one-way ANOVA with post-hoc comparison with control condition using Dunnett's multiple comparison test. * $P < 0.05$, ** $P < 0.01$.

Supplementary methods

Reagents. All reagents were purchased from Sigma unless specified otherwise. Hemin (ferriprotoporphyrin IX chloride), tin protoporphyrin IX (SnPP), and cobalt (III) protoporphyrin IX chloride (CoPP) were obtained from Frontier Scientific, were protected from light and prepared by dissolving in 0.2 M NaOH, diluted to the desired concentration in PBS and buffered carefully to pH 7.5 with HCl. Hemin solutions were subsequently filtered through a 0.2 µm acrodisc syringe filter unit (Pall Corporation) and the concentration of the filtered solution determined using a Quantichrom Heme assay (BioAssay Systems) according to the manufacturer's instructions. SnPP and CoPP solutions were not filtered but were prepared using sterile reagents and technique. Aliquots of hemin (3.26 mg ml⁻¹), SnPP (3 g ml⁻¹) and CoPP (1 mg ml⁻¹) were stored at -80 °C until use. Phenylhydrazine hydrochloride 25 mg ml⁻¹ solution was freshly prepared immediately prior to injection by dissolving in PBS, buffering to pH 7.4 with NaOH, and filtering through a 0.2 µm syringe unit.

Salmonella enterica serovar Typhimurium 12023 (*S. typhimurium*) constitutively expressing GFP was a gift from Prof. David Holden (Imperial College, London, UK). Bacteria were grown to late log phase in static culture in Luria-Bertani (LB) broth with 50 µg ml⁻¹ carbenicillin. Bacteria were frozen in aliquots in 10% Glycerol and stored at -80 °C until required for use. The concentration of stock bacteria was determined by dilution cultures on LB Agar, and reconfirmed for each aliquot at the time of use. Prior to inoculation, the Salmonella stock was defrosted, washed twice in PBS and diluted to the desired concentration in sterile PBS. For *in vitro* experiments Salmonella were opsonized in 20% normal mouse serum at 37°C for 30 min prior to inoculation. For quantitation of bacterial loads from organs of Salmonella infected mice, cell suspensions were prepared by disruption of tissue with a syringe plunger, passage through a 70 µm nylon cell strainer (BD), and resuspension as 10% solution by weight in sterile PBS. 10 fold-dilutions of organ homogenates and whole blood were made in 1% Triton X-100, plated onto LB agar and incubated for 18 h before counting the number of colony forming units (CFUs).

Animals. Animal experimentation conformed with UK Home Office Regulations and was approved by Institutional ethical review. Female, 6–10 week old C57BL/6 mice were obtained from Harlan and Charles River, UK and maintained under barrier conditions. Frozen stocks of blood-stage *Plasmodium yoelii* 17X Non-Lethal (Py17XNL) were inoculated in passage mice. Blood was collected after 5-7 days and experimental animals were infected by intraperitoneal (i.p.) injection of 10⁵ parasitised red blood cells (pRBCs). Parasitemia was determined by examination of Giemsa-stained thin blood smears. Erythrocyte count was determined using a Z2 Coulter particle counter (Beckman Coulter). Parasitemia, erythrocyte count and, where relevant, body weight were monitored at least twice a week. To induce acute hemolysis, mice were treated with phenylhydrazine (125 µg g⁻¹ body weight) by subcutaneous (s.c.) injection. Hemin was administered by i.p. injection (50 µmol kg⁻¹ body weight per dose) in two doses 12 h apart. Salmonella infections were initiated by i.p. inoculation of 10⁵ CFU of *S. typhimurium* in 200 µL PBS, on day 15 of Py17XNL infection or 6 h after PHZ or first dose of hemin treatment. To inhibit heme oxygenase activity, SnPP (40 µmol kg⁻¹ dose⁻¹) was administered by i.p. injection 48, 24 and 8 h before *S. typhimurium* infection of Py17XNL infected mice, 2 h before PHZ treatment, or 8 h before *S. typhimurium* alone. The HO-1 inducer CoPP (10mg kg⁻¹) was administered i.p. 6 h prior to *S. typhimurium* infection. Control animals received injections of equivalent volumes of PBS. After infection with *S. typhimurium*, mice were monitored 6 hourly until displaying signs of illness (clinical stage 2, Supplementary Methods Table 1) and then every 1-2 h until they reached clinical stage 4, at which point they were euthanized. Since progression from stage 4 to death is extremely rapid in Salmonella and rodent malaria infections, and using death as an experimental endpoint was considered unethical, the humane endpoint - time of reaching stage 4 - was used for survival analysis. In each experiment a group of PBS-treated Salmonella-infected mice was sacrificed at the same time as the treatment groups to allow comparison of bacterial loads. In all experiments with groups of Salmonella-infected mice, animals were killed by injection of pentobarbital. In all other experiments mice were killed with CO₂ inhalation. Immediately after death, under aseptic technique, blood was collected by cardiac puncture into heparinised syringes and tissues were removed into ice cold RPMI and stored on ice, protected from light, until processing.

Table 1. Clinical scale used to determine disease severity in mice:

1. no signs
2. ruffled fur and/or abnormal posture and/or minor weight loss (<15%)
3. lethargy and/or moderate weight loss (≥ 20%)
4. reduced response to stimulation and/or ataxia and/or respiratory distress/hyperventilation
5. prostration and/or paralysis and/or convulsions and/or severe weight loss (>25%)
6. Death

The humane endpoint was defined as mice reaching stage 4

Flow cytometry. Antibodies used are shown in Supplementary Methods Table 2. For all experiments except oxidative burst assays, cells were incubated for 5 min with red blood cell lysing buffer (Sigma), washed, and resuspended in FACS buffer prior to staining. Cells from *S. typhimurium* infected mice were fixed in 4% formaldehyde prior to surface staining. Cells were incubated with cocktails of antibodies for 30 min at room temperature in the dark and washed twice before analysis.

Intracellular staining for HO-1 was based on the method of Ewing *et al.*⁵⁶. Briefly, cells were fixed in 2% formaldehyde for 10 min at 37°C, centrifuged at 500 g for 5 min at 4°C, resuspended in ice-cold 90% methanol and incubated on ice for an additional 30 min. After washing with FACS buffer, cells were resuspended in FACS buffer containing 1% normal mouse serum (Southern Biotech) and Mouse Fc Block (BD Biosciences) or PE conjugated antibody against CD16/32 and incubated for 5 or 30 minutes at room temperature respectively. Cells were then centrifuged at 1000g for 2 minutes and resuspended in FACS buffer containing rabbit polyclonal antibody against HO-1 or an equivalent concentration of normal rabbit polyclonal antibody as a control for 30 min at 4°C. Cells were washed twice in FACS buffer before resuspension with FITC conjugated secondary antibody and a cocktail of surface marker antibodies and incubation at 4°C for 30 min, or room temperature for 90 min when the antibody against CD34 was used, followed by 2 final washes.

A BD FACSCalibur flow cytometer was used to acquire all samples except those for analysis of bone marrow progenitor populations (which were acquired using a BD LSR-II) and data were analysed using FlowJo version 7.6 (Tree Star, Inc.).

Microscopy. Chamber slides were protected from light and air-dried for 2 h before nuclear staining and mounting with DAPI dissolved in confocal matrix (Micro-Tech-Lab). Slides were examined using a Zeiss axiovert confocal microscope with a Plan-Apochromat 63x oil immersion lens and Zeiss LSM510 analysis software. For quantitative assessment of phagocytosis, cells with overlapping GFP⁺ *S. typhimurium* were assessed further by 0.5 µm interval Z-stack imaging to determine whether bacteria were intracellular or adherent to the cell surface. For light microscopy, thin blood films were fixed with methanol and air dried before staining with May-Grünwald Giemsa stain according to the manufacturer's instructions. Images were acquired using a Zeiss Axioplan2 microscope with CP Apochromat 100x oil immersion lens, and images were obtained with a Retiga 2000R camera (QImaging) and analysed using Volocity 5.5.1 software (PerkinElmer).

Salmonella phagocytosis and killing assays. Ex-vivo phagocytosis and killing of *S. typhimurium* by blood granulocytes and monocytes were assessed in a gentamicin protection assay and quantified by flow cytometry, confocal microscopy and bacterial culture. Following red blood cell lysis, CD11b⁺ cells were isolated from murine whole blood using anti-CD11b magnetic beads (Miltenyi), according to the manufacturers' instructions. After washing twice in DMEM (Gibco), cells were resuspended at 5.6×10^5 ml⁻¹ in DMEM without antibiotics and seeded at 1×10^5 per well in flat bottomed 96-well plates or at 2×10^5 per well in 8-well chamber slides (Labtek). Plates and slides were incubated at 37 °C and 5% CO₂ for 20 min prior to addition of *S. typhimurium* at a multiplicity of infection (MOI) of 10 *S. typhimurium*: 1 CD11b⁺ cell. To control for autofluorescence and binding of opsonised *S. typhimurium* to the cell surface without phagocytosis, wells containing uninfected cells (negative control), or wells in which both cells and bacteria were fixed with 2% formaldehyde (fixed controls), were incubated in parallel. Plates were incubated for 15 min before addition of gentamicin to a final concentration of 100 µg ml⁻¹ to kill remaining extracellular bacteria (i.e. those which had not been phagocytosed) and incubation for another 30 min. To assess bacterial phagocytosis, after 30 min cells were washed twice with warm sterile PBS and either harvested for flow cytometry, fixed *in situ* for confocal microscopy, or lysed for bacterial culture. Alternatively, to assess bacterial killing, cells were washed twice with warm medium, then reincubated for 2 h in medium containing 10 µg ml⁻¹ gentamicin to prevent extracellular growth of *S. typhimurium*. To assess phagocytosis by flow cytometry, cells were gently scraped from wells (on ice) and resuspended in PBS with 2% formaldehyde before staining with APC-conjugated antibody against Gr-1 and PE-Cy7-conjugated antibody against CD11b. Phagocytosis was quantified as the proportion of GFP⁺ cells after subtraction of the proportion of GFP⁺ cells in the fixed control samples. To assess phagocytosis and bacterial survival by culture, cells were washed twice in warm sterile PBS to remove gentamicin, then lysed with 1% Triton X-100 and 10-fold dilutions were plated onto LB agar, incubated for 18 h at 37°C and colonies counted. To assess phagocytosis by confocal microscopy, cells were fixed with 2% formaldehyde for 15 min at room temperature, then washed twice in PBS containing 5% fetal calf serum, and allowed to air dry before staining and mounting.

Oxidative burst and degranulation assay. Neutrophil oxidative burst was assessed using a modification of the flow cytometric assay described by Richardson *et al.*³⁴. 50 μ L aliquots of fresh whole blood, or bone marrow suspension (cells from 1 femur resuspended in 500 μ L RPMI), were mixed with either 50 μ L of PMA solution (stimulated samples, 25 μ M for whole blood, 2.5 μ M for bone marrow) or sterile PBS (unstimulated) and incubated for 15 minutes in a 37°C water bath. Next 25 μ L of a staining cocktail containing dihydrorhodamine 123 and fluorochrome- conjugated antibodies to cell surface markers in PBS was added and cells reincubated for 5 min at 37°C. 2 ml of red blood cell lysis buffer was added to each tube and incubated in the dark for 15 min at room temperature then centrifuged at 1000 g for 1 min. Cells were washed again with PBS, and resuspended in PBS with 1% paraformaldehyde. The magnitude of the oxidative burst was assessed by rhodamine median fluorescence intensity (MFI) measured in the FL-1 channel, analysed on the same day. Degranulation was measured by the percentage increase in the MFI of surface CD11b expression in stimulated versus unstimulated samples. In each experiment at least three control samples (from age-matched, healthy, uninfected mice) were assayed in parallel with experimental samples. For longitudinal assessment of the oxidative burst and degranulation at different time points in the same experiment, rhodamine fluorescence values were normalized to the average value for the control samples at each time point.

Measurement of heme and hemoglobin. Standard hemoglobin solution was prepared by 0.2 μ m filtration of lysed red blood cell supernatant. The concentrations of standard solutions of hemin and hemoglobin were determined using Quantichrom Heme and Hemoglobin assay kits (BioAssay Systems) in accordance with the manufacturer's instructions. Protein bound plasma heme was quantified by the spectrophotometric method of Shinowara and Waters⁵⁷ using a NanoDrop ND1000 spectrophotometer (Nanodrop Technologies). Briefly heparinised whole blood was centrifuged at 500g for 5 minutes, then plasma was removed and subjected to centrifugation at 15000g for a further 7 minutes to pellet any remaining red blood cells. Two μ L of the remaining plasma was used to determine absorbance at 562, 578, 598, 615 and 675nm wavelengths. Using the absorption values at 562, 578 and 598nm the concentration of plasma hemoglobin was determined by the method of Khan *et al.*⁵⁸. The effect of plasma hemoglobin on the difference in absorption 615–675 nm was determined by preparation of a standard curve of hemoglobin in plasma, and the absorption difference 615–675nm was corrected for the effect of plasma hemoglobin. The corrected absorption at 615–675nm was used to determine plasma heme from a standard curve for hemin in plasma.

Hmox1 expression, HO activity and HO-1 protein assays. *Hmox1* mRNA expression in liver was determined from fresh snips of liver which were snap frozen in liquid nitrogen and stored at –80°C until processing. RNA was extracted (RNAeasy, Qiagen) and DNase1- treated prior to cDNA synthesis. cDNA was quantified using pre-validated inventoried Taqman gene expression assays for *Hmox1* (Mm00516004_m1), *Gapdh* (Mm99999915_g1) and *Tbp* (Mm00446973_m1), and an ABI Prism 7000 sequence detection system (Applied Biosystems). cDNA expression for each sample was standardized using the housekeeping genes *Gapdh* and *Tbp* and expressed as relative fold change compared to healthy control samples. HO activity was measured in whole liver homogenates after RBC lysis, using the method of Foresti *et al.*⁵⁹. To allow comparison between experiments, HO activity was normalized to the average value of at least three control samples in each experiment. Plasma HO-1 was measured by ELISA using a HO-1 Immunoset (Assay Designs) performed in accordance with the manufacturer's instructions.

Statistical analysis. Statistical analysis was performed using Graph Pad Prism 5 software. All statistical analyses were pre-planned and used an alpha value of 0.05. Survival analysis was performed using the Log Rank Mantel Cox test. Continuous data which were approximately normally distributed were analysed using two-sided unpaired or paired student's t-test for pairwise comparisons, or one-way ANOVA with post-hoc testing using Dunnett's multiple comparison test for comparison with the control group, Tukey's multiple comparison test for comparison between multiple groups, and Bonferroni's multiple comparison test for comparison between two or more selected pairs. All data relating to bacterial loads were log₁₀-transformed prior to analysis. Comparison of proportions between groups was performed using Fisher's exact test.

Supplementary Methods Table 2: antibodies used for flow cytometry

| Antigen (clone) | Manufacturer | Fluorochrome |
|--|--------------------|-----------------------------------|
| Gr-1 (RB6-8C5) | eBioscience | FITC, PE, PE-Cy7, APC, efluor 450 |
| CD11b (M1/70) | eBioscience | PE, PE-Cy7 |
| Ly6G (1A8) | Miltenyi Biotec | APC |
| F4/80 (BM8) | eBioscience | FITC, APC |
| CD68 (FA-11) | AbDSerotec | AlexaFluor 647 |
| Ly6C (HK1.4) | Abcam Biolegend | FITC APC |
| Ly6B.2 (7/4) | AbDSerotec | FITC |
| CD115 (AFS98) | eBioscience | PE, APC |
| CD34 (RAM34) | eBioscience | eFluor 660 |
| CD16/32 (93) | eBioscience | PE |
| CD127 / IL-7R α (A7R34) | eBioscience | PERCP-Cy5.5 |
| Sca-1 / Ly6A/E (D7) | eBioscience | PE-Cy7 |
| c-Kit / CD117 (ACK2) | eBioscience | APC-eFluor 780 |
| Mouse haematopoietic lineage cocktail | eBioscience | eFluor 450 |
| HO-1 (SPA-895, polyclonal rabbit) | Assay Designs | None, primary antibody |
| Polyclonal rabbit serum | Covance | None, primary antibody |
| F(ab') ₂ Anti-Rabbit IgG (secondary antibody) | eBioscience | FITC, secondary antibody |

Chapter 6. Do children with malaria have evidence of heme- and heme oxygenase-1 related neutrophil dysfunction?

Prolonged neutrophil dysfunction following Plasmodium falciparum malaria is related to hemolysis and heme oxygenase-1 induction.

The material presented in this chapter represents an article that has been submitted for peer-reviewed publication. In this article we describe neutrophil function in Gambian children with acute *P. falciparum* malaria, and relate this to indices of hemolysis and heme oxygenase-1 induction. Patients were recruited and samples were collected in the context of a larger ongoing, multi-purpose study platform evaluating the immunological, parasitological, genetic and clinical associations of mild and severe malaria. A large team of clinical staff, field workers, clinical laboratory staff and support staff contributed to the process of subject recruitment, sample collection, processing of clinical samples, clinical care of the subjects, and follow-up. Although I routinely performed all the described assays myself, I received additional assistance as necessary from laboratory technicians, Madi Njie and Simon Correa, who were employed by The Medical Research Council Laboratories (The Gambia) to facilitate laboratory aspects of the study platform. Under my direct supervision, they assisted with sample preparation and data acquisition for all of the flow cytometry based assays when multiple assays needed to be performed at the same time, and also assisted with the CRP ELISA. Extraction of RNA from whole blood and PfHRP2 ELISAs were performed in conjunction with another PhD student, Sarah Nogaro, as some samples were to be utilised in both of our PhD projects.

Cover sheet for each 'research paper' included in a research thesis

1. For a 'research paper' already published

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2. For a 'research paper' prepared for publication but not yet published

2.1. Where is the work intended to be published? Journal of Immunology

2.2. List the paper's authors in the intended authorship order

Aubrey J. Cunnington, Madi Njie, Simon Correa, Ebako N. Takem, Eleanor M. Riley, and Michael Walther

2.3. Stage of publication – Undergoing revision from peer reviewers' comments

3. For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)

I had the idea for the study, designed and supervised the running of the study, conducted the laboratory work, analysed and interpreted the results and wrote the manuscript.

Candidate's signature _____

Supervisor or senior author's signature to confirm role as stated in (3)

**Prolonged Neutrophil Dysfunction Following *Plasmodium falciparum* Malaria is
Related to Hemolysis and Heme Oxygenase-1 Induction¹**

Running Title: Impaired neutrophil function in malaria

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Abstract:

It is not known why people are more susceptible to bacterial infections such as non-Typhoid Salmonella (NTS) during and after a malaria infection but, in mice, malarial hemolysis impairs resistance to NTS by impairing the neutrophil oxidative burst. This acquired neutrophil dysfunction is a consequence of induction of the cytoprotective, heme degrading enzyme heme oxygenase-1 (HO-1) in neutrophil progenitors in bone marrow. In this study, we assessed whether neutrophil dysfunction occurs in humans with malaria and how this relates to hemolysis. We evaluated neutrophil function in 58 Gambian children with *Plasmodium falciparum* malaria, and examined associations with anemia, haptoglobin, hemopexin, plasma heme, expression of receptors for heme uptake, and HO-1 induction. Malaria caused the appearance of a dominant population of neutrophils with reduced oxidative burst activity, which gradually normalized over 8 weeks of follow-up. The degree of neutrophil impairment correlated significantly with markers of hemolysis and HO-1 induction. HO-1 expression was increased in blood during acute malaria, but at a cellular level HO-1 expression was modulated by changes in surface expression of the haptoglobin receptor (CD163). These findings demonstrate that neutrophil dysfunction occurs in *P. falciparum* malaria and support the relevance of the mechanistic studies in mice. Furthermore, they suggest the presence of a regulatory pathway to limit HO-1 induction by hemolysis in the context of infection, and indicate new targets for therapeutic intervention to abrogate the susceptibility to bacterial infection in the context of hemolysis in humans.

Introduction

Plasmodium falciparum malaria caused an estimated 655,000 deaths and 216 million cases globally in 2010 (1), but this almost certainly underestimates the indirect health burden (2) which includes increased susceptibility to Gram negative bacterial infections (3-4), particularly non-Typhoidal Salmonella (NTS)³(5-6). In areas with high malaria transmission, these indirect effects of malaria infection may explain more than half of the child mortality (2) and community acquired bacteremia (4). The incidence of NTS closely reflects that of malaria (4, 6-7) and there is compelling evidence that *P. falciparum* malaria increases susceptibility to NTS bacteremia in humans. In The Gambia, the incidence of NTS bacteremia has declined dramatically over the past thirty years, mirroring the decline in the incidence of malaria (7); this observation has since been confirmed in Kenya (4). In the pre-antibiotic era, malaria therapy for treatment of neurosyphilis was frequently complicated by NTS bacteremia even when NTS infection was otherwise very rare (8), and quinine alone often cured endemic malaria-NTS co-infection (9). NTS bacteremia incidence was found to be more closely related to malaria incidence than to stool carriage of NTS (6), and in Kenyan children sickle cell trait was found to have a protective effect against bacteremia, which was entirely dependent on the protection it affords against malaria (4). Several studies have shown that susceptibility to NTS is greatest in the context of severe malarial anemia (5-6), while others have found that the greatest risk occurred in children with recent rather than current malaria infection (10-11).

Studies in mice have confirmed that hemolysis - caused by malaria or in any other way - increases susceptibility to NTS and some other bacterial infections, whereas blood loss alone does not (12-14). We have recently shown in a mouse model of malarial anemia that resistance to *S. typhimurium* is impaired as a result of neutrophil dysfunction caused by liberation of heme during hemolysis and by induction of the cytoprotective heme catabolising enzyme heme oxygenase-1 (HO-1) (15). In this model system, HO-1 induction in myeloid progenitor cells in the bone marrow leads to production of granulocytes with reduced oxidative burst activity, and their mobilization into the blood is enhanced by both hemolysis-derived heme and the response to bacterial co-infection. This results in the accumulation of functionally impaired granulocytes in the circulation which are able to phagocytose *S. typhimurium* but not able to kill the bacteria effectively, providing a new niche for bacterial replication. We found that normal resistance to *S. typhimurium* was restored by inhibition of heme oxygenase with the competitive inhibitor tin protoporphyrin, a drug that can be used to treat hyperbilirubinemia in newborns (16), suggesting that HO inhibitors might represent a novel therapeutic intervention to abrogate the susceptibility to NTS induced by malaria.

Humans and mice with genetic deficiency of subunits of the phagocytic NADPH oxidase, a complex enzyme that catalyzes the generation of superoxide radicals in phagocytic cells, are known to be susceptible to NTS infection (17-18), and the importance of the neutrophil oxidative burst for killing of serum opsonized *Salmonella*

by blood leukocytes from African children has been demonstrated *in vitro* (19).

Impairment of the neutrophil oxidative burst in humans with malaria would thus be a compelling explanation for susceptibility to NTS bacteremia. In the current study we investigated whether the same mechanism may apply in humans, by examining neutrophil function in a cohort of children with predominantly uncomplicated malaria. Despite the fact that this population would not be considered at high risk of NTS co-infection, we found that malaria caused a marked abnormality of function in a large proportion of neutrophils, with impairment of oxidative burst capacity but not degranulation. The severity of the impairment of the neutrophil oxidative burst was strongly associated with hemolysis and prior induction of HO-1, but the duration of impairment was much longer than expected, lasting up to 8 weeks after infection.

Materials and Methods

Study subjects and procedures

The study and all procedures were approved by the Gambian Government / Medical Research Council Laboratories Joint Ethics Committee, and the London School of Hygiene and Tropical Medicine Ethics Committee. All human samples were collected with written informed consent from the participant or from the parent or legal guardian of child participants. Between September and December 2010, 58 Gambian children with *P. falciparum* malaria (defined by compatible clinical symptoms and >5000 asexual parasites/ μ L blood) were recruited within a longitudinal study investigating clinical, immunological and parasitological factors in mild and severe malaria, details of which have been published (20). Briefly, subjects were recruited, without selection for disease severity, from three peri-urban health centres: The Medical Research Council Gate Clinic, Brikama Health Centre and The Jammeh Foundation for Peace Hospital, Serekunda. Initial parasitemia (to determine eligibility for inclusion in the study) was estimated from Field's stained thick blood films and subsequently accurately counted from 50 fields on Giemsa stained thin blood smears. All children underwent full clinical examination and were managed in accordance with Gambian government guidelines. Severe malaria was defined using modified WHO criteria (21): severe anemia, defined as hemoglobin < 6g/dL; lactic acidosis defined as blood lactate > 7mmol/L; cerebral malaria defined as a Blantyre coma score \leq 2 in the absence of hypoglycaemia, with the coma lasting at least for 2 hours; severe

prostration (SP) defined as inability to sit unsupported (children > 6 months) or inability to suck (children \leq 6 month). Children suspected to have concomitant bacterial infections were excluded. For this study children underwent standardized assessment on the day of presentation (day 0) and days 7, 28 and 56, and blood was collected for: thick blood film, full blood count (EDTA), immunological assays (sodium heparin), and RNA (PaxGene tube) (days 0 and 28). On day 0 a thin blood film was prepared and sickle cell status, blood lactate, and glucose were determined. Blood samples were transported to the laboratory on ice within 2 hours of sample collection. Full blood count was performed using a Medonic instrument (Clinical Diagnostics Solutions, Inc). Sickle cell status was determined by metabisulfite test and confirmed on cellulose acetate electrophoresis. Heparinised whole blood was used for assessment of neutrophil oxidative burst and degranulation (350 μ L), intracellular and cell surface flow cytometry (400 μ L), and for neutrophil isolation (1.25mL). On some occasions there was insufficient blood available to perform all assays. Single blood samples were obtained from 6 healthy Gambian children and 10 healthy Gambian adults, all without current or recent malaria, recruited from Brefet village where malaria transmission is now extremely low (22).

Laboratory reagents

All reagents were obtained from Sigma unless specified otherwise. GFP-expressing *Salmonella enterica* serovar Typhimurium pfpv 12023 (*S. typhimurium*) was a gift from Prof. David Holden (Imperial College London, UK), grown to late log-phase in Luria

Bertani (LB) broth supplemented with ampicillin, and kept as frozen stock at -70°C in 10% glycerol.

Neutrophil oxidative burst and degranulation assays

The neutrophil oxidative burst was assessed in minimally manipulated whole blood using a modification of the assay described by Richardson et al. (23). Briefly, 50µL aliquots of blood were mixed with 50 µL of PMA (final concentration 1µM) or PBS (as control) and incubated for 15 min at 37°C in a water bath. Next 25 µL of PBS (unstained sample) or staining cocktail (Dihydrorhodamine 123, PECy7 anti-CD11b (eBioscience, ICRF44), and APC anti-CD15 (Miltenyi Biotec, VIMC6)(unstimulated and stimulated samples)) was added and incubated for 5 min at 37°C in the dark. Ammonium chloride RBC lysis buffer was added for 5 min at room temperature, shielded from light, before washing in PBS and resuspending cells in 1% paraformaldehyde in PBS. Samples were stored at 4°C protected from light, and analysed on the day of collection, using a 3 laser/9 channel CyAn™ ADP flowcytometer with Summit 4.3 software (Dako), after calibration of the FL-1 voltage with fluorescent beads (Spherotech). Data were analysed in FlowJo 7.6 (Tree Star, Inc, OR). The magnitude of the oxidative burst was quantified by the rhodamine median fluorescence intensity (MFI), and degranulation was quantified by the fold increase in surface CD11b MFI from the unstimulated to the stimulated sample. Neutrophils were divided into rhodamine^{Hi} and rhodamine^{Lo} populations at the mid-point of the nadir between peaks.

Flow cytometry for cell surface receptors and intracellular HO-1 expression

Whole blood was subjected to ammonium chloride RBC lysis and, after washing, cell pellets were resuspended in surface marker antibody cocktail (FITC anti-CD91 (AbD Serotec, A2Mr α -2), PE anti-CD16b (BD Pharmingen, CLB-gran11.5), PERCP anti-CD14 (BD Pharmingen, M ϕ P9), APC anti-CD163 (R&D Systems, 215927)) or a similar cocktail instead containing the corresponding manufacturer-matched isotype-control antibodies for CD91 (Mouse IgG1) and CD163 (Mouse IgG1, 11711). Cells were permeabilized with CytoFix/CytoPerm (BD) before intracellular staining with polyclonal anti-HO-1 (Assay Designs, SPA-895) or an equivalent concentration of polyclonal control rabbit serum (Covance), followed by PE-Cy7 conjugated secondary antibody (F(ab')₂ anti-rabbit IgG, Santa Cruz Biotechnology). The expression of HO-1, CD163 and CD91 were quantified as the ratio of MFI to the respective isotype control antibody for the same sample.

Neutrophil isolation and Salmonella phagocytosis and killing assays

CD15⁺ cells were isolated from whole blood, after red blood cell lysis and labelling with APC anti-CD15, using anti-CD15 magnetic beads and MS columns (all from Miltenyi Biotec) according to the manufacturer's instructions. CD15⁺ cells were resuspended in RPMI + 2mM L-Glutamine at a concentration of 10⁷/ml. GFP-expressing *S. typhimurium* (concentration confirmed by serial dilution) were opsonised in 10% pooled healthy Gambian adult serum (derived from 10 donors, as has been described by others (19)) for 20 min in the dark at room temperature. Neutrophils and *S. typhimurium* were

mixed continuously at a ratio of 50:1 at 60 rpm at 37°C. Bacterial counts were assessed at time 0 and 120 min by 10-fold dilutions of aliquots of the neutrophil-*S. typhimurium* suspension in 1% Triton, and plating onto LB agar, with colony forming units counted 16-18 h later. Bacterial killing was quantified as the percentage reduction in bacterial count between time 0 and 120 min. Phagocytosis was assessed after 15 min of incubation, by removing the neutrophil-*S. typhimurium* suspension directly into PBS 4% paraformaldehyde and analysing by flow cytometry. To control for autofluorescence and surface binding of bacteria without phagocytosis, control samples were prepared in an identical manner except that neutrophils and *S. typhimurium* were both fixed with 4% formaldehyde before mixing together. The proportion of cells phagocytosing bacteria was determined by subtraction of the proportion of GFP⁺ cells in the fixed-control samples from that in the respective unfixed sample.

ELISAs. Plasma levels of *P. falciparum* histidine rich protein-2 (PfHRP-2) (Cellabs), hemopexin, haptoglobin (both Genway), C-Reactive protein (CRP, R&D systems) and HO-1 (Enzo Life Sciences) were measured by ELISA. All ELISA assays were performed according to the manufacturer's instructions and samples for each assay were performed in a single batch.

Heme Assay

Total plasma heme (that is plasma hemoglobin plus free- and protein bound-heme) was measured using a colorimetric heme assay kit (QuantiChrom heme, BioAssay Systems).

Quantitative RT-PCR

Total RNA was extracted from PAX tubes using PAXgene blood RNA kits (Qiagen) according to the manufacturer's instructions, and converted into cDNA using a reverse transcription reagent kit (Invitrogen). *HMOX1* (141250) gene expression was determined by qRT-PCR on a DNA Engine Opticon® (MJ Research) using a TaqMan® Probe kit with primers (all Metabion) as described by Hirai et al. (24). 18S rRNA was used as an endogenous reference gene since its expression has been shown to be stable in acute and convalescent samples from malaria cases regardless of disease severity (20), and was amplified with a commercial kit (rRNA primers and VIC labeled probe, Applied Biosystems). Data were analysed using Opticon Monitor 3™ analysis software (BioRad). *HMOX1* expression was quantified as the ratio of the transcript number of the *HMOX1* to 18S rRNA.

Estimation of total parasite biomass

Total parasite biomass was calculated from plasma PfHRP-2 concentration using the method of Dondorp et al. (25). This assumes that PfHRP-2 concentration is an integral of all PfHRP-2 released in preceding rounds of schizogony (when infected erythrocytes rupture to release merozoites), and is therefore a reliable indicator of cumulative

hemolysis since the start of the infection (25). We modified the calculation to account for the relatively higher blood volume at lower body weight in small children (26). To account for variation in size of children, parasite biomass was expressed as parasites per kg body weight.

Statistics

The study was designed to detect a 30% difference in neutrophil oxidative burst activity between samples at day 0 and day 28 with 80% power at the 0.05 significance level, allowing for 15% loss to follow up. Statistical analysis was performed using PASW Statistics 18 (SPSS Inc). Variables were examined for normality of distribution, and most were found to be non-normal. Two-tailed non-parametric tests appropriate for paired or related repeated measures were used to compare longitudinal data at different time points, and correlation was tested with Spearman's rho correlation. In order to normalize distribution for general linear model analysis, some variables were Log_{10} transformed or converted to binary variables. Haptoglobin concentrations showed a bimodal distribution, and were thus converted to a binary variable (<0.349 mg/mL, the lowest value observed in healthy control samples, or ≥ 0.349 mg/mL). Sample volumes did not allow for some assays to be performed at time points after day 0, in which case values from six healthy control children were presented for comparison, but not for formal statistical analysis.

Results

Subjects

58 children with *P. falciparum* malaria were recruited to the study, 55 (95%) of whom had uncomplicated malaria (Table I). Four children had recurrent episodes of parasitemia during the course of follow-up and were excluded from longitudinal analyses; another 13 children were lost to follow-up or withdrew consent. Thus, at days 7, 28, and 56 the number (%) of children in follow-up were 52 (89.6%), 46 (79.3%), and 41 (70.1%) respectively.

Prolonged impairment of the neutrophil oxidative burst

We assessed the PMA-stimulated oxidative burst of neutrophils using a whole blood flow cytometric assay based on the oxidation of dihydrorhodamine 123 to its fluorescent derivative rhodamine, where the magnitude of the oxidative burst is quantified by the rhodamine fluorescence intensity (23). The assay was modified to allow simultaneous assessment of degranulation based on upregulation of CD11b (27), and surface staining of CD15 to identify neutrophils. We found that neutrophils (Fig. 1A) from subjects with acute malaria (day 0) showed an abnormal, bimodal, distribution of oxidative burst activity (Fig. 1B), with distinct populations of rhodamine^{Hi} and rhodamine^{Lo} cells, whereas CD11b expression showed a unimodal distribution (Fig. 1C). Overall, neutrophil rhodamine median fluorescence intensity (MFI) increased significantly over time (Fig. 1D), but in view of the bimodal distribution of neutrophil rhodamine fluorescence, we also compared the proportion of cells that

were rhodamine^{Lo} and the rhodamine MFI of the rhodamine^{Lo} cells over time. The proportion of rhodamine^{Lo} cells decreased significantly during the convalescent period (Fig. 1E) but remained above that of healthy controls for at least 56 days; the rhodamine MFI of the rhodamine^{Lo} cells also significantly increased over time and was similar to that of healthy controls by day 56 (Fig. 1F). In contrast, there was no evidence of abnormalities in neutrophil degranulation as assessed by CD11b expression (Fig. 1G). Of interest, the rhodamine MFI of the rhodamine^{Hi} cells was higher on days 0 and 7 after presentation ($P=0.04$, and $P<0.001$ respectively, Wilcoxon matched pairs test), than on day 56 (Fig. 1 H), suggesting that the oxidative burst is primed in these neutrophils (28). These findings are consistent with our observations in mice that hemolysis and infection can prime the oxidative burst of mature, circulating neutrophils whilst simultaneously mobilizing immature neutrophils with impaired oxidative burst activity from the bone marrow (15). However the duration of these neutrophil abnormalities following *P. falciparum* infection was longer than we expected.

Hemolysis and neutrophil dysfunction

We have previously shown that hemolysis-derived heme impairs neutrophil function during malaria infection in mice through two related mechanisms: mobilization of functionally immature neutrophils from bone marrow, and impairment of the oxidative burst capacity of developing neutrophils due to HO-1 induction in bone marrow progenitors (15). The severity of malarial hemolysis can be inferred from levels of the plasma proteins haptoglobin and hemopexin, which provide sequential lines of

defence against heme-mediated toxicity by binding cell-free hemoglobin and cell-free heme respectively (29). Only once haptoglobin is depleted do levels of hemopexin begin to fall, indicating that heme is being released from cell-free hemoglobin (30).

To assess the extent of hemolysis in study participants we measured erythrocyte count and total parasite biomass (Table I), total plasma heme (Fig. 2A), haptoglobin (Fig. 2B) and hemopexin (Fig. 2C), and examined their correlation with the proportion and function of rhodamine^{Lo} neutrophils (Table 2). As expected, total plasma heme levels were significantly greater on day 0 than on day 28 (Fig. 2A). Levels of haptoglobin showed a bimodal distribution (Fig. 2B), consistent with the expected depletion of haptoglobin by hemolysis in some subjects (30), but also increased production of haptoglobin as part of the acute phase response in other subjects (31). Hemopexin levels, however, were relatively normally distributed and very similar to healthy controls (Fig. 2C), suggesting that in these subjects with predominantly uncomplicated malaria, hemolysis does not liberate sufficient cell-free heme to deplete plasma hemopexin (29-30).

Since rhodamine^{Lo} cells may be similar to the functionally immature granulocytes released into the circulation during malaria and NTS infection in mice (15), we assessed whether their frequency was associated with markers of inflammation and hemolysis (Table II). The proportion of rhodamine^{Lo} cells was significantly correlated with C-reactive protein (CRP) concentration, which would be consistent with their

mobilization as part of an inflammatory response (32), but did not correlate directly with measures of hemolysis. By contrast, the magnitude of the oxidative burst among rhodamine^{Lo} neutrophils (rhodamine^{Lo} MFI) showed significant univariate correlations with erythrocyte count and haptoglobin concentration, and significant negative correlations with total parasite biomass, CRP, and total plasma heme. Since these variables are likely to be highly correlated with each other, we analysed their effects on rhodamine^{Lo} neutrophil MFI using a general linear model. After stepwise elimination of the least significant variables in the model, only parasite biomass remained significantly associated with rhodamine^{Lo} neutrophil MFI ($F=16.036$, $P < 0.001$). Overall these results are consistent with the acute phase inflammatory response being the primary determinant of release of rhodamine^{Lo} neutrophils into the circulation in children with uncomplicated malaria, but parasite burden and consequent hemolysis being the major determinant of the impairment of the oxidative burst in rhodamine^{Lo} neutrophils.

Factors associated with HO-1 induction

In malaria-infected mice we found that heme-mediated HO-1 induction in neutrophil progenitors in bone marrow was necessary to impair the oxidative burst of developing neutrophils (15). Since there were no clinical indications for bone marrow aspiration in any of the study subjects, our analysis of the HO-1 pathway was restricted to parameters measurable in peripheral blood, namely plasma HO-1, whole blood *HMOX1* gene expression and HO-1 protein expression in peripheral blood cells. Also, since the induction of cellular HO-1 by haptoglobin-hemoglobin or heme-hemopexin

complexes depends on the presence of surface receptors for their uptake (CD163 and CD91 respectively) (31, 33-34), we examined CD163 and CD91 expression on monocytes and neutrophils.

As previously reported in mice (15) and humans (35), HO-1 expression (assessed by fluorescence intensity) in circulating neutrophils was not increased in acute malaria infection compared with convalescence (data not shown). In contrast, monocyte HO-1 expression was higher on day 0 than on days 7 or 28 (Fig. 3A). Plasma HO-1 was higher in subjects on day 0 than in healthy control children (Fig. 3B), and as we have previously reported (35), whole blood *HMOX1* expression was significantly higher on day 0 than following recovery on day 28 (Fig. 3C).

In control subjects, CD163 and CD91 were expressed on the surface of monocytes but were not detectable on neutrophils (Fig. 3D). In children with malaria, monocyte CD163 expression was significantly lower at day 0 than on days 7 and 28 (Fig. 3E), whereas CD163 remained undetectable on neutrophils from most subjects (Fig. 3F). CD91 expression did not change significantly over time on monocytes (Fig. 3G) or neutrophils (Fig. 3H), although when all subjects were considered together, there did appear to be very low level CD91 expression on neutrophils at all time points.

To further explore the likely pathways of HO-1 induction during malaria infection, we constructed a simple conceptual model (Fig. 4), beginning with the malaria parasite as the cause of hemolysis, inflammation, and tissue hypoxia/ischemia (36) - all of which may induce HO-1 expression (37) - and analysed univariate correlations between the various measures of HO-1 induction. As expected, parasite biomass was strongly correlated with total plasma heme and CRP. Plasma HO-1 correlated much more

strongly with CRP and lactate than with plasma heme, supporting the idea that it may be released in response to harmful and inflammatory stimuli. Surprisingly, however, neither whole blood *HMOX1* expression nor monocyte specific HO-1 correlated significantly with total plasma heme, CRP, or lactate. As the haptoglobin receptor CD163 has been reported to be down regulated during inflammation (38), and to be shed from the cell surface during acute, uncomplicated malaria infections (39), we explored whether monocyte specific HO-1 expression might be confounded by changes in expression of CD163, using a general linear model controlling for the effect of CD163 expression. This revealed a significant interaction between total plasma heme and CD163 expression, but strong independent associations between monocyte HO-1 and total plasma heme ($F=15.1$, $P < 0.001$) and monocyte HO-1 and CD163 expression ($F=9.378$, $P = 0.003$). In other words, monocyte HO-1 induction would closely correlate with total plasma heme, but down regulation of surface CD163 prevents the uptake of hemoglobin-haptoglobin complexes and hence limits HO-1 induction. This explains in part the discordance between different measures of the HO-1 induction pathway in blood. In summary, however, this analysis demonstrates that HO-1 protein expression in myeloid cells can be increased by hemoglobin and heme liberated during malarial hemolysis, although this effect is limited by reductions in surface CD163 expression.

Neutrophil oxidative burst and prior HO-1 induction

In mice we had observed that suppression of the oxidative burst of circulating neutrophils by hemolysis required the release of immature neutrophils from bone

marrow, requiring either a lag time, or an additional stimulus (such as NTS infection), to cause these cells to rapidly enter the circulation (15). Having observed that an abnormal population of neutrophils was present for prolonged time following *P. falciparum*, we looked for evidence of an association between HO-1 induction on day 0, and neutrophil oxidative burst on day 7. Using the ratio of day 0 to day 28 whole blood *HMOX1* expression as an indicator of induction in acute malaria, we found a significant inverse correlation with the rhodamine MFI of rhodamine^{Lo} neutrophils on day 7 (Spearman's correlation coefficient = -0.352, *P* = 0.028, n=39), whereas there was no significant correlation with the proportion of rhodamine^{Lo} cells on day 7 (Spearman's correlation coefficient = -0.101, *P* = 0.542, n=39). Although the kinetics of the process of HO-1 induction, suppression of oxidative burst capacity in developing neutrophils, and subsequent release of the functionally immature neutrophils into the circulation are unknown, the observed association between HO-1 induction during acute disease and neutrophil dysfunction during early convalescence is consistent with this sequence of events.

Salmonella phagocytosis and killing

Since we have observed that neutrophil killing (but not phagocytosis) of *S. typhimurium* is defective in malaria-infected mice (15), we assessed the *ex-vivo* killing and phagocytosis of serum-opsonised *S. typhimurium* by neutrophils isolated from whole blood of subjects and controls from whom sufficient blood remained after the preceding assays (Fig. 5A–C). Bacterial killing, calculated as the reduction in the viable bacterial count 2 hr after co-culture, was slightly higher on day 0 than at subsequent

time points (Fig. 5A). There was no significant correlation between bacterial killing on day 0 and either the proportion of rhodamine^{Lo} neutrophils, the rhodamine MFI of the rhodamine^{Lo} neutrophils, total plasma heme or parasite biomass (data not shown). Bacterial phagocytosis, determined by flow cytometric analysis of the proportion of GFP⁺ neutrophils after 15 min co-culture (Fig. 5B), did not vary significantly over time following infection (Fig. 5C). However phagocytosis at day 0 was inversely correlated with parasite biomass (n=26, Spearman's correlation coefficient = -0.512, P=0.008), and with total plasma heme (n=28, Spearman's correlation coefficient = -0.441, P=0.019) (Fig. 5D). Taken together with the neutrophil oxidative burst assay data (Fig. 1 F,H), these data indicate that although there may be some degree of (heme-mediated) priming of neutrophil function in children with acute malaria (day 0) which enhances bacterial killing during acute illness (40), phagocytosis of *S. typhimurium* by circulating neutrophils becomes increasingly impaired with increasing parasite burden and increasingly severe hemolysis, and that the ability of neutrophils to kill *S. typhimurium* once they are phagocytosed might also become impaired.

Discussion

Although the association between malaria infection and susceptibility to NTS bacteremia has been recognized for almost a century (9), the mechanism has been elusive. The strongest association is with severe malarial anemia (5-6) and at least two other conditions associated with hemolytic anemia - sickle cell disease (41) and acute bartonellosis (42) - also predispose to NTS bacteremia. This is likely a result of both the

nature of the defect in host-defence induced by malaria, and also the prevailing epidemiology of invasive bacterial infection: NTS is one of the most common causes of bacteremia in Sub-Saharan Africa (43-44). In a mouse model we recently showed that hemolysis due to malaria or phenylhydrazine treatment impaired resistance to *S. typhimurium*, which could be recapitulated by treatment with hemin, and abrogated by treatment with the HO inhibitor tin protoporphyrin (15). We found that bacteria accumulated in circulating neutrophils, and that these neutrophils were defective in killing *S. typhimurium*, associated with impairment of their oxidative burst response which is an essential mechanism for killing *S. typhimurium* (18). This was due to heme-mediated induction of HO-1 in granulocyte precursors in bone marrow, causing immature neutrophils leaving the bone marrow to have a reduced capacity to mount an effective oxidative burst. It is not known whether bacteria accumulate preferentially in neutrophils in humans with malaria and NTS co-infections, but in the pre-antibiotic era neutrophils and NTS were often found co-localized in abscesses which formed at the site of intramuscular quinine injection in co-infected individuals (9). Although neutrophil function has not been extensively studied in malaria, there are several case reports of patients with severe malarial hemolysis spontaneously developing fungal sepsis (45-47), which is typically associated with neutropenia and neutrophil dysfunction.

The present study was designed to determine whether Gambian children with *P. falciparum* malaria have evidence of neutrophil dysfunction similar to that observed in mice infected with *P. yoelli* 17XNL. We hypothesized that the neutrophil oxidative burst would be impaired in children with malaria, and the severity of this impairment would be related to hemolysis and HO-1 induction. However, we also predicted that

the impairment of neutrophil function would be relatively mild, because declining malaria transmission in The Gambia has led to a decrease in the incidence of severe malarial anemia and malaria-NTS co-infection (7). Overall, our results are consistent with our hypothesis: the oxidative burst activity of circulating neutrophils was profoundly abnormal in subjects with acute *P. falciparum* malaria, and most severely impaired in children with the highest parasite burdens and greatest hemolysis, albeit the magnitude of this impairment did not translate into a clinically significant defect in neutrophil killing of *S. typhimurium in vitro*. We also found that these abnormalities persisted for at least 56 days, and that bacterial phagocytosis and killing appeared to deteriorate during the early convalescent period (up to 28 days), findings which may be consistent with descriptions of increased susceptibility to NTS bacteremia in children who have recently had malaria (6, 10), and the gradual emergence of dysfunctional neutrophils from bone marrow following HO-1 induction during the acute infection (15). To facilitate comparison with our studies in mice (15), we assessed neutrophil function using PMA as the stimulus for the oxidative burst. Although this is not a physiological stimulus, the advantages of this method are: i) that it produces a strong oxidative burst (48-49) which is clearly distinguished from any low-level activation caused by malaria infection *per se*, ii) it is not dependent on phagocytosis (which might also be impaired by malaria) (48-49), and iii) variations in the magnitude of the PMA-induced oxidative burst are directly related to the ability of humans to survive infections (50).

Consistent with data from mice and humans (15, 35, 51), we observed induction of HO-1 during acute malaria. Although dissecting the causal and consequential pathways of HO-1 induction is difficult in an observational study, we constructed a conceptual

model of likely pathways leading to HO-1 induction based on existing literature (Figure 4) (37), and used this model to guide our statistical analysis. Plasma HO-1 levels correlated more strongly with plasma lactate and CRP concentrations than with plasma heme concentrations, suggesting that plasma HO-1 may be predominantly a response to inflammation and hypoxia and a marker of cell damage. As noted previously, intracellular HO-1 protein expression was not significantly upregulated in acute malaria in circulating neutrophils (35), presumably because they lack CD163 expression, whereas monocytes did show evidence of increased HO-1 protein expression in acute malaria. However there was not a significant univariate association between total plasma heme concentration and HO-1 expression in monocytes, which could be explained statistically by the reduced levels of monocyte surface CD163 expression in acute malaria. This explanation is fully consistent with the subjects in this study having only mild hemolysis (none had severe malarial anemia and only half had low haptoglobin levels) and with the assumption (as hemopexin levels were not depleted) that very little of the total circulating heme represents cell-free heme. In this case, HO-1 induction due to hemolysis would be expected to proceed predominantly through CD163-mediated uptake of haptoglobin-hemoglobin complexes, and reduction in surface CD163 would be expected to limit HO-1 induction (38). In contrast, severe hemolysis would be expected to generate cell free heme, and lead to HO-1 induction and heme catabolism in cells expressing the surface receptor (CD91) for heme-hemopexin complexes, which appears to be invariantly expressed during infection. Indeed, we previously found elevated carboxyhemoglobin levels, an indirect measure of HO activity, only in children with severe malarial anemia suggesting that heme catabolism is constrained in acute malaria and only detectably increased in cases with

the most severe hemolysis (52). It is conceivable that by reducing CD163 expression in the context of infection, monocytes are rendered relatively resistant to HO-1 induction by hemolysis, perhaps preventing HO-1-mediated impairment of their normal inflammatory responses (38, 53). However, it is presently unknown whether either CD163 or CD91 expression are required for HO-1 induction in immature myeloid cells and their progenitors in human bone marrow. If HO-1 induction in bone marrow is responsible for the observed neutrophil dysfunction (as it is in mice (15)), it may be either independent of CD163, or CD163 may not be down regulated in the bone marrow to the same extent as in blood monocytes. We did not examine the effect of *HMOX1* promoter (GT)_n length polymorphisms in this study because the majority of subjects had uncomplicated malaria with mild hemolysis and we expected that under these circumstances genetic polymorphisms would have a relatively small effect and a much larger sample size would have been required (35).

In apparent contradiction of our findings in mice, killing of *S. typhimurium* by neutrophils was not noticeably impaired on day 0, but deteriorated over the next 4 weeks. A possible explanation is that, despite the accumulation of a rhodamine^{Lo} neutrophil population, the oxidative burst of the rhodamine^{Hi} neutrophil population was higher on days 0 and 7 than at later time points, and enhanced bactericidal activity among rhodamine^{Hi} cells may initially compensate for the lack of killing among the rhodamine^{Lo} population, particularly if the rhodamine^{Hi} cells showed preferential phagocytosis of the opsonised *S. typhimurium*. This finding is perhaps not surprising because none of the children had severe hemolysis or severe anemia, which are the major risk factors for malaria-NTS coinfection (5-6), suggesting that their bactericidal capacity should not be seriously impaired. We predict, however, that bacterial killing

would be seriously impaired in children with severe hemolytic anemia. The acquired defect of neutrophil function that we observed in children with malaria in this study might be considered analogous the neutrophil defect observed in female carriers of X-linked chronic granulomatous disease, where around 50% of neutrophils have defective oxidative burst activity due to random inactivation of the X-chromosome, but there is not increased susceptibility to infection (54) and *in vitro* bactericidal activity may be normal (55). However in some carriers, inactivation of the X-chromosome becomes skewed, and when less than 15% of neutrophils are able to make a normal oxidative burst, susceptibility to infections is markedly increased (17). Consistent with this, in a large European registry of chronic granulomatous disease patients, *Salmonella* has been reported as by far the most common cause of septicemia (whilst fungi and *Staphylococcus aureus* are the most common causes of chronic lung and deep tissue infections, due to the persistent defect in oxidative burst activity) (17). We propose that in patients with malaria a threshold proportion of abnormal neutrophils in blood may be required to produce susceptibility to NTS, and when this threshold is exceeded the degree of susceptibility may then also be determined by the magnitude of the impairment of oxidative burst capacity. Factors which may determine whether the proportion of dysfunctional neutrophils exceeds this putative threshold may include: the duration of infection and the severity of the inflammatory response, which may both influence the mobilization of dysfunctional neutrophils from bone marrow (15); the severity of hemolysis, since cell-free heme itself promotes neutrophil mobilization (15); and possibly host factors such as age and genetic background. The strong correlation we observed between impaired neutrophil oxidative burst and total parasite biomass allows us to predict that children with high parasite burden (who are

also most likely to have severe hemolysis) would have the most impaired oxidative burst. These children may also have depleted hemopexin levels and accumulate cell free heme, which can itself mobilize neutrophils from bone marrow (15), perhaps increasing the proportion of abnormal neutrophils above a threshold required to induce susceptibility to NTS.

The prolonged duration of abnormal neutrophil oxidative burst activity, extending up to 8 weeks after acute infection in some subjects, was unexpected. It seems unlikely that these children would all have an underlying defect in neutrophil function, but one possibility that deserves consideration is that the duration of neutrophil impairment may be linked to persisting hemozoin. Hemozoin is an insoluble hemin polymer, the end product of hemoglobin digestion inside the parasitised red cell, which is able to induce HO-1 (and impair the oxidative burst in phagocytes) but is not catabolised by it (56-57), resulting in prolonged persistence in the circulation, reticuloendothelial system, and bone marrow, of hemozoin-laden phagocytes (58).

The major limitations of our study are that most subjects had relatively mild hemolysis and that we did not have bone marrow samples to confirm HO-1 induction in neutrophil progenitors. To recruit a significant number of subjects with severe malarial hemolysis would require a much larger study, probably conducted in a higher transmission setting. To study prospectively whether the severity of neutrophil dysfunction at recruitment correlated with susceptibility to NTS bacteremia during convalescence would require an even larger and more logistically complex study. To obtain bone marrow aspirates from children with malaria would be difficult to justify ethically unless appropriate sedation and analgesia (usually requiring a general

anaesthetic) could be provided without placing them at additional risk of complications.

Nevertheless, our findings have a number of important implications. First we show that the oxidative burst capacity of a large proportion of neutrophils is markedly abnormal in children with *P. falciparum* malaria, supporting the translation of findings in a mouse model (15). Second, neutrophil function recovers only very slowly over the two months after treatment, providing an explanation for the association of susceptibility to NTS with recent malaria (10-11). In the mouse model, hemolysis-induced neutrophil dysfunction could be abrogated by competitive inhibition of HO with tin protoporphyrin (15), but using this treatment in acute malaria would be challenging because HO-1 is also important for tolerance to cytotoxic effects of cell free heme in mouse models (51, 59). Alternative therapeutic strategies would be administration of tin protoporphyrin upon completion of antimalarial treatment, with the aim of restoring neutrophil function during convalescence and preventing the susceptibility to NTS caused by recent malaria, or prioritisation of children at greatest risk of persistent neutrophil dysfunction for prophylactic antibiotic treatment. Third, we propose the down-regulation of the haptoglobin receptor CD163 on the surface of blood monocytes during acute *P. falciparum* malaria as a novel host-protective homeostatic response to hemolysis and inflammation, which may prevent HO-1 induction from impairing monocyte function. Further experimental studies are needed to confirm the effects of manipulating CD163 expression during infections, but manipulation of this axis would hold promise for both the modulation of inflammation and optimization of iron re-utilization during chronic infections.

Acknowledgements: We are grateful to David Conway who helped to set up and manage the recruitment process used in this study, and to the clinic, laboratory, and administrative staff, field workers, and subjects who participated in the study.

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Footnotes

¹ This study was supported by a clinical research training fellowship from the Medical Research Council (UK) (G0701427) to A.J.C. and by MRC Institute funding to the MRC Laboratories, The Gambia.

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³Abbreviations used in this paper: NTS, non-Typhoid Salmonella; HO-1, heme oxygenase-1; MFI, median fluorescent intensity; CRP, C-reactive protein

FIGURE 1. *P. falciparum* malaria causes prolonged impairment of the neutrophil oxidative burst. (A) Representative FACS plots showing the gating of the neutrophil population based on forward scatter and side scatter characteristics followed by selection of single cells based on pulse width and forward scatter, and selection of the CD15⁺ population. (B,C) Rhodamine (B) and CD11b (C) fluorescence of unstimulated (filled histogram) and PMA-stimulated neutrophils (unfilled histogram) on days 0, 7, 28 and 56 after presentation with *P. falciparum* malaria. Representative plots from a healthy control child are also shown for comparison. Rhodamine^{Hi} and rhodamine^{Lo} populations of neutrophils were defined for each sample by partition at the nadir of the bimodal distribution, and percentages of the total cells in each population are shown (B). (D-H) Longitudinal analyses of neutrophil function, compared using Friedman's two way ANOVA for all subjects with valid data at all time points. Healthy controls are also shown for comparison, but not included in the statistical analysis (D,E,G). Horizontal lines represent medians. n stated for valid data at every time point. (D) Rhodamine MFI for all neutrophils (rhodamine^{Hi} and rhodamine^{Lo} considered together as a single population); n=32. (E) Proportion of neutrophils that are rhodamine^{Lo}; n=28. (F) Rhodamine MFI of rhodamine^{Lo} neutrophils; n=28. (G) Degranulation of all neutrophils, assessed by fold change in surface CD11b (PMA stimulated CD11b MFI: unstimulated CD11b MFI); n=29. (H) Rhodamine MFI of rhodamine^{Hi} neutrophils; n=32.

FIGURE 2. Indicators of hemolysis in subjects with *P. falciparum* malaria. (A) Total plasma heme on day 0 and day 28, compared using Wilcoxon matched pairs test for those with data at both time points ($n=32$). (B) Distribution of plasma haptoglobin levels at day 0, $n=57$. For comparison levels in six healthy control children are also shown. (C) Distribution of plasma hemopexin levels at day 0, $n=49$. For comparison levels in six healthy control children are also shown.

FIGURE 3. Factors associated with heme oxygenase-1 expression in *P. falciparum* malaria. (A-C): heme oxygenase-1 induction. (A) Representative FACS analysis of HO-1 induction in monocytes showing exclusion of red blood cells, gating on single cells, and subsequent definition of monocytes as CD14⁺. Histograms show fluorescence of monocytes stained intracellularly with control antibody (filled) or anti-HO-1 antibody (unfilled) followed by a secondary detection antibody. Quantitative data for HO-1 expression in monocytes (ratio of anti-HO-1 to control antibody fluorescence) for all subjects on days 0, 7 and 28 are shown in the right hand panel, compared using Friedman's two way ANOVA (*n*=20). (B) The distribution of Plasma HO-1 on day 0, *n*=57. For comparison levels in six healthy control children are also shown. (C) Whole blood *HMOX1* RNA expression, measured by qRT-PCR, compared between samples on day 0 and day 28 using Wilcoxon matched pairs test for those with data at both time points (*n*=42). (D-H) CD163 and CD91 expression on monocytes and neutrophils. (D) Representative flow cytometry analysis of healthy control subject neutrophils (CD14⁻CD16b⁺) and monocytes (CD14⁺CD16b⁻), showing staining with respective control (filled histogram) and anti-CD91 or anti-CD163 antibodies (unfilled histograms). Quantitative data for expression (ratio to control antibody fluorescence) of CD163 in monocytes (E) and neutrophils (F), and CD91 in monocytes (G) and neutrophils (H), compared using Friedman's two way ANOVA for all subjects with valid data at all time points (CD163, *n*=19; CD91 *n*=20). Horizontal bars represent medians. *n* stated for those with valid data at all time points.

FIGURE 4. A conceptual model of the pathways leading to HO-1 induction in acute *P. falciparum* malaria. The biomass of *P. falciparum* parasites within the subject was considered to be the quantifiable cause of hemolysis, inflammation and tissue hypoxia / ischemia, all of which are stimuli for induction of HO-1. The variable measured to quantify each step in the pathway is indicated in italics. Associations between variables were tested using Spearman's correlation as indicated by lines with arrows showing the hypothesized relationship from cause to effect. Significant correlations are denoted by solid lines, and line thickness indicates the significance of the correlation (thin line $0.01 \leq P < 0.05$, medium thickness line $0.001 \leq P < 0.01$, heavy line $P < 0.001$), whereas non-significant correlations are denoted by dashed lines. The strength of correlation is indicated by Spearman's rho adjacent to significant correlation lines.

FIGURE 5. *Ex-vivo* killing and phagocytosis of *S. typhimurium* by neutrophils from children with *P. falciparum* malaria. (A) Killing of *S. typhimurium*. Neutrophils isolated on days 0, 7, 28 and 56 after presentation with *P. falciparum* malaria were mixed with *S. typhimurium* at ratio of 50:1 and killing expressed as the percentage reduction in bacterial numbers after 2 h incubation. Statistical comparison using Friedman's two way ANOVA for all subjects with valid data at all time points, $n=18$. Data from control subjects shown for comparison. (B) Representative flow cytometry plots showing phagocytosis of GFP⁺ *S. typhimurium* by neutrophils isolated on day 0. RBCs and debris were excluded based on forward scatter and side scatter characteristics, then single cells were selected based on pulse width and forward scatter characteristics (upper row). The proportion of GFP⁺CD15⁺ cells was determined in samples where both neutrophils and *S. typhimurium* were fixed in 4% formaldehyde prior to incubation (to control for surface binding without phagocytosis), and in unfixed samples (lower row). (C) Phagocytosis of *S. typhimurium*. Neutrophils isolated on days 0, 7, 28 and 56 after presentation with *P. falciparum* malaria were mixed with *S. typhimurium* at a ratio of 50:1 and phagocytosis expressed as the percentage GFP⁺ neutrophils after 15 min incubation. The percentage GFP⁺ cells was calculated by subtracting the proportion of GFP⁺ cells in formaldehyde fixed samples from that in unfixed samples. Statistical comparison using Friedman's two way ANOVA for all subjects with valid data at all time points, $n=7$. Data from control subjects shown for comparison. (D) Correlation of phagocytosis (on day 0) with parasite biomass on day 0 (left hand panel, $n=26$) and total plasma heme on day 0 (right hand panel, $n=28$).

Table I. Demographic, clinical and laboratory characteristics at recruitment.

| Variable | Category | n*(%) | Median (IQR) |
|---|--|--------------|--|
| Sex | Male | 36 (62) | |
| | Female | 22 (38) | |
| Ethnicity | Mandingo | 25 (43) | |
| | Fula | 10 (17) | |
| | Wolof | 7 (12) | |
| | Manjago | 5 (9) | |
| | Jola | 5 (9) | |
| | Serere | 4 (7) | |
| | Aku | 1 (2) | |
| | Fanti | 1 (2) | |
| Age (years) | | 58 | 8 (4-12) |
| Severity | Mild | 55 (95) | |
| | Severe | 3 (5) | |
| | prostration | 2 | |
| | lactic acidosis | 1 | |
| Plasmodium species | <i>P. falciparum</i> | 55 (95) | |
| | <i>P. falciparum</i> and <i>P. malariae</i> | 3 (5) | |
| | | | |
| Sickle cell screen | Negative | 53 (91) | |
| | AS | 1 (2) | |
| | Not done | 4 (7) | |
| Hemoglobin, g/dl | | 58 | 11.5 (9.98-12.5) |
| Erythrocyte count, x10 ¹² /L | | 58 | 4.26 (3.83-4.66) |
| Mean corpuscular volume, fL | | 56 | 76.9 (73.5-80.7) |
| Leukocyte count, x10 ⁹ /L | | 57 | 8.50 (6.74-10.3) |
| Granulocyte count, x10 ⁹ /L | | 57 | 6.21 (3.91-7.85) |
| Parasite density, parasites/μL | | 57 | 92800 (28200-219000) |
| Parasite biomass, parasites/kg | | 55 | 1.23x10 ¹⁰ (5.21x10 ⁹ -2.14x10 ¹⁰) |
| C-reactive protein, mg/L | | 46 | 106.4 (64.5-234.3) |
| Lactate, mmol/L | | 42 | 2.0 (1.6-2.45) |

* Data were not available for every variable for every subject. IQR, Interquartile range.

Table II. Association of neutrophil dysfunction with hemolysis on day 0.

| Variable | % Rhodamine ^{Lo} Neutrophils | | | Rhodamine MFI of Rhodamine ^{Lo} Neutrophils | | |
|---------------------|---------------------------------------|-------------|-------|--|-------------|--------|
| | n* | Correlation | | n* | Correlation | |
| | | coefficient | P | | coefficient | P |
| Parasite biomass/kg | 53 | 0.208 | 0.134 | 53 | -0.539 | <0.001 |
| Erythrocyte count | 56 | -0.001 | 0.992 | 56 | 0.280 | 0.037 |
| Haptoglobin | 55 | 0.004 | 0.976 | 55 | 0.292 | 0.031 |
| Total plasma heme | 54 | 0.222 | 0.106 | 54 | -0.268 | 0.050 |
| C-reactive protein | 44 | 0.350 | 0.020 | 44 | -0.452 | 0.002 |

Indicators of hemolysis and inflammation were assessed for correlation with the proportion of rhodamine^{Lo} neutrophils and with the rhodamine MFI of rhodamine^{Lo} neutrophils on day 0 using Spearman's correlation. *Data were not available for every variable for every subject.

Figure 1

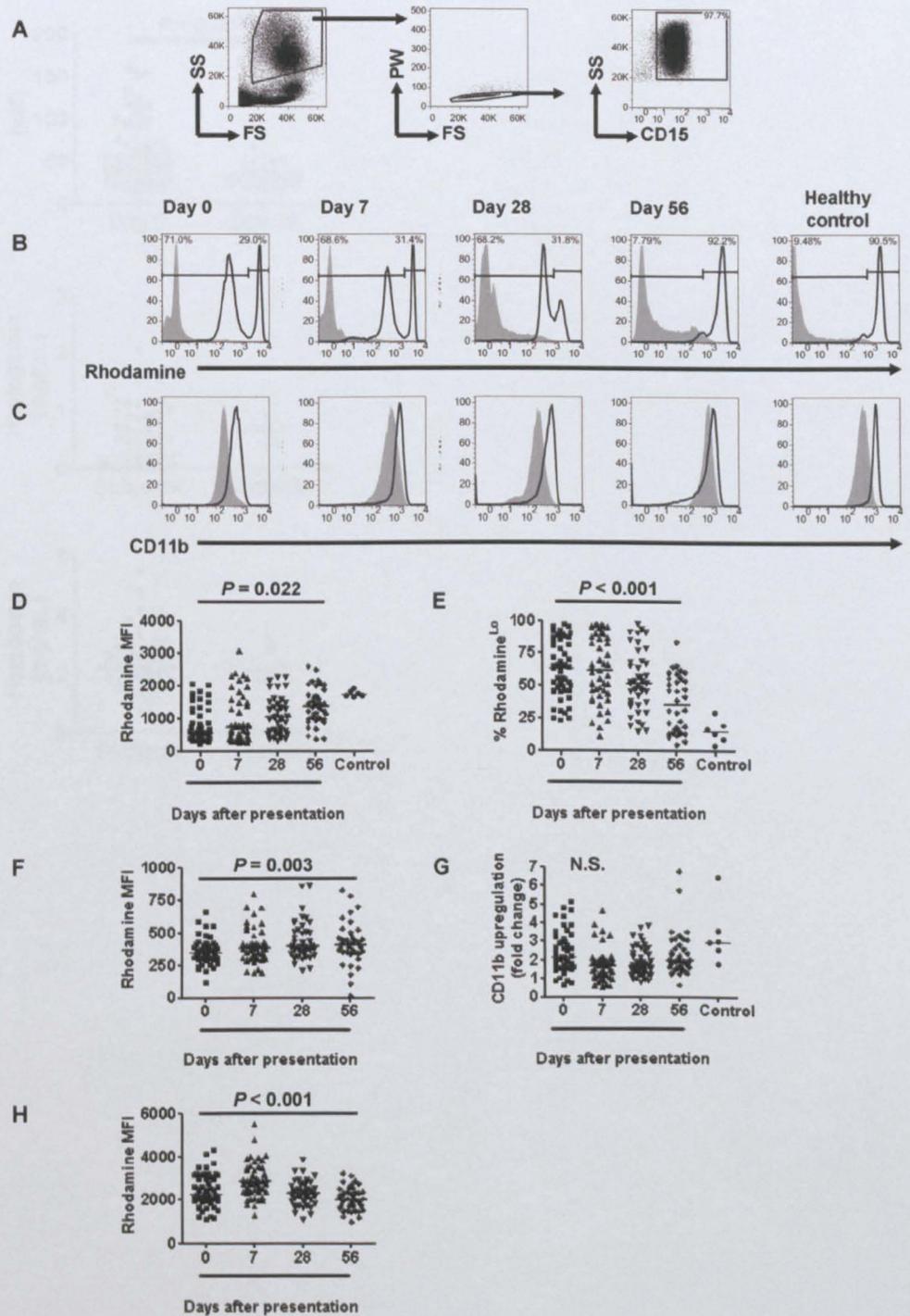


Figure 2

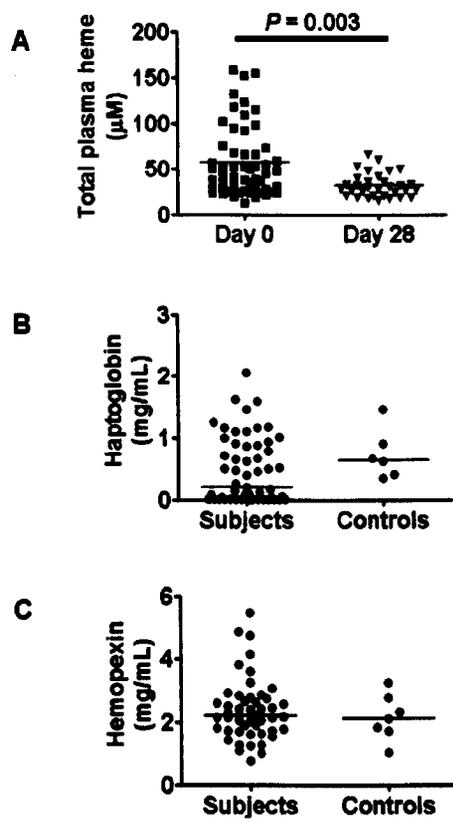


Figure 3

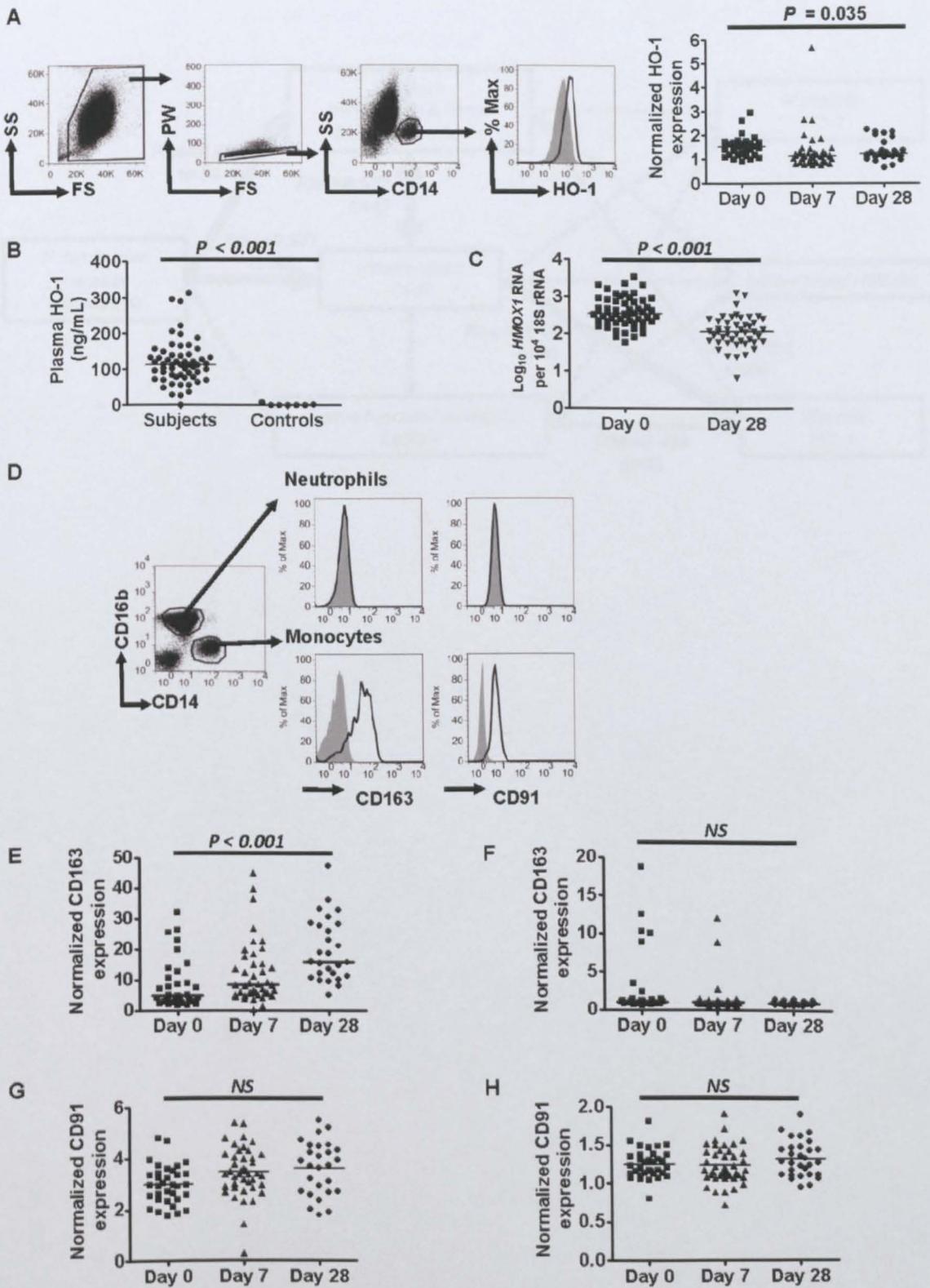


Figure 4

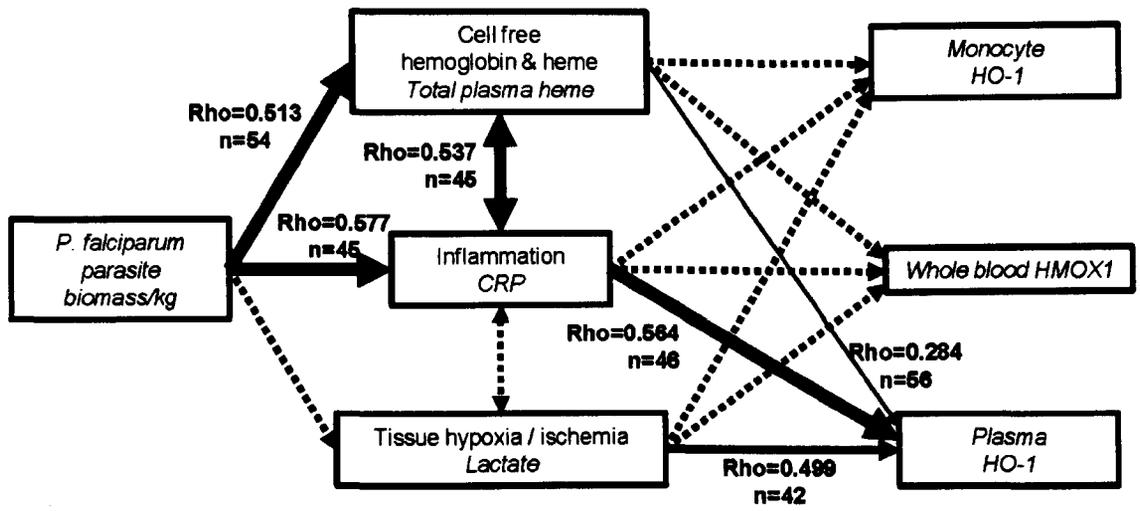
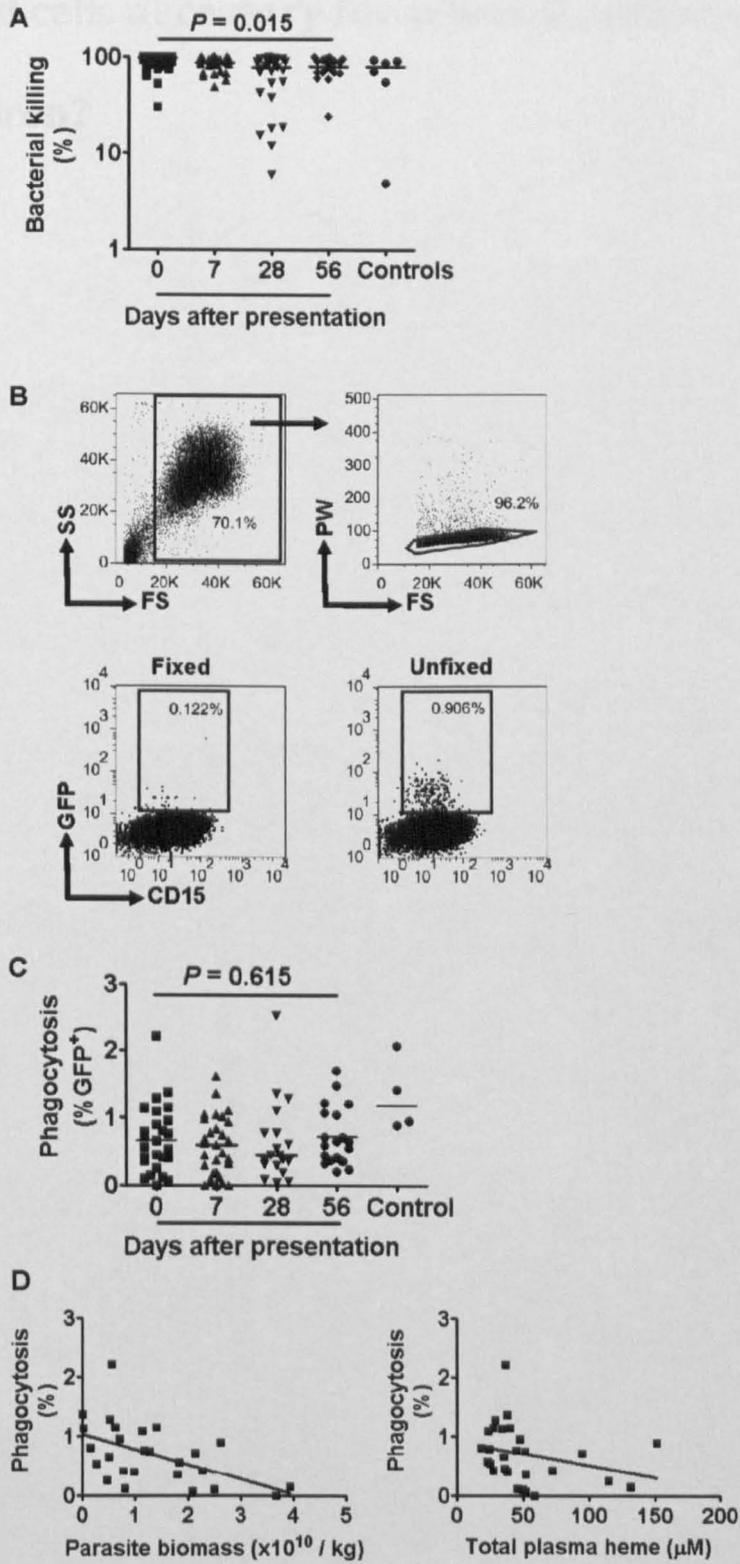


Figure 5



Chapter 7. Is extensive sequestration of parasitized red blood cells necessary for severe *P. falciparum* malaria in children?

Extensive parasite sequestration is not necessary for severe disease in Gambian children with Plasmodium falciparum malaria

The material presented in this chapter represents an article that has been submitted for peer reviewed publication. This chapter does not obviously follow from the rest of the thesis so far, but arose from an analysis of the relationship between parasitemia and PfHRP2-derived total parasite biomass, which has been suggested to be a better marker of the parasite burden within an individual with *P. falciparum* malaria.²⁸⁸ In the preceding chapter, parasite biomass was presented using estimates derived from the concentration of PfHRP2 in plasma. The material in this chapter explores the association of PfHRP2 and the derived parameter, sequestered parasite biomass, with severe malaria in Gambian children. The subjects included in this study are those from the same data collection platform described in Chapter 6. The assays for PfHRP2 in plasma were performed with Sarah Nogaro. Analysis of possible confounding factors (requiring advanced statistical techniques) was performed by Dr Michael Bretscher, who also provided general statistical advice on the analysis and interpretation of results from this study. The conclusion of this article, that extensive sequestration of parasites is not necessary for severe malaria, suggests that other factors mentioned in Chapter 1, such as hemolysis and immune activation, may be more important in triggering severe malaria, and these aspects will be considered in more detail in Chapter 8. The relationship between hemolysis, heme, HO-1 induction and severe malaria is discussed further in Chapter 9.

Cover sheet for each 'research paper' included in a research thesis

1. For a 'research paper' already published

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Aubrey J. Cunnington, Sarah I. Nogaro, Michael T. Bretscher, Eleanor M. Riley, Michael Walther

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3. For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)

I conceived the study, supervised some of the data collection, performed the laboratory work, analysed and interpreted the data and wrote the manuscript.

Candidate's signature _____

Supervisor or senior author's signature to confirm role as stated in (3)

**Extensive Parasite Sequestration is Not Necessary for Severe Disease in Gambian Children
with *Plasmodium falciparum* Malaria**

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Abstract

Background: Intravascular sequestration of parasitized red blood cells (pRBC) is thought to play a central role in the pathogenesis of severe *Plasmodium falciparum* malaria (SM), but current evidence does not allow resolution of whether sequestration is the cause or a consequence of SM.

Methods: Parasite biomass estimates were compared in 296 Gambian children with *P. falciparum* malaria, 120 (40.5%) of whom had one or more indicators of SM. Circulating parasite biomass was calculated from blood film parasitaemia, whilst total parasite biomass was estimated from plasma levels of *P. falciparum* histidine rich protein 2 (PfHRP2). Evidence of sequestration was defined as the PfHRP2-derived biomass estimate being greater than circulating biomass.

Findings: In uncomplicated malaria (UM) the PfHRP2-derived parasite biomass and circulating biomass estimates were remarkably similar. Both total and circulating parasite biomass estimates were significantly greater in children with SM than in those with UM. However, there was no significant difference between PfHRP2-derived and circulating parasite biomass estimates in children with SM, or in any subgroup of children with SM classified by clinical and laboratory features, except those with severe anaemia (SA). Furthermore, the calculated sequestered parasite biomass did not differ significantly between UM and SM, except in those with SA. Both estimates of parasite biomass correlated equally well with blood lactate, a well-established marker of disease severity, whereas there was no significant correlation between lactate and sequestered parasite burden.

Interpretation: Extensive parasite sequestration is not necessary to cause most manifestations of SM in Gambian children. Extensive sequestration seen in post mortem studies of children with malaria may be a consequence of severe disease rather than the initiating factor.

Funding: This study was supported by core funding from the Medical Research Council (MRC, UK), The Gambia, and a MRC clinical research training fellowship (AJC, G0701427).

Introduction

Current dogma places the extensive sequestration of pRBC within the microvasculature at the centre of the pathogenesis of SM,¹ based on the following compelling observations:

Plasmodium falciparum, the cause of most SM, is the only *Plasmodium* species exhibiting extensive sequestration in humans;² post mortem studies have shown the presence of sequestered pRBCs in patients dying from malaria;^{1,3-4} reversible obstruction of blood flow in retinal vessels occurs in patients with cerebral malaria (CM);⁵ and adults with SM have a calculated total parasite biomass much higher than that estimated from peripheral blood smears.⁶ Whilst the association of sequestration with SM is beyond doubt, these observations fall short of proving that sequestration is the cause of, rather than a consequence of SM. Indeed, other observations raise questions about this dogma: *P. vivax*, which exhibits much less pRBC sequestration, is increasingly recognized as a cause of severe disease;⁷ a significant proportion of CM cases do not exhibit retinopathy;⁸ and human and animal studies have implicated numerous other pathways that can lead to organ damage and dysfunction in malaria without the need for pRBC sequestration.⁹⁻¹¹ To prove that pRBC sequestration is necessary to cause SM in humans is extremely challenging. However, evidence that SM does not require extensive sequestration might be more readily obtained if SM occurs with sequestered parasite burdens similar to those in UM. PfHRP2, a soluble parasite molecule released predominantly at schizogony (when pRBCs rupture, releasing merozoites to infect

new RBC), has been used to provide an indirect estimate of total (sequestered plus unsequestered) parasite biomass.⁶ Sequestered biomass can be estimated from the difference between PfHRP2-derived parasite biomass and circulating parasite biomass (estimated from the unsequestered parasites visible on a peripheral blood film). In this study in Gambian children we found that there was remarkably good concordance of population estimates of parasite burden between the two methods in both UM and SM, but little evidence of extensive sequestration of pRBCs in SM.

Methods

The study was approved by the Gambia Government / MRC Laboratories Joint Ethics Committee. All samples were collected with informed consent from the child's parent or legal guardian. During each malaria season between August 2007 and January 2011, Gambian children with *P. falciparum* malaria (defined by compatible clinical symptoms and ≥ 5000 asexual parasites/ μL blood) were recruited within a longitudinal study investigating clinical, immunological and parasitological factors in UM and SM.¹² Briefly, subjects were recruited from three peri-urban health centres, The MRC Gate Clinic, Brikama Health Centre and The Jammeh Foundation for Peace Hospital, Serekunda. All children underwent full clinical examination and were managed in accordance with Gambian government guidelines. Children suspected to have concomitant bacterial infections were excluded. SM was defined using modified WHO criteria:¹³ SA = Hb $\leq 6\text{g/dL}$; lactic acidosis (LA) = blood lactate $\geq 7\text{mmol/L}$; CM = a Blantyre coma score ≤ 2 for at least 2 hours in the absence of hypoglycaemia; severe prostration (SP) = inability to sit unsupported (children > 6 months) or inability to suck (children ≤ 6 month). Children fulfilling the criteria for both SP and SA, LA, or CM were classified as having SA, LA, or CM. Outcome was assessed by survival to discharge from hospital. On presentation, capillary blood was used for lactate, glucose, thick and thin blood

films; venous blood was collected for sickle cell screen, full blood count (EDTA), RNA (PAX tubes), and plasma (heparin). Initial parasitaemia (to determine eligibility) was estimated from Field's stained thick blood films and subsequently accurately counted from 50 fields on Giemsa stained thin blood smears by an experienced microscopist. Samples were transported to the laboratory on ice within 2 hours of collection. Plasma was separated on arrival in the laboratory and stored at -70°C until analysis was performed. Full blood count was performed using a Medonic instrument (Clinical Diagnostics Solutions, Inc).

PfHRP-2 was measured in duplicate in plasma by ELISA kit (Cellabs). A standard curve was constructed using serial dilutions of the PfHRP-2 standard and run with every plate. Each plate contained a mixture of samples from UM and SM arranged in order of enrollment. Circulating, PfHRP2-derived, and sequestered parasite biomass estimates were calculated as described by Dondorp et al.⁶ To account for the higher blood volume relative to weight in small children we modified the blood volume term in the equation as follows: males, blood volume (mL) = $312 + (63 \cdot 11 \times \text{body weight (kg)})$; females, blood volume = $358 + (62 \cdot 34 \times \text{body weight (kg)})$.¹⁴ To account for variation in size of children, parasite biomass was expressed as parasites per kg body weight.

The presence of metabolically active bacteria in blood was determined using quantitative PCR on cDNA with species-specific primers for *Streptococcus pneumoniae*¹⁵ and non-Typhoid Salmonella (NTS),¹⁶ the two most common causes of invasive bacterial infection.¹⁷

Statistical analysis was performed using PASW statistics 18 (SPSS Inc.), GraphPad Prism (GraphPad Software Inc.) and the R-statistical software (R Foundation). Variables of interest were log₁₀ transformed to achieve normal distribution prior to parametric analysis, except sequestered biomass (which had both positive and negative values) which was analysed with non-parametric tests. Unpaired and paired sample t-tests were used to compare continuous variables between independent and related groups respectively. Sequestered parasite biomass

was analysed using the Mann-Witney U test for group comparisons, and the Scheirer-Ray-Hare test and quantile regression were used to assess confounding. Correlation was assessed using Spearman's rank correlation coefficient. To allow for the multiplicity of tests resulting from multiple responses and multiple comparisons within a response, a false discovery rate (FDR) of 5% was assumed, using the Benjamini and Hochberg approach.¹⁸

Role of the funding source. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Results

Plasma samples, sufficient data for calculation of total and circulating parasite biomass, and full clinical data were available from 315 children. In 19 cases PfHRP-2 measurements were below the detection limit of the assay (0.4ng/mL), and these were excluded from further analysis. In total, data from 296 children (Tables 1 and 2), 120 (40.5%) with SM (Figure 1), were analysed.

Children with SM had higher parasite biomass than children with UM, regardless of which estimate of parasite biomass was used (Figure 2 and Table 2). As expected, the children with SM were younger, more anaemic, had lower platelet counts and had higher blood lactate, parasitaemia and parasite density than UM cases.

We assessed the extent of parasite sequestration in two ways: first, PfHRP-2-derived parasite biomass was compared with the circulating biomass within groups of subjects (with a significantly higher PfHRP-2-derived biomass indicating sequestration of pRBC); second sequestered parasite biomass estimates for each subject were compared between groups. Using the first method, the two parasite biomass estimates did not differ significantly for either UM or SM, or for any of the subgroups of SM except SA (Table 2). Since a large

proportion (74 out of 120, 61.6%) of SM cases had prostration alone, which is associated with a lower risk of mortality than the other indicators of severity,¹⁹ we reanalyzed the data for subjects with SM excluding those with prostration alone. Even in this group of 46 children with the most severe manifestations of malaria, there was no significant difference between estimates of parasite burden. Reanalysis using a less stringent definition of hyperlactataemia (> 4mmol/L, as used by Dondorp et al.)⁶ increased the numbers of children classified as having SM, SM excluding prostration, and SM with LA to 142, 106 and 100 respectively, but did not change the significance or inference from the comparisons between the different estimates of parasite burden (Table 2). Similarly, in the small group of children who died, there was no significant difference between estimates of parasite burden. We next calculated sequestered parasite biomass: for all subjects taken together this revealed a roughly symmetrical distribution of values about zero, and there was no significant difference in sequestered parasite biomass between UM and SM groups, or between UM and any individual severe manifestations except SA (Table 2). When we calculated sequestered parasite burden in subjects with SM, excluding those with prostration alone, the geometric mean value was 3.0×10^9 parasites/kg, which is equivalent to just 9.9% and 11% of the geometric mean total parasite biomass and circulating biomass respectively. Only subjects with SA had clear evidence of what might be considered extensive sequestration, with a PfHRP2-derived biomass greater than the circulating biomass. Taken together, these results provide no evidence of extensive parasite sequestration associated with SM except in a small subgroup of children with SA.

Elevated venous blood lactate is strongly associated with mortality in *P. falciparum* malaria,^{6, 20} and has been reported to correlate strongly with PfHRP2-derived parasite biomass, but not circulating parasite biomass in adults.⁶ This has been taken as further evidence of the importance of parasite sequestration in causing microvascular occlusion and tissue hypoxia. We found that lactate correlated significantly with both estimates of parasite burden, and the

correlation coefficients were very similar (Table 3). Furthermore, there was no significant correlation between sequestered parasite biomass and lactate (Spearman $r = -0.084$, $P = 0.151$). These results suggest that PfHRP2-derived biomass provides a good estimate of circulating parasite biomass but, in contrast to findings in adults, does not support extensive parasite sequestration as a cause of hyperlactataemia.

Potential confounding by age was considered because it is related to parameters used to calculate parasite biomass and differs significantly between UM and SM subjects (Table 2).

Both estimates of parasite biomass showed significant and similarly strong negative correlation with age (Table 3), whereas sequestered biomass did not correlate significantly with age (Spearman $r = 0.051$, $P = 0.381$). Confounding by age was assessed by performing a two-way ANOVA on the rank-transformed data (Scheirer-Ray-Hare test), which found no significant effect of age ($P=0.641$), severity (UM vs. SM, $P=0.304$) or an interaction of the two ($P=0.565$) on sequestered biomass. This result was confirmed by step-wise selection of the best-fitting quantile regression model (performed using the “quantreg” package in R). Another confounding factor may be misclassification of subjects with another severe illness and coincidental parasitaemia as having SM. Since bacterial sepsis would be the most likely illness to present in this way, PCR was performed to detect NTS or *S. pneumoniae* bacteremia in 160 (54.1%) study subjects (92 of 176 (52.3%) uncomplicated and 68 of 120 (56.7%) severe cases). No subjects (95% CI 0-2.3%) had evidence of bacterial co-infection.

Discussion

Eradication of malaria is now high on the global health agenda,²¹ yet it is predicted that as malaria becomes less common, the proportion of cases with severe disease will increase.²²

Understanding the pathophysiology of SM is important for developing new strategies to prevent malaria deaths. Progress in this field is difficult because studies in humans with SM can only describe associations with severe disease, and cannot prove causality. Post-mortem

studies are of limited value because they describe findings in the minority of patients dying from SM and it is not possible to determine whether the same pathological processes occur in the majority who survive. Animal models allow experimental manipulation of factors which may be essential for different manifestations of SM, but there has been much debate about whether they recapitulate the features of severe disease in humans, particularly parasite sequestration.²³ Sequestration of pRBC may cause microvascular occlusion and lead to hypoxic and ischaemic tissue damage²⁴ but is assumed to be beneficial to malaria parasites by preventing their clearance by the reticuloendothelial system.²⁵ *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) variant surface antigens are expressed on the surface of infected erythrocytes and can bind to molecules on the host vascular endothelium such as CD36 and ICAM-1, allowing cytoadherence and sequestration.²⁴ Expression of ICAM-1 is upregulated during inflammation, possibly enhancing pRBC sequestration.²⁴ However others have suggested that inflammatory mediators, released in response to parasite factors, might themselves cause the clinical manifestations of severe malaria and in this scenario, sequestration may be a consequence rather than a cause of severe disease.²⁶

The present study was undertaken to assess whether extensive parasite sequestration occurs in Gambian children with malaria at the time of clinical presentation.⁶ We found that PfHRP2 derived parasite biomass estimates were remarkably similar to the circulating biomass calculated from blood parasitaemia. All indices of parasite biomass (parasitaemia, parasite density, PfHRP2 concentration, circulating parasite biomass, and PfHRP2-derived parasite biomass) were significantly higher in SM compared with UM. Considering UM and SM separately, and all subgroups of severe disease, we only found significant evidence of sequestration in subjects with SA. Furthermore, PfHRP2-derived biomass estimates did not correlate any better with lactate (an important marker of severity),²⁰ than circulating biomass estimates. These findings are in contrast to a similarly large study conducted in adults in

Thailand, which found that peripheral blood parasitaemia was a poor indicator of parasite burden in SM, and provided evidence of extensive parasite sequestration in patients with severe disease. The simplest interpretation of these results is that in our study population, circulating *P. falciparum* biomass provides a good estimate of total parasite biomass because there is little sequestration. From this we infer that extensive parasite sequestration is not necessary to cause SM in these children.

In assessing the validity of our findings we must consider methodological issues which might influence our results. First, our study was conducted in children, whereas the model relating PfHRP-2 concentration to parasite biomass was derived from data in adults.⁶ In order to account for physiological and size differences between children and adults we modified the model slightly to improve the accuracy of the term for calculation of blood volume, and we report parasite burden data relative to body weight. Overall estimates of PfHRP2-derived parasite biomass are remarkably consistent with those obtained in adults: among non-prostrated SM subjects we found a geometric mean of 3.02×10^{10} (95% CI $1.98-4.61 \times 10^{10}$) parasites/kg, whilst Dondorp et al. found a geometric mean of 1.7×10^{12} ($1.3-2.3 \times 10^{12}$) parasites per body in SM subjects,⁶ equivalent to 3.4×10^{10} ($2.6-4.6 \times 10^{10}$) parasites/kg for a body weight of 50kg. However this compelling consistency of numerical estimates of parasite burden, across measurement techniques and study populations, should not create the false impression that correctness of the model is required to conclude that there is no difference in sequestration between UM and SM subjects: this conclusion simply requires that any estimation error be the same in each group. Second, it could be argued that our study was conducted in a setting where previous exposure to malaria may result in acquisition of antibodies that reduce circulating levels of PfHRP2. The Gambia has experienced a remarkable and sustained decline in malaria transmission over the last decade,²⁷⁻²⁸ and young children are the least likely to have experienced prior malaria infections and the least likely to have

antibodies to *P. falciparum* antigens.²⁸ Moreover, children with SM were much younger than those with UM, and so it is very unlikely that anti-PfHRP2 antibodies caused us to underestimate the total parasite burden in SM cases. We found no evidence of any interaction between age, severity and sequestered parasite burden. Third, parasite multiplication rate (the number of new parasites produced from each parasite in the preceding generation) is one of the most significant parameters in the model,⁶ and may be reduced at increasing parasite densities,²⁹ resulting in over estimation of the total parasite biomass.⁶ In our study, children with SM had higher circulating parasite densities than those with UM, and so overestimation of multiplication rate would lead us to overestimate the extent of parasite sequestration in SM, rather than underestimate it. Fourth, our patients with SM had only a 4.2% mortality rate compared with 17% in the adult study,⁶ which might suggest the children were 'less' seriously ill than the adults. However, in both studies similar percentages of SM cases (44% in adults and 48.3% in children) had lactate levels above 5 mmol/L, and the average lactate levels in SM cases were similar in both studies. To try to improve comparability between the two studies we excluded children classified as SM due to prostration alone and reanalyzed our data; although mortality was 8.7% in this group of children there was no significant difference between biomass estimates. We also reanalyzed our data using a lower cut-off for hyperlactataemia, but this did not alter the results. After considering these methodological issues, we believe our findings are robust.

How should our results be interpreted? Considering we had at least 80% power to detect a 2-fold difference in parasite biomass estimates in the subgroup of 46 children with SM excluding prostration alone, our findings do not exclude lower levels of parasite sequestration, or more extensive sequestration limited to specific vascular beds, such as the retinal vessels. Rather we find no evidence for extensive generalized parasite sequestration. Due to the small number of deaths in this study, we cannot exclude that sequestration plays a role in the progression from severe to fatal malaria. The evidence of sequestration in children with SA is plausible, because

extensive sequestration might exacerbate anaemia. However, artefactual overestimation of total parasite burden in these children by PfHRP2 measurements, due to decreased multiplication rate when the percentage parasitaemia is high,^{6, 29} cannot be ruled out.

The findings of this study have important implications. In Gambian children, circulating parasite burden rather than sequestration is associated with severe disease. Genetic, physiological, immunological and parasitological differences may explain why extensive sequestration is apparent at the time of presentation in Thai adults with severe malaria⁶ but not in Gambian children. If extensive sequestration is not essential for the development of severe malaria then this implies that other mechanisms may be more important, and parasite sequestration may be a consequence rather than cause of severe disease. For example, free heme released during malarial hemolysis has been proposed to cause severe malaria in mice,¹⁰ and in humans cell free hemoglobin impair the function of endothelial cells,³⁰ which may be both target and effector cells at the interface between the parasites in the blood and organ tissue damage.³¹ pRBC sequestration may appear intimately related to SM without being the proximate cause, because the processes that cause endothelial activation will also increase binding of pRBC to endothelium.²⁴ In adults, perhaps sequestration is necessary to allow total parasite biomass to rise to sufficient levels to trigger severe disease, whereas in children this may not be necessary, explaining the dissociation of extensive sequestration from SM in this study. If our findings can be generalized to other children in Sub-Saharan Africa, then they suggest that the research agenda should be refocused beyond parasite sequestration in the search for new methods to prevent and treat SM.

Contribution:

AJC, SIN and MW conceived the study and collected the data; AJC, MTB and MW analysed the data; all authors contributed to the interpretation of the data; AJC, EMR and MW wrote the manuscript; all authors contributed to critical revision of the manuscript.

Conflicts of interest:

The authors declare that they have no conflicts of interest.

Acknowledgements:

We are grateful to the subjects who participated in this study, and for the assistance provided by Mathew Edwards, Madi Njie, Simon Correa, Lamin Manneh, Ebako Takem, Augustine Ebonyi, Brigitte Walther, David Conway, and the clinical, laboratory, field work and administrative staff of the MRC Laboratories (UK) The Gambia, the MRC Gate clinic, the Jammeh Foundation for Peace Hospital and Brikama Health Centre.

Table Legends

Table 1. Characteristics of children enrolled in the study

Table 2. Comparison of indicators of severity, parasite biomass, and sequestered parasite

biomass between children with different manifestations of malaria. Severe malaria cases

were grouped according to syndrome (as shown in Figure 1). Severe malaria syndromes were

considered both exclusively (every combination of syndromes considered as a distinct group)

and inclusively (subjects with combinations of syndromes considered within each syndrome

group). Data represent geometric mean (95% CI), except in the case of sequestered parasite

burden where data represent median (interquartile range, or range if $n=3$), and the P value for

comparison with the uncomplicated malaria group using unpaired t-test on \log_{10} -transformed

data. For each manifestation of malaria, circulating and PfHRP2-derived biomass estimates

were compared using the paired sample t-test. CM, cerebral malaria, LA, lactic acidosis, SA,

severe anaemia. * Platelet count was not available for four subjects with severe malaria (all

prostration alone). Only tests with a P -value at or below 0.024 have a false discovery rate of

5% or less using the Benjamini and Hochberg method to control for multiple comparisons and

are considered significant (P -value in bold type).

Table 3. Correlation of blood lactate and age with estimates of parasite biomass.

Figure Legends

Figure 1. Manifestations of severe malaria. Venn diagram showing the number of children with each severe malaria syndrome.

Figure 2. Severe malaria is associated with high circulating and PfHRP-2-derived parasite biomass estimates. Parasite biomass estimates for children with mild malaria (blue boxes, n=176) and severe malaria (red boxes, n=120) calculated from blood film parasitaemia (circulating biomass) and plasma PfHRP2 concentration (PfHRP2-derived biomass). Comparison between mild and severe cases for each biomass estimate using the unpaired t test.

Table 1.

| | | n | % |
|---------------------------------------|----------|----------|----------|
| Sex | Male | 169 | 57.1 |
| | Female | 127 | 42.9 |
| Ethnicity | Mandingo | 117 | 39.5 |
| | Wollof | 28 | 9.5 |
| | Fulla | 58 | 19.6 |
| | Jola | 50 | 16.9 |
| | Serehuli | 2 | 0.7 |
| | Serere | 10 | 3.4 |
| | Manjago | 13 | 4.4 |
| | Aku | 1 | 0.3 |
| | Other | 10 | 3.4 |
| | Unknown | 7 | 2.4 |
| Severity | Mild | 176 | 59.5 |
| | Severe | 120 | 40.5 |
| Survival to hospital discharge | Alive | 291 | 98.3 |
| | Died | 5 | 1.7 |

Table 2.

| Clinical manifestation | n (%) | Age years | Hemoglobin g/dL | Platelets $\times 10^9/L$ | Lactate mmol/L | Parasitaemia % | Parasite density $\times 10^3/\mu L$ | Plasma PfPR-2 ng/mL | Circulating parasites $\times 10^3/\mu g$ | PfPR2 derived parasites $\times 10^3/\mu g$ | Circulating vs. PfPR2 derived biomass | Sequestered Parasites $\times 10^3/\mu g$ | | | |
|----------------------------------|-----------------------------------|-------------------|-----------------------------|---------------------------|--------------------|-------------------|--------------------------------------|---------------------|---|---|---------------------------------------|---|-------------------|-------------------|------------------|
| Uncomplicated | 176 (59.5) | 6-37 (5-81-6-99) | 11.1 (10.80-11.42) | 110 (99.0-122) | 2.19 (2.04-2.34) | 3.2 (2.7-3.8) | 1.3 (1.1-1.6) | 106 (88-4-127) | 1.09 (0.91-1.29) | 0.96 (0.71-1.03) | P=0.0357 | -0.13 (-1.5-0.62) | | | |
| Severe | All severe | 120* (40.5) | 4-24 (3.87-4.66) | 9-12 (8.68-9.59) | 59-0 (50.5-68.7) | 4-76 (4.26-5.32) | 7-8 (6.3-9.6) | 2-7 (2.2-3.4) | 245 (198-303) | 2-35 (1.92-2.87) | 2-24 (1.79-2.81) | P=0.7543 | -0.46 (-2.2-1.6) | | |
| | | Prostration alone | 74* (25.0) | 4-50 (4.03-5.04) | 9-86 (9.43-10.32) | 61-7 (52.1-73.0) | 3-64 (3.23-4.11) | 6-6 (5.0-8.7) | 2-5 (1.9-3.3) | 210 (165-268) | 2.14 (1.63-2.82) | 1.86 (1.45-2.39) | P=0.4319 | -0.72 (-2.6-0.31) | |
| | | | Excluding prostration alone | 46 (15.5) | 3-85 (3.27-4.54) | 8-05 (7.29-8.89) | 55-1 (40.7-74.7) | 7-32 (6.29-8.52) | 10-1 (7.4-13.8) | 3-2 (2.4-4.2) | 313 (210-466) | 2.73 (2.03-3.67) | 3.02 (1.98-4.61) | P=0.6116 | 0.30 (-1.6-3.8) |
| Severity Category (exclusive) | CM | 7 (2.4) | 3-41 (2.64-4.40) | 10-31 (8.65-12.28) | 40-5 (7.62-215.7) | 4-47 (3.22-6.20) | 7-8 (3.1-19.4) | 3-0 (1.2-7.2) | 190 (63-9-567) | 2-62 (1.08-6.34) | 1-73 (0.55-5.45) | P=0.5309 | -1.4 (-2.9-1.5) | | |
| | | LA | 19 (6.4) | 4-78 (3.81-6.01) | 9-82 (8.95-10.76) | 71-5 (47.6-108) | 9-39 (8.32-10.60) | 9-3 (5.1-17.1) | 3-6 (2.1-6.2) | P=0.2185 | P=0.0528 | P=0.1540 | P=0.0615 | P=0.6546 | |
| | | | 8 (2.7) | 2-30 (1.25-4.22) | 5-16 (4.62-5.77) | 71-1 (45.0-112.3) | 3-76 (2.66-5.31) | 7-5 (2.8-20.0) | 1-6 (0.6-4.2) | P=0.0360 | P=0.0005 | P=0.0226 | P=0.0386 | P=0.2578 | |
| | CM+LA | 6 (2.0) | 5-08 (3.36-7.70) | 8-77 (7.69-10.01) | 35-4 (15.0-83.8) | 9-96 (7.72-12.85) | 16-6 (9.0-30.7) | 5-2 (3.1-8.7) | 670 (280-1600) | 4-37 (2.66-7.16) | 6-37 (2.81-14.4) | P=0.2234 | 2.9 (-1.7-6.9) | | |
| | | LA+SA | 3 (1.0) | 3-21 (2.41-4.26) | 5-24 (3.31-8.30) | 29-9 (0.89-1006) | 9-02 (4.63-17.56) | 19-0 (9.1-39.3) | 4-4 (1.8-10.1) | 537 (211-1370) | 3-96 (1.91-8.21) | 6-14 (2.17-17.4) | P=0.3698 | 2.0 (-1.1-6.5) | |
| | | | 3 (1.0) | 3-56 (0.55-23.11) | 5-41 (3.47-8.44) | 49-0 (14.6-164.7) | 12-44 (5.45-28.40) | 12-9 (8.0-20.9) | 2-7 (2.3-3.1) | 1013 (24.2-42400) | 2-31 (1.77-3.02) | 11-0 (0.29-42.4) | P=0.2285 | 17.6 (-0.53-30) | |
| | Severity Category (Inclusive) | CM | 16 (5.4) | 3-99 (3.16-5.05) | 8-60 (7.38-10.02) | 39-9 (20.5-77.9) | 7-31 (5.48-9.75) | 11-4 (7.4-17.4) | 3-6 (2.4-5.3) | 417 (212-823) | 3-10 (2.11-4.55) | 3-99 (1.98-8.03) | P=0.4992 | 1.0 (-1.8-6.2) | |
| | | | LA | 31 (10.5) | 4-52 (3.81-5.38) | 8-53 (7.66-9.50) | 55-3 (39.8-77.0) | 9-72 (8.84-10.69) | 11-5 (7.8-16.9) | 3-8 (2.7-5.3) | 328 (189-571) | 3-07 (2.29-4.54) | 3-07 (1.72-5.49) | P=0.8218 | -0.36 (-1.7-3.3) |
| | | | | 14 (4.7) | 2-71 (1.87-3.91) | 5-23 (4.82-5.68) | 54-5 (34.4-86.4) | 5-86 (4.04-8.50) | 10-3 (5.9-17.9) | 2-2 (1.3-3.9) | 521 (323-840) | 2-01 (1.16-3.48) | 5-93 (3.70-9.50) | P=0.0054 | 2.5 (0.78-6.5) |
| Non-survivors | 5 (1.7) | 6-26 (3.40-7.36) | 9-31 (6.79-12.76) | 26-0 (18.3-36.9) | 9-31 (5.90-14.69) | 14-4 (5.2-40.0) | 5-1 (2.3-11.6) | 763 (163-3580) | 4-14 (1.82-9.38) | 6-62 (1.20-36.4) | P=0.4659 | 6.9 (-2.2-17.2) | | | |
| LA redefined as lactate >4mmol/L | Uncomplicated | 154 (52.0) | 6-68 (6.06-7.36) | 11-34 (11.04-11.66) | 110-6 (99.2-123.4) | 1-95 (1.84-2.07) | 2-9 (2.4-3.5) | 1-2 (1.0-1.5) | 99-1 (82.0-120) | 1-00 (0.83-1.20) | 0-79 (0.65-0.96) | P=0.0626 | -0.13 (-1.3-0.62) | | |
| | | Severe | 142 (48.0) | 4-29 (3.93-4.69) | 9-19 (8.79-9.60) | 64-5 (56.0-74.4) | 4-77 (4.34-5.23) | 7-5 (6.2-9.0) | 2-7 (2.2-3.2) | 231 (189-283) | 2-29 (1.91-2.75) | 2-11 (1.71-2.60) | P=0.4872 | -0.4 (-2.3-1.3) | |
| | Severe excluding prostration only | | 106 (35.8) | 4-21 (3.81-4.67) | 8-80 (8.35-9.29) | 67-1 (56.7-79.5) | 5-96 (5.52-6.43) | 8-4 (6.8-10.3) | 2-9 (2.4-3.6) | 260 (202-334) | 2-51 (2.04-3.08) | 2-42 (1.86-3.14) | P=0.7826 | -0.3 (-2.2-1.5) | |
| | | LA | 100 (33.8) | 4-28 (3.84-4.75) | 8-97 (8.52-9.43) | 65-7 (55.1-78.3) | 6-22 (5.79-6.68) | 8-7 (7.0-10.7) | 3-1 (2.5-3.8) | 263 (202-341) | 2-65 (2.15-3.26) | 2-43 (1.85-3.19) | P=0.5277 | -0.38 (-2.3-1.5) | |

Table 3.

| | n | Circulating Biomass | | | PfHRP-2-derived Biomass | | |
|----------------|-----|---------------------|------------------|-------------------|-------------------------|------------------|-------------------|
| | | Spearman r | 95% CI | P | Spearman r | 95% CI | P |
| Lactate | 296 | 0.496 | 0.402 to 0.579 | <0.0001 | 0.437 | 0.337 to 0.528 | <0.0001 |
| Age | 296 | -0.284 | -0.388 to -0.172 | <0.0001 | -0.291 | -0.395 to -0.180 | <0.0001 |

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Figure 1.

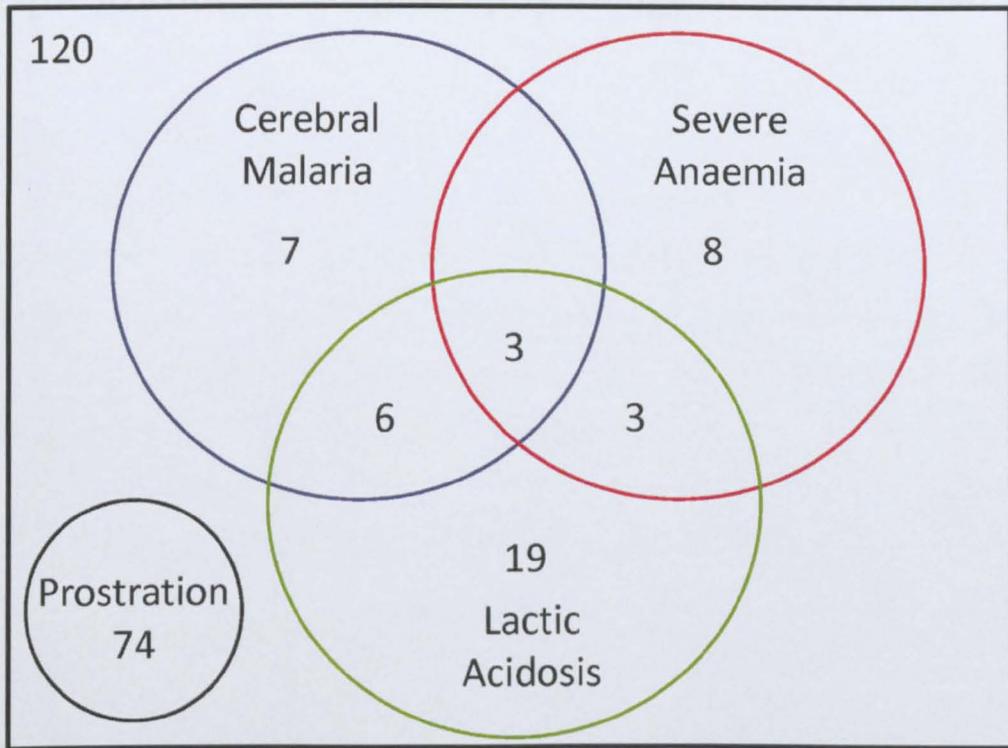
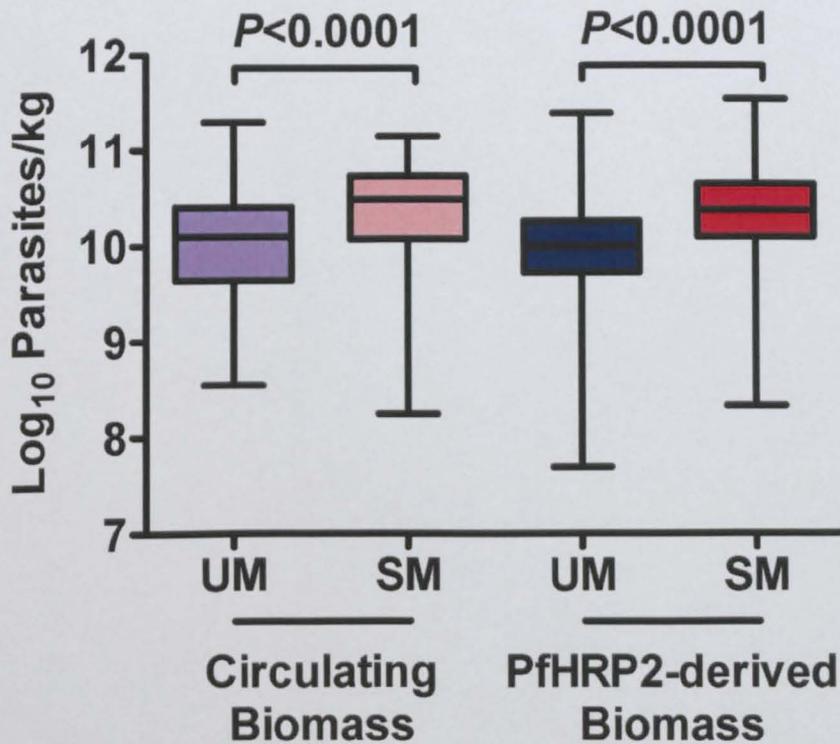


Figure 2.



Chapter 8. The role of parasitized red blood cell sequestration in the pathophysiology of severe malaria.

Stuck in a rut? Moving beyond sequestration of parasitized red blood cells as the cause of severe malaria.

The material presented in this chapter represents an uncommissioned article that is in the process of being submitted for peer reviewed publication. In this “Opinion Article” my co-authors and I discuss whether parasitized red blood cell sequestration is a cause or a consequence of other factors that cause severe malaria. The review of the literature and formulation of hypotheses was undertaken with advice and critical comments from Prof. Eleanor Riley and Dr. Michael Walther.

Stuck in a rut? Moving beyond sequestration of parasitized red blood cells as the cause of severe malaria.

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Summary

The sequestration of parasitized red blood cells (pRBCs) in the microvasculature is widely assumed to be the proximal cause of severe *Plasmodium falciparum* malaria. Obstruction of blood flow by sequestered pRBCs is believed to cause tissue hypoxia and endothelial damage, resulting in the metabolic, neurological, respiratory and other features that constitute the different manifestations of severe *P. falciparum* malaria. There is abundant circumstantial evidence linking sequestration of pRBCs with severe and fatal *P. falciparum* malaria, but the association is not absolute and there is accumulating evidence that sequestration is neither necessary nor sufficient to cause severe malaria. Here we critically review the evidence associating pRBC sequestration with severe malaria, review sequestration-independent processes which are also associated with severe disease and propose a unifying hypothesis which explains the features of severe malaria caused by various *Plasmodium* species in humans and in animal models. This hypothesis invokes endothelial dysfunction and microcirculatory failure as the proximal causes of severe malaria and implies that sequestration is a specific consequence of these events during *P. falciparum* infections. Testing of this hypothesis may help to progress the development of new interventions to prevent and treat severe malaria.

Malaria

Malaria remains a major cause of morbidity and mortality in tropical regions of the world with estimates of 700,000¹ to 1.24 million deaths from malaria in 2010².

Plasmodium falciparum infections account for the majority of clinical cases and deaths, but *P. vivax* also causes a large burden of disease³, including severe disease⁴⁻⁵, and *P. knowlesi* is an emerging cause of severe malaria in south east Asia⁶. Clinical symptoms of malaria are exclusively associated with the asexually replicating red blood cell stage of infection. Whilst uncomplicated malaria is characterized by fever, myalgia and headache, severe malaria may result in severe anemia, coma, respiratory distress, multi-organ failure and death⁷. Although the pathogenesis of severe malaria is uncertain, many authorities cite microvascular occlusion by sequestered parasitized red blood cells (pRBCs) as the proximate cause of severe *P. falciparum* disease⁸⁻¹⁰ and severe *P. falciparum* malaria is widely attributed to pRBC sequestration in medical text books¹¹⁻¹².

What is sequestration and how does it occur?

Sequestration is the adherence of pRBC to the luminal walls of small blood vessels (particularly capillaries and venules)¹⁰ or their retention within the slow open circulation of the spleen¹³ and is a characteristic feature of *P. falciparum* infections where erythrocytes containing mature trophozoites, schizonts and developing gametocytes (sexual stages) are rarely detected on blood films. Sequestration of pRBC in the capillaries is believed to offer a survival advantage to *Plasmodium* species by avoiding passage through the spleen where distorted or rigid pRBC are cleared¹³. Hence, sequestration may facilitate replication of asexual parasites, extending the duration of the infection and increasing both the generation and survival of sexual (transmissible) stages¹³⁻¹⁶. However, whilst sequestration appears to be a universal trait of *P. falciparum*, *P. vivax* is only weakly adherent and mature forms of *P. vivax* are routinely detected in the circulation of infected patients¹⁷. Although sequestration has been observed in one post-mortem human *P. knowlesi* case¹⁸, and in a small study *P. knowlesi* isolates were found to bind to variable degrees to human endothelial cell receptors¹⁹, trophozoites and schizonts are usually visible on peripheral blood films²⁰, and coma is not a feature of *P. knowlesi* infections²¹⁻²³. Sequestration has not been reported for *P. malariae* and *P. ovale* infection, although for the latter, rosetting – a

phenomenon associated with sequestration – has been described²⁴. Further, some rodent and non-human primate malaria species do sequester²⁵⁻³⁰ suggesting that this is an ancient and relatively conserved trait.

Adhesion of pRBC to vascular endothelium appears analogous to the early phases of leukocyte recruitment to a site of inflammation, involving first rolling adhesion and then static adhesion³¹. Adhesion is mediated by specific binding of parasite-derived ligands on the surface of pRBCs to host receptors expressed on vascular endothelial cells³¹ and may be facilitated by aggregation of platelets, fibrin and other components of the coagulation system³². Reduced deformability of pRBCs³³⁻³⁴ and pRBC aggregation into rosettes³⁵ also contribute to the mechanical retention of pRBCs within the microvasculature. In the specific case of malaria in pregnancy, sequestration may also occur in the placenta, and in this circumstance parasites may be undetectable in peripheral blood whilst multiplying and causing chronic infection in the placental vascular bed³⁶. There are several excellent reviews of malaria in pregnancy, in which sequestration in the placenta clearly plays an important role³⁶⁻³⁸, and this will not be considered further in this article.

The best characterized adhesive ligands are members of the highly polymorphic *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) variant surface antigen (VSA) family³⁹. Different VSAs are able to bind to different host receptors and, since VSA expression can vary over the course of an infection due to sequential expression of different VSA family members, the cytoadhesive phenotype may vary as infection progresses⁴⁰. Moreover, since different host receptors are differently expressed in different tissues, sites of sequestration may vary according to parasite VSA genotype and expression patterns. The best characterized host receptors, which are probably most important for static cytoadhesion, are CD36 and intercellular adhesion molecule-1 (ICAM-1, CD54)^{31, 41}. CD36 is a scavenger receptor which is widely expressed on vascular endothelium (except the brain), and also on platelets, monocytes and macrophages⁴². ICAM-1, an immunoglobulin super family member, is expressed on endothelial cells (including the brain capillaries) and leukocytes⁴³. Whilst most PfEMP1 variants can bind to CD36, only a subset of variants (encoded by group B var genes)

can bind to ICAM-1³¹. Other host molecules that may mediate adhesion include thrombospondin, P-selectin, platelet endothelial cell adhesion molecule-1 (PECAM-1, CD31), E-selectin, VCAM-1 (CD106) and chondroitin sulphate A (CSA)³¹. In addition, adhesion of pRBC to CD36 on platelets, and the adhesion of platelets to von Willebrand factor at sites of vascular endothelial injury, may serve to amplify sequestration and allow sequestration in endothelial beds where appropriate host receptors are not expressed⁴⁴. Expression of host receptors on endothelium is a dynamic process, and changes dramatically in response to infection⁴⁵. ICAM-1 in particular is upregulated by inflammation, whereas CD36 is constitutively and relatively invariantly expressed^{42-43, 46}.

The molecular basis of sequestration is less well understood for other Plasmodium species but the limited cytoadherence of *P. vivax* is believed to be mediated by binding to ICAM-1 and CSA¹⁷, whereas clinical isolates of *P. knowlesi* have shown variable binding to ICAM-1 and VCAM but not CD36¹⁹. On the other hand, some rodent malaria parasites bind extensively to CD36^{16, 25, 47}.

How can sequestration be measured?

Examination of serial blood films from *P. falciparum* infected individuals reveals far fewer circulating late stage trophozoites and schizonts than would be expected from the subsequent increases in parasitaemia⁴⁸ suggesting that these mature forms are present in the body but not visible in the peripheral blood⁴⁹. Although pRBC sequestration can be directly assessed in animals - by histology, intravital microscopy⁵⁰ or bioluminescent imaging of whole animals infected with genetically modified (luminescent) parasites²⁵, direct quantification of sequestration in humans is limited to histological analysis of biopsy samples - such as muscle, skin and subcutaneous tissue - in live subjects⁵¹⁻⁵² or at post-mortem in those dying from severe malaria^{32, 53-55}. Histological assessment allows the exact anatomical location, number and maturational stage of sequestered parasites to be quantified and is the gold standard, but is considerably limited by the requirement for tissue samples which

in most cases will come only from subjects who die and not those who survive severe malaria, and may not be representative of the situation preceding death.

Given this limitation, several indirect methods to quantify pRBC sequestration have been developed. Although these methods can be very useful, it needs to be borne in mind that their validation is also hindered by the lack of direct measurements.

Mathematical models have been developed to estimate the sequestered parasite burden, fitting data from parasitemia measurements in subjects receiving successful drug treatment in clinical trials⁵⁶⁻⁵⁷ or, occasionally, following ineffective drug treatment⁴⁸. A more direct way to determine the total parasite biomass in an individual, and hence to calculate the sequestered parasite biomass, is measurement of soluble parasite molecules – such as *P. falciparum* histidine rich protein 2 (PfHRP2) – which are released into the circulation. PfHRP2 is a relatively large molecule, assumed to remain mostly in the intravascular compartment, released predominantly at schizogony⁵⁸ and eliminated with first order kinetics, not dependent on renal or liver function⁹. These characteristics make it possible to relate PfHRP2 concentration to total parasite biomass within the body⁹. Subtraction of circulating parasite biomass (calculated from parasite density in blood and estimated blood volume) from the total body parasite biomass (calculated from PfHRP2 concentration) reveals the sequestered parasite burden. Using this methodology, the sequestered biomass in Thai adults with severe malaria was estimated to be 10 times higher than in those with uncomplicated malaria⁹. Alternatively, parasite-derived lactate dehydrogenase has been used to quantify the biomass of parasites in human tissues collected at postmortem⁵⁹. Observation of blood flow in human retinal and rectal blood vessels, using fluorescein angiography⁶⁰ or orthogonal polarization spectroscopy⁶¹ has provided estimates of vascular occlusion as a proxy measure of sequestration.

What is severe malaria?

The term “severe malaria” is applied to a variety of clinical manifestations of malaria which are associated with an increased risk of death or other adverse outcomes such as neurological sequelae. The definition of severe *P. falciparum* malaria is based on

criteria published by the WHO⁶²⁻⁶³, often modified to suit local circumstances⁷ or improve specificity of the diagnosis⁶⁴⁻⁶⁵, which attempt to identify patients at increased risk of death. The WHO criteria include numerous clinical and laboratory features, although the vast majority of children who will die from malaria can be recognized from the presence of the following clinical syndromes (which may occur alone or in any combination): impaired consciousness, severe respiratory distress or severe anaemia⁶⁶. The spectrum of severe disease can be different in adults, with more frequent jaundice, renal impairment and pulmonary edema, and less frequent hypoglycaemia⁶², but acidosis and reduced consciousness still predict the majority of deaths⁶⁷. The WHO criteria are sensitive for identification of those at risk of death but are not specific since other illnesses can cause similar clinical features and malarial parasitemia may be a coincidental finding⁶⁴. For research purposes, the definition can be improved by setting a threshold level of parasitemia (as the likelihood of the illness being attributable to malaria increases with the parasitemia) and use of simple clinical and laboratory tests to exclude other illnesses⁶⁴⁻⁶⁵.

Although *P. falciparum* is by far the most common cause of malaria deaths, fatal cases of *P. vivax* and *P. knowlesi* infection are well recognized^{6, 68}. The definitions of severe disease due to these *Plasmodium* species have largely been extrapolated from those for *P. falciparum*^{5, 21-22, 69}. In Papua New Guinea, where both *P. vivax* and *P. falciparum* infections are common, the proportion of *P. vivax* cases fulfilling the definition of severe disease is similar to that for *P. falciparum*; all three severe disease syndromes have been observed although *P. vivax* is more likely to cause respiratory distress^{5, 70}. Similarly severe *P. knowlesi* disease appears to manifest most frequently as respiratory distress; renal failure and shock are also common but coma has not been reported in human hosts^{21-22, 69}. For all *Plasmodium* species, severe malaria is the exception rather than the rule: the vast majority of cases are uncomplicated^{5, 66, 71}.

An important, and unresolved, issue is whether the different severe malaria syndromes are distinct entities with distinct pathophysiology, or are simply different manifestations of the same underlying process. Similarly, it is assumed, but not proven, that the pathophysiological processes are the same for the different *Plasmodium* species. A number of observations support the possibility of distinct

pathophysiologies for different syndromes. For example, the median age of children with severe anemia is significantly lower than that of children with respiratory distress or cerebral malaria⁷², profiles of pro-inflammatory and anti-inflammatory cytokines differ between severe disease manifestations⁷³⁻⁷⁵, and sequestration of pRBCs is markedly less - not only in brains but also in most other organs - when compared between fatal cases of non-CM and CM malaria⁵⁴. However, the inherent limitations of studying disease causation and progression in humans make it impossible to discern whether these differences reflect different pathological processes or different stages of the same process.

The association between sequestration and severe malaria

It is widely reported that pRBC sequestration leads to microvascular obstruction and resultant tissue ischemia and hypoxemia and thus, especially, CM^{8, 61, 76-77}. However, there is an evolving opinion that adherent pRBC also trigger signaling events in vascular endothelial cells and cause release of inflammatory mediators within the blood vessels^{41, 78}. Either, or both, of these scenarios place pRBC sequestration as the proximal event in the pathogenesis of severe malaria: without sequestration there should not be severe disease. Indeed, there is abundant evidence to support the association of pRBC sequestration with severe *P. falciparum* malaria but, understandably – given the limitations of clinical studies, there is a lack of data showing a causal association. The limitations of the various forms of evidence are summarized in Table 1.

Postmortem studies examining the gross, microscopic and ultrastructural characteristics of organs from individuals dying of severe malaria have tended to focus on the pathology of cerebral malaria. In children the post-mortem hallmarks of CM are swelling and edema of the brain, sequestered parasites in blood vessels, hemozoin-containing macrophages, and petechial (ring) hemorrhages¹⁰. In adults cerebral edema is less common and not associated specifically with CM⁷⁹ but other features are similar. However there is considerable variability between individuals in the pathological findings in CM and the actual cause of death is often difficult to establish (reviewed

in¹⁰). There is frequently striking evidence of pRBC sequestration in the majority of small vessels in the brains of patients dying from cerebral malaria^{32, 55, 59, 80-81}, with all stages of the asexual lifecycle detectable at up to 40-times higher concentrations than in the peripheral blood⁸⁰. However, the extent of sequestration is variable⁵⁵: there is considerable overlap in the extent of sequestration in brains of patients dying with CM and that in brains of patients dying with other severe malaria syndromes^{32, 54-55} and some studies suggest that CM can occur with minimal sequestration or that extensive sequestration may not always be associated with CM^{32, 55, 80}. Sequestration of pRBC is associated with localised endothelial activation (increased expression of ICAM-1, VCAM-1 and E-selectin)⁸²⁻⁸³; iNOS⁸⁴⁻⁸⁵, EpoR and CD131⁸⁶ expression and adjacent diffuse axonal injury^{81, 87}, but absence of an inflammatory infiltrate or necrosis⁸¹⁻⁸². In children^{81, 83} (and probably to a lesser extent in adults⁷⁹), intercellular junction and blood brain barrier breakdown coincides with sequestration. Sequestration also occurs in other tissues of patients with CM^{10, 32, 59, 82, 88}, and so is not specific for the cerebral vasculature, and is associated with endothelial activation in these other sites^{82, 88}.

Despite demonstrating the intimate association between sequestration and pathology of CM, these studies are limited by the static nature of the histopathological assessment - making it impossible to differentiate vascular blockage from vascular stasis or post-mortem pooling of pRBC in certain sites. More importantly, the single snap-shot of data and the lack of data from appropriate controls (those without CM, those with CM who recover) makes it impossible to determine the sequence of events, or to assess whether sequestration is either necessary or sufficient for CM. We speculate that terminal events in patients dying with CM (in children this is most often respiratory arrest with sustained cardiac output⁸⁹) may artefactually increase the accumulation of pRBCs in cerebral blood vessels due to their greater resistance to flow and increased adhesive properties³⁴. This might explain why muscle biopsies of African patients with both CM and non-CM malaria showed extensive vascular endothelial activation but no pRBC sequestration, whereas necropsy specimens of muscle did show sequestration⁸⁸. Despite the higher mortality rate in African children with malaria and severe respiratory distress (SRD) than with CM⁶⁶, the association of pRBC

sequestration with SRD, or with the related condition of metabolic acidosis, has not been specifically assessed in postmortem studies.

Malarial retinopathy, which in postmortem studies is strongly associated with pRBC sequestration in the retinal vessels^{10, 53}, is a frequent observation in clinically defined CM and can help to distinguish CM from other causes of encephalopathy in parasitemic children^{53, 90}. Its hallmark features are retinal whitening, vessel discoloration, hemorrhages, and papilloedema⁹¹; retinal whitening corresponds with areas of capillary non-perfusion⁶⁰. Retinopathy is most likely to be seen in fatal cases⁹²⁻⁹³, but adults and children with uncomplicated malaria and with non-CM severe malaria may have a similar retinopathy, albeit less frequently than in CM⁹²⁻⁹⁶. Unfortunately, examination of the retina *in vivo* does not allow visualization of individual RBCs so quantification of sequestration is impossible, and is inferred from the pattern of retinopathy or by correlation at postmortem⁹⁰. Impairment of microvascular blood flow has also been observed *in vivo* by imaging of the rectal mucosal capillaries and correlated with markers of disease severity such as lactate and parasitemia, but again the contribution of sequestration cannot be directly assessed⁶¹. Other attempts to measure *in vivo* sequestration by biopsy of superficial tissue have yielded conflicting results: one study found no evidence of sequestration in skeletal muscle⁸⁸ whilst another found that sequestration in muscle was inconsistent and rather limited to CM cases⁵¹. In another small study, sequestered pRBCs were much more frequent in dermal specimens from severe than uncomplicated malaria cases⁵².

Indirect estimates of total parasite burden based on mathematical models of parasite clearance curves^{49, 56-57} have indicated that pRBC sequestration is greater in fatal malaria than in severe non-fatal malaria, and lower again in uncomplicated malaria, and that sequestered parasite burden is predictive of a fatal outcome⁵⁶. However, again, there is considerable heterogeneity in these data, especially amongst African children with CM⁵⁶⁻⁵⁷, with estimates of sequestered parasite burden being quite modest in surviving children treated for CM in the Gambia (approximately one third of the circulating parasite burden⁵⁶) and considerably lower than this in some children⁵⁶. In line with this we recently reported that estimates of sequestered parasite burden

derived from plasma PfHRP-2 from 296 Gambian children did not differ significantly between cases of uncomplicated and severe malaria, allowing the conclusion that in these children extensive parasite sequestration is not a prerequisite to develop severe disease (Cunnington *et al.*, manuscript under review). This contrasts with the very high estimates of sequestered parasite burden in Thai adults (obtained using the same method), being double the circulating parasite biomass in survivors and four times higher in fatal cases⁹.

In summary, therefore, although the association between pRBC sequestration and severe malaria is incontrovertible, a causal association has not been demonstrated. PRBC sequestration could be either the cause of severe malaria or a consequence of other underlying pathologies. Even with the advent of non-invasive imaging techniques, establishing the sequence of events in humans is impossible because it is unethical to allow uncomplicated malaria infections to progress to severe disease.

Sequestration does not fully explain severe malarial pathology

Rapid clinical recovery without persistent neurological symptoms is the most common outcome of CM following treatment⁷⁷. Focal necrosis is not a feature of CM brains postmortem¹⁰ and acidosis can be rapidly reversed following initiation of antimalarial therapy⁹⁷. It is hard to reconcile these observations with the dramatic obstruction of vessels seen at postmortem in CM or with the assumption that microvascular obstruction by pRBCs is the proximal cause of disease. Such extensive pRBC sequestration would not be expected to show such rapid reversibility and would be expected to leave some irreversible endothelial damage. Extensive microvascular obstruction may explain cases where neurological defects persist after treatment⁹⁸, but this is the exception rather than the rule.

That CM can occur with low sequestered parasite burdens^{56, 59, 82} and without retinopathy⁹⁹ indicates the extensive sequestration of pRBCs is not an absolute prerequisite, and our data from Gambian children suggest that severe disease occurs in the presence of high parasitemia, but with relatively little sequestration (Cunnington *et*

al., manuscript under review). Although sequestered biomass was a better predictor of death than peripheral parasitemia in Thai adults⁹, peripheral parasitemia is a strong predictor of mortality among non-immune adult travelers returning to Europe¹⁰⁰⁻¹⁰¹. Also, in the Thai study, total parasite biomass was not significantly predictive of death after adjustment for level of consciousness, renal function and lactate indicating that sequestration may not, in fact, be the major determinant of fatal outcome⁹.

It is also hard to reconcile microvascular obstruction with the well recognized possibility of clinical deterioration after the onset of treatment (most often with progression to coma)¹⁰⁰⁻¹⁰³. Since quinine and artesunate preferentially kill late ring and early trophozoite parasite forms (which would be the pool of parasites most likely to contribute to further sequestration)⁴⁹ treatment should limit further pRBC sequestration, but will not inhibit the rupture of schizonts already sequestered in microvasculature. In a Gambian study, deterioration to death was associated with an increase in parasitemia (implying rupture of schizonts and release of merozoite)⁵⁶, and is thus more easily explained by a pathological effect of schizogeny (and release of pRBC contents) than by additional sequestration.

Furthermore, sequestration in all organs (i.e. not just the brain) is usually more extensive in CM cases than in non-CM cases at post mortem⁵⁴ but case fatality rates are actually lower for CM than for severe respiratory distress in pediatric studies⁶⁶, indicating that the extent of sequestration is poorly correlated with outcome. Consistent with these observations, neither cytoadhesion properties of parasite isolates nor genetic polymorphisms of host receptors have shown consistent associations with severe disease (reviewed in^{31, 104}). In contrast, vascular endothelium from CM patients shows greater activation in response to inflammatory cytokines than that of controls¹⁰⁵, indicating that vascular endothelial activation may be more important than sequestration in determining outcome of infection.

Finally, both *P. vivax* and *P. knowlesi* infections can cause severe malaria and severe disease has been reported in isolated cases with *P. malariae* infection^{5, 21, 106} but there is no obvious correlation with pRBC sequestration. *P. vivax* shows limited cytoadherence to host vascular endothelium, but all stages of the asexual lifecycle are

regularly seen on blood films and pRBC deformability is increased compared with uninfected RBCs, making microvascular sequestration and obstruction extremely unlikely explanations for its pathogenesis⁶⁸. There are, however, no modern comprehensive postmortem studies of fatal *P. vivax* malaria which would allow comparison with equivalent manifestations of severe *P. falciparum* malaria⁶⁸. On the other hand, *P. vivax* induces a relatively greater inflammatory response and more endothelial activation than *P. falciparum*¹⁰⁷, which might explain its ability to cause severe disease at lower parasitemia⁵. *P. knowlesi* may have the capability to cytoadhere, judged by the (variable) binding to ICAM-1 and VCAM but not CD36¹⁹, but peripheral parasitemia predicts severity of infection²¹ and CM has not been described⁶. Intriguingly, postmortem of an adult patient infected with *P. knowlesi* who died from circulatory collapse showed petechial hemorrhages and pRBC accumulation in the brain remarkably similar to that in *P. falciparum* CM, however there was no evidence of endothelial activation (assessed by ICAM-1 expression) and the patient did not have coma preceding circulatory collapse¹⁸. This suggests that the pRBC accumulation in the brain is not sufficient for the development of CM. There is no evidence that *P. malariae* sequesters but it does occasionally cause very severe illness^{106, 108}.

Experimental models of severe disease and sequestration

The use of experimental animal models to investigate the pathophysiology of severe malaria has recently come under intense scrutiny and fuelled a highly polarized debate^{8, 109}. In particular, the well characterized model of experimental cerebral malaria (ECM), *P. berghei* ANKA (PbA) infection in susceptible inbred strains of mice¹¹⁰⁻¹¹¹, has been criticized⁸ for failing to recapitulate “key” pathological features of human CM, such as extensive pRBC sequestration. However, throughout this debate, the assumption that pRBC sequestration is the proximal cause of human CM has not been questioned. In fact, PbA pRBCs do sequester, most notably in lungs and adipose tissues, as evidenced by examination of serial blood films and bioluminescent imaging²⁵. Sequestration is CD36-dependent²⁵ and confers a growth advantage to parasites¹⁶. Nevertheless, CD36^{-/-} mice (in which sequestration is abolished) are fully

susceptible to ECM implying that sequestration is not a prerequisite for ECM. Conversely, acute lung injury is reduced in PbA-infected CD36^{-/-} mice⁴⁷ which may be due to lack of sequestration or to lower total body parasite biomass.

An alternative, inflammatory, etiology has been well described for ECM and other severe pathologies caused by rodent malaras, that involves endothelial activation, CD4⁺ and CD8⁺ T-lymphocytes, inflammatory cytokines (reviewed in ¹¹¹⁻¹¹³) and heme¹¹⁴⁻¹¹⁵. Moreover, despite the lack of overt vascular obstruction, there is evidence of microcirculatory dysfunction and impaired blood flow, resulting in cerebral hypoxia and ischemia¹¹⁶⁻¹¹⁸. *In vitro* experiments support the assertion that inflammation rather than adherence is a pre-requisite for many of the pathological features of CM. For example, *P. falciparum* pRBCs decrease the integrity of monolayers of human brain microvascular endothelial cells¹¹⁹, cause proinflammatory endothelial activation¹²⁰ and upregulation of ICAM-1¹¹⁹. These effects are largely independent of pRBC cytoadherence and involve soluble mediators¹¹⁹⁻¹²¹ such as histones, which are released from ruptured pRBCs¹²². Furthermore, soluble inflammatory mediators liberated from pRBC-activated endothelium can cause astrocyte activation and neuronal axonal damage¹²³.

An alternative etiology for severe malaria

If the proximal role of pRBC sequestration in severe malaria is to be challenged, alternative explanations which better explain the features of different severe malaria syndromes in children, adults and experimental models across the spectrum *Plasmodium* species must be proposed, and these explanations must account for the strong association of sequestration with severe disease in humans. The most convincing hypotheses relate to endothelial activation and dysfunction, and have been formulated in a variety of different forms to either supplement or replace the role of pRBC sequestration^{41, 78, 123-126}. Inflammatory activation of the vascular endothelium and disruption of the blood brain barrier may be mediated by molecules released at pRBC rupture, such as hemoglobin-derived heme^{114, 127-128} and soluble parasite

products¹¹⁹⁻¹²¹ including histones¹²², and by circulating cytokines such as TNF- α ¹²⁹ without the need for cytoadhesion.

The vascular endothelium is intimately involved in the control of microcirculatory blood flow and oxygen delivery, mainly through nitric oxide production¹³⁰⁻¹³¹, and represents the physical barrier and point of interaction between pRBC and tissues⁴¹. Profound abnormalities of microcirculatory function, due to reduced nitric oxide bioavailability, have been observed in patients with severe malaria^{97, 132-134} and in mice with experimental cerebral malaria¹¹⁸, and correlate with disease severity and measures of hemolysis^{97, 132, 134-137}. Mechanistically this involves reduced availability of the essential substrate for NO synthesis (arginine)¹³², quenching of nitric oxide by cell free hemoglobin which is released as a result of intravascular hemolysis¹³⁴, and increases in endothelial activating molecules such as angiopoietin-2^{133, 136-137}. On this basis, it has been hypothesized that nitric oxide administration may be of benefit as an adjunctive therapy in severe malaria¹³⁸, and a clinical trial has been initiated¹³⁹.

Microcirculatory dysfunction causes heterogeneity of blood flow across vascular beds and regional hypoxia^{130, 140}. Regional hypoxia is evident from elevated cerebrospinal fluid lactate concentrations in adults and children with CM¹⁴¹⁻¹⁴² and increased lactate in the brain parenchyma in ECM¹¹⁷; and almost certainly contributes to elevated blood lactate levels seen in CM and other manifestations of severe malaria¹⁴³⁻¹⁴⁴.

Heterogeneous patterns of microcirculatory flow, with adjacent areas of stagnant and hyperdynamic flow, have been visualized in the rectal mucosa of patients with severe malaria⁶¹; patchy capillary non-perfusion in the retina and postmortem congestion of cerebral vessels (which would not normally be patent)^{55, 60} are indicative of microcirculatory disturbance in the CNS. These features are identical to those of distributive shock associated with sepsis, where they are attributed to endothelial dysfunction rather than mechanical obstruction of vessels¹³⁰. Microcirculatory dysfunction due to low nitric oxide bioavailability is also causally associated with ECM^{116, 118}.

Thus many of the features of CM and severe non-CM can be explained without sequestration. However, as a consequence of endothelial activation, increased

expression of adhesion molecules such as ICAM-1¹⁴⁵ will facilitate pRBC sequestration and select for parasite clones expressing relevant sequestration ligands, and may explain why sequestration is seen in patients with the most severe endothelial dysfunction. Additionally, where microcirculatory dysfunction leads to reduced rates of flow, pRBCs would be expected to accumulate due to their reduced deformability or weak cytoadhesion³⁴.

Concluding remarks

We propose that vascular endothelial dysfunction provides a unifying explanation for the pathophysiology of severe malaria across *Plasmodium* species, host species and host age ranges (Figure 1, Box). Endothelial dysfunction can occur in all host species, in response to any type of malaria infection, irrespective of the ability of the pRBCs to cytoadhere and sequester. Endothelial dysfunction will lead to systemic microcirculatory disturbance, tissue hypoxia and lactate accumulation. Importantly, however, all of these effects are rapidly reversible once the proximal cause – the infected red blood cells – are removed. For those malaria species where cytoadherence does occur, endothelial activation will facilitate pRBC sequestration in a dose-dependent manner. In other words, the more severe the endothelial dysfunction, the more sequestration there will be. Since the extent of endothelial dysfunction is expected to correlate with disease severity, sequestration is also expected to correlate with disease severity. In particularly severe cases, pRBC sequestration leading to complete vascular obstruction may be the final straw - leading to irreversible damage to vital organs and thus to death. Such a scenario is entirely consistent with the post-mortem findings in the small proportion of severe malaria cases that are fatal.

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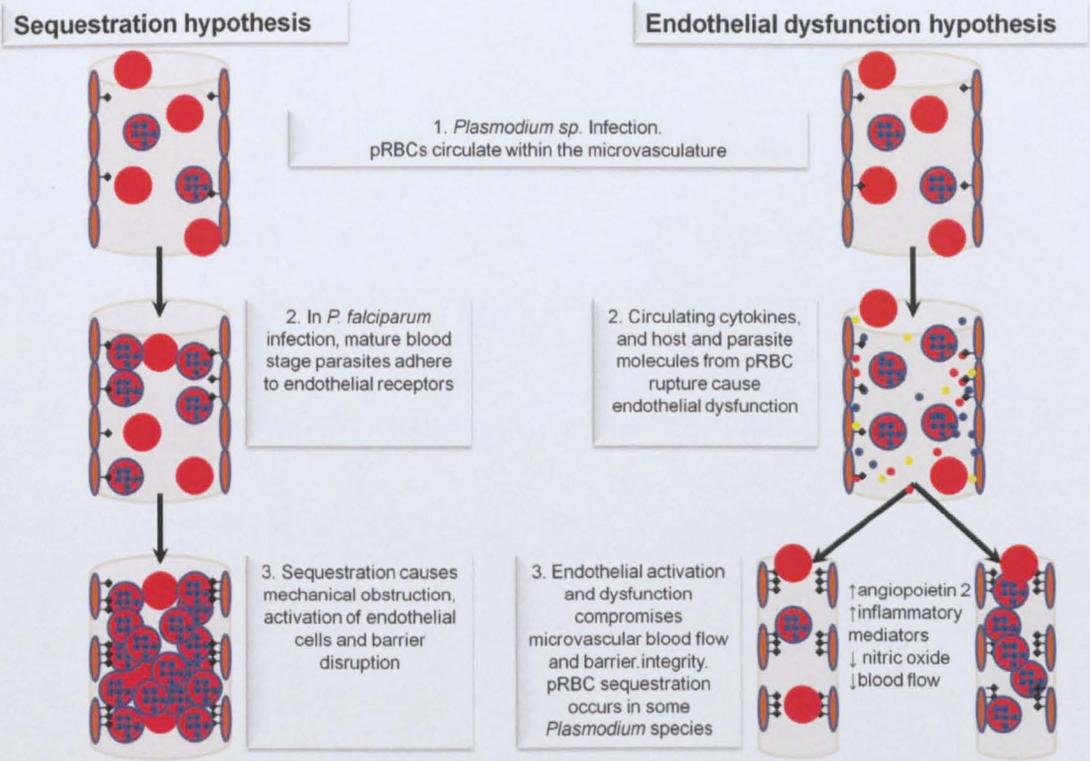
Table 1. Pitfalls in studies designed to assess whether sequestration causes severe disease

| Study Design | Problems | Potential Bias |
|---|--|---|
| All studies | Specificity of diagnosis of severe malaria | Inadvertant inclusion of patients dying from other causes, with incidental parasitemia will make interpretation more difficult |
| Postmortem | Static assessment | Sequence of events cannot be established: necessity of pathological findings to cause severe disease cannot be established |
| | Selection of control groups | Comparison with uncomplicated malaria cases cannot be performed: sufficiency of pathological findings to cause severe disease cannot be established |
| | Observer bias | Without blinding pathological findings may be over-interpreted |
| Tissue biopsy | Limited availability of tissues from live subjects | Tissues of interest in pathogenesis of severe disease may not be accessible |
| | Selection of controls | Failure to match controls for equivalent peripheral parasitemia |
| Visualisation of obstructed blood flow | | Failure to compare severe with uncomplicated malaria |
| | Limited availability of vascular beds | May not be representative of microcirculation in the brain or viscera |
| | Only vessel patency / perfusion can be assessed | Flow abnormalities may be due to microcirculatory dysfunction independent of sequestration |
| Biochemical assessment eg PfHRP2 | Assumption based model to calculate sequestration | Incorrect estimation of model parameters may cause systematic bias in biomass estimates |
| Genetic studies | Linkage dysequilibrium | Effects of polymorphisms in different genes in linkage disequilibrium may increase or decrease the effect of the gene of interest |
| | Large sample size required to determine effect | Insufficient sample size increases likelihood of finding no effect |
| | May be influenced by parasite as well as host genetics | May reduce power to find a significant effect |
| Parasite binding studies | Parasites isolated from blood may differ from those sequestered in the tissues | May reduce power to detect association between parasite binding phenotype and severe disease |
| Animal models | Different host / parasite species combinations | Models selected for specific disease features may not represent the natural interaction of parasite and host |
| | Possibility of different pathogenesis | Conclusions about causality from experimental manipulation may only be valid to the specific model system |

Box: A unifying hypothesis

1. The severity of endothelial dysfunction is determined by the combined effects of cytokines, soluble host and parasite-derived molecules, and anatomical and genetic factors which influence the response to these stimuli in specific vascular beds. These effects are not anticipated to be uniform
2. The rate of expansion of the parasite biomass and rupture of pRBCs influences the magnitude of the stimulus provoking endothelial dysfunction, and also determines whether regulatory host mechanisms can maintain homeostasis or whether decompensation occurs and severe disease develops. Parasite growth is restricted by splenic clearance and innate and adaptive immune responses
3. pRBC sequestration helps parasites to avoid clearance by the spleen, facilitating more rapid expansion of the parasite biomass, and also results in higher local concentrations of soluble mediators presented to the endothelium when pRBCs rupture
4. Reduced nitric oxide bioavailability due to the effects of hemolysis, and reduced arginine availability, causes impaired distribution of microvascular blood flow and results in reversible regional hypoxia, and anaerobic tissue metabolism
5. In the brain, regional hypoxia, blood brain barrier disruption, and elaboration of soluble inflammatory mediators from brain microvascular endothelial cells cause variable degrees of edema and axonal damage
6. Progressive endothelial dysfunction is accompanied by increased expression of adhesion receptors and thus increased pRBC cytoadherence, hence sequestration becomes intimately associated with severe or fatal malaria. As pRBCs increasingly pack vessels in the dysfunctional microcirculation they may further reduce blood flow through increased viscosity and direct physical obstruction
7. Progressive hypoxia, ischemia and inflammation cause worsening endothelial dysfunction in a self-amplifying cycle, and result in the manifestations of severe malaria eventually progressing to death if untreated

Figure 1.



Chapter 9. Discussion.

The roles of hemolysis, heme and HO-1 in the direct and indirect burden of malaria disease

In the preceding chapters the results, their limitations and implications have already been discussed in some detail. In this chapter the discussion will focus on how these findings contribute to understanding several conundrums associated with malaria: immunosuppression, susceptibility to bacterial infection, the role of HO-1 in severe malaria, and iron metabolism during malaria infection.

Immunosuppression by malaria

The literature review presented in Chapter 1 demonstrated that immunosuppression is probably a misleading term for the immunological consequences of malaria. Malaria is specifically associated with impaired resistance to EBV and Gram negative bacteremia, and with impaired vaccine responses to predominantly T-independent antigens.

Impaired resistance to EBV appears to be related to the intensity of malaria exposure¹¹¹. Recent studies in The Gambia following a sustained decline in malaria transmission over two decades have shown that the impaired T-cell responses to EBV described in the context of intense exposure are no longer seen.⁷⁵ Similarly, in both The Gambia and Kenya, declining malaria transmission has been associated with large reductions in the incidence of NTS bacteremia.¹¹⁸⁴ Using the malaria-dependent protective effect of sickle cell trait on risk of Gram negative bacteremia to control for confounding by other common factors related to both malaria and NTS-risk, the Kenyan study was able to prove (as far as is possible for an epidemiological study) that the decline in malaria transmission was the cause of the declining incidence of Gram negative bacteremia.¹¹ These findings again suggest that intensity of exposure may play a major role in susceptibility. The effect of malaria on vaccine responses was also generally greatest in studies in the highest intensity transmission settings.²¹

The very specific nature of the defects in immune function associated with malaria indicate that malaria does not cause a generalized immunosuppression. They also raise the issue of whether a single mechanism or disparate mechanisms are responsible. If

there was a generalized immunosuppression then initially one may hypothesise a unifying mechanism as the most likely cause. However, the limited impairment of resistance to other infections must make it at least as likely that different mechanisms are responsible for impaired resistance to EBV and Gram negative bacteremia and impaired vaccine responses. Possible explanations for impaired vaccine responses have already been discussed in Chapter 3, and the favoured hypothesis would involve both splenic dysfunction impairing T-independent responses, possibly with an additional contribution from polyclonal B cell activation generally impairing the ability to mount specific antibody responses.²¹ Impaired resistance to EBV may share some of these mechanisms, since acquisition of anti-EBV antibodies may be impaired, and polyclonal B-cell proliferation will promote EBV replication and expand the target cell pool. However, impaired T-cell responses to EBV may only be significantly impaired when the EBV viral load becomes extremely high and there is sustained by-stander activation by prolonged and intense exposure to malaria resulting in functional T-cell exhaustion. Alternatively, malaria may impair T-cell priming through effects on dendritic cells, or malaria induced regulatory T-cells may produce bystander suppression of EBV-specific response. Experimental data to support these hypotheses is not yet available. In contrast, susceptibility to gram negative bacterial infections may involve very different mechanisms as discussed below.

Susceptibility to bacterial infection

In the preceding chapters I have provided evidence to suggest that susceptibility to NTS bacteremia specifically may be caused by neutrophil dysfunction as a consequence of hemolysis, release of erythrocytic heme, induction of HO-1 and consequent impairment of neutrophil function.²⁷⁶ Of note, these studies did not seek to exclude the contribution of other mechanisms, however they do suggest that hemolysis may play a major role in susceptibility. By inference from previous studies in mice, the same mechanisms may also account for the susceptibility to other Gram negative bacteria, whereas in mice hemolysis did not impair susceptibility to *S.pneumoniae*,¹⁰² which shows no association with malaria in human studies.¹¹ We found that even humans with uncomplicated malaria have evidence of impaired neutrophil function, but in

keeping with the clinical studies suggesting that this population would be a low risk of NTS bacteremia, the magnitude of the defect in oxidative burst was not sufficient to impair bactericidal function. Additional studies in the highest risk populations (i.e. children with severe anemia) will be necessary to establish that neutrophil dysfunction is indeed related to susceptibility to NTS bacteremia in humans.

A number of other mechanisms, which may be relevant in malaria patients, have been proposed to cause susceptibility to NTS and warrant additional consideration alongside the experimental findings described in this thesis. The entry of NTS and other Gram negative bacteria into the body is the first step in causing bacteremia, and is a prerequisite even before the interaction of bacteria with macrophages or neutrophils. As these are all enteric organisms, increased bacterial translocation from the gut into the bloodstream has been proposed,¹⁰ due to impaired integrity of the gut mucosa,²⁸⁹ possibly associated with sequestration of pRBCs in the gut microvasculature.²⁹⁰

Others have suggested macrophage dysfunction as the explanation for susceptibility to NTS in malaria, above and beyond any contribution made by hemolysis.^{105 256} This may be explained by macrophage dysfunction as a result of hemozoin ingestion,²⁵⁶ erythro-/hemo-phagocytosis,^{105 291} or reduced production of cytokines such as IL-12 (which is necessary to facilitate the killing of intracellular NTS).¹⁰⁵ Whilst these may all be contributing factors, that may explain the formation of localized NTS abscesses which are frequently seen in patients with macrophage dysfunction due to IL-12/IL-23 signalling defects,²⁹² they do not easily explain the clinical association of malaria with NTS bacteremia. In fact, NTS is far more common as a cause of bacteremia than of abscesses in patients with chronic granulomatous disease where both neutrophil and macrophage function are abnormal.²⁶¹ Furthermore, the macrophage hypothesis is limited by the lack of any direct evidence to date showing that macrophages are the main cell type within which NTS are replicating *in vivo* in humans or animals with malaria co-infection.

Perhaps a more important mechanism, which has received little attention, is the effect of malaria on the natural acquisition of antibodies against NTS. Antibodies play a clear role in protection against NTS bacteremia, and are usually acquired during the second

year of life in African children.⁹⁷ Recurrent episodes of malaria during early childhood might suppress natural acquisition of antibodies to NTS, similar to the suppression of antibody responses to Salmonella capsular polysaccharide vaccine.²¹ Although the mouse model developed in the studies presented in this thesis is assumed to be antibody independent because the mice are Salmonella naive and infection progresses too rapidly for antibody production to make a significant contribution, in humans a lack of antibody may exacerbate the severity of defects in neutrophil oxidative burst.⁹⁴

Another factor worth considering is the recently described role of erythropoietin in impairment of resistance to NTS in mice.²⁹³ Erythropoietin is the main regulator of erythropoiesis in bone marrow, but its receptors are also expressed on other cell types and appear, amongst other effects, to regulate the inflammatory function of macrophages.²⁹⁴ Consistent with this, erythropoietin levels are generally elevated in severe malarial anemia, the major risk factor for NTS,⁸¹ as would be expected as part of the homeostatic response to severe anemia.²⁹⁵ However, evidence is lacking that erythropoietin is associated with susceptibility to infection in humans. In a large prospective study in renal dialysis patients, therapeutic erythropoietin administration was not a risk factor for bacterial infection.²⁹⁶

The experimental findings presented in this thesis are remarkably consistent with the clinical observations of susceptibility to NTS infection in children with either severe malarial anemia, or recent malaria. Although the study in Gambian children provides preliminary evidence and proof of principle for a relationship between hemolysis, HO-1 induction and neutrophil dysfunction, it does not prove that this is associated with clinically significant increase in risk of NTS infection. Ideally this requires a very large prospective study of children with malarial anemia, to have neutrophil function and indices of hemolysis and HO-1 induction assessed at the time of clinical presentation with malaria and at regular intervals after anti-malarial treatment, and to be followed up during convalescence for acquisition of NTS bacteremia. At the time of diagnosis with NTS bacteremia, it would be very useful to again assess neutrophil function, evidence of hemolysis and HO-1 expression, and also to determine the cellular location of NTS in blood. If such a study confirmed that children at highest risk of NTS infection had the most severely impaired neutrophil function, then targeted treatment to

reduce this risk may be justified. This might be antibiotic prophylaxis, or perhaps administration of a HO-1 inhibitor prior to discharge from hospital following successful treatment for malaria. HO-1 inhibition has been successfully performed in human neonates with severe hyperbilirubinaemia secondary to hemolysis, and proved very safe.²⁹⁷⁻²⁹⁹ In children who have already commenced effective treatment for malaria and who are no longer at risk of deterioration, administration of a drug like SnPP is likely to be safe. Similarly it might be administered as an adjunctive treatment to children who present with NTS infection in the context of a negative malaria blood film, but positive antigen based rapid diagnostic test.

The exact mechanism by which HO-1 induction leads to impairment of the oxidative burst in maturing neutrophils was not elucidated in the studies presented in this thesis. Existing literature affords speculation about several mechanisms which might be investigated in further studies. The most direct mechanism simply involves catabolism of cellular heme, such that little is available for incorporation into the gp91^{phox} subunit of the NADPH oxidase complex.²²³ The main problem with this explanation is that induction of HO-1 by heme is likely to also provide adequate heme for gp91^{phox} maturation, unless the increased HO-1 activity persists long enough to catabolise the cellular heme pool as well as the finite amount of administered heme. Others have shown that bilirubin²¹⁸ and CO³⁰⁰ can inhibit NADPH oxidase activity, whilst Nakahira and colleagues have proposed that direct binding of CO to the heme moiety of gp91^{phox} interferes with the assembly of the enzyme complex.²¹⁰ In a different model system involving priming of neutrophil oxidative burst activity in rats by alcohol intoxication, HO-1 induction with CoPP decreased the expression of p47^{phox} and p67^{phox}, but the molecular basis of this was not investigated.²¹⁷ At present the exact mechanism by which heme-induced HO-1 might suppress neutrophil oxidative burst activity remains to be clarified. Any mechanism identified must also explain why only a proportion of neutrophils appear to have abnormal oxidative burst activity in the study in Gambian children with malaria. In acute malaria this might be proposed to be due to the mobilisation of immature granulocytes with an impaired oxidative burst, but during convalescence this is unlikely to be the sole explanation. As proposed in Chapter 6, the persistence of hemozoin in bone marrow may cause prolonged

induction of HO-1 in myeloid lineage cells and perhaps influence the oxidative burst capacity of only a proportion of developing granulocytes.

The roles of HO-1 in malaria

The results of the studies presented in this thesis also need to be considered in the wider context of the possible roles of HO-1 in malaria. Elegant studies in mice have clearly demonstrated that the ability to upregulate HO-1 is essential for survival in experimental severe malaria infections.¹¹²⁻¹¹⁴ However, HO-1 induction also promotes the survival of liver stage malaria parasites in experimental infections in mice, which in theory might lead to a greater inoculum of merozoites and more rapid ascent of blood stage parasitemia.²⁵⁵ In humans, it is much harder to isolate the effects of HO-1 induction from other factors which might also determine susceptibility to severe malaria, but it remains at best uncertain whether the results obtained in mice may be of relevance. The most common strategy to find evidence that HO-1 induction is causally related to susceptibility to severe malaria has been to examine the association with genetic polymorphisms which determine HO-1 expression. The most consistent finding has been an association between the polymorphic (GT)_n repeat in the HMOX1 promoter and disease severity.^{158 163-164} Contrary to expectations, short (GT)_n repeats, which confer greater *HMOX1* mRNA synthesis in response to heme, are more common in patients with severe malaria than uncomplicated *P. falciparum* malaria.^{158 163-164} One other study has reported that long (GT)_n repeats, associated with lower HO-1 protein expression, were seen more frequently in subjects with symptomatic than asymptomatic (predominantly *P. vivax*) malaria.³⁰¹ However this study had significant methodological limitations, using a different definition for long (GT)_n repeats and comparing alleles rather than genotypes between subjects. Discounting the findings of the latter study, a reasonable explanation for the somewhat contradictory observations in mice and humans is that the relationship between HO-1 levels and malaria severity is "U" shaped: whilst some HO-1 induction is desirable to protect the host against malaria, excessive HO-1 induction can also become harmful. In inbred mice there are no polymorphisms which influence HO-1 expression and the increase in *hmx1* expression required to protect from severe malaria is only 4-5 fold,¹¹² whereas

in humans with severe malaria *HMOX1* expression may show up to a 10-fold increase.¹⁵⁸ It is possible that beyond a certain point, excessive HO-1 induction may cease to prevent oxidative damage and actually begin to enhance it by increasing intracellular ferrous iron availability.³⁰² Consistent with this, neutrophils incubated overnight with a high concentration of hemin showed an enhanced oxidative burst upon PMA stimulation, which was abrogated by co-incubation with SnPP.¹⁵⁸

The findings in mice and humans presented in this thesis become particularly interesting when considered alongside the possibility that extent of HO-1 induction determines whether production of reactive oxygen species is limited or exacerbated. That induction of HO-1 expression in progenitor cells in bone marrow leads to reduced oxidative burst activity, may represent an adaptive mechanism to limit the consequences of excessive HO-1 induction elsewhere, by reducing the production of reactive oxygen species.²⁷⁶ Similarly, downregulation of surface CD163 expression on monocytes and presumably macrophages in acute malaria, may also serve to limit excessive HO-1 induction. Although this might limit the immunomodulatory effect of modest HO-1 induction, it might also prevent the potential pro-oxidative effect of excessive HO-1 induction. Further studies to prospectively assess the potential role of HO-1 in human malaria are clearly needed before serious consideration is given to the manipulation of HO-1 expression, or administration of carbon monoxide, as potential therapies in severe malaria. These studies will need to consider the many different stimuli for HO-1 induction, the numerous factors that control the availability and cellular delivery of the substrate heme, and the potential modifying effect of other changes within host cells that may influence oxidative and non-oxidative cell damage.

Heme and iron metabolism in malaria

The role of HO-1 induction in malaria must not only be considered in the context of protection from the toxicity of free heme and reactive oxygen species,³⁰³ but also in the context of its crucial role in heme catabolism and the coordinated regulation of heme iron recycling and redistribution.³⁰⁴ The increase in erythrocyte destruction in malaria, either by rupture of infected erythrocytes, intravascular hemolysis or erythrophagocytosis by macrophages of the reticuloendothelial system results in a

huge amount of heme iron being directed primarily to macrophages.³⁰⁵ Under normal conditions hemoglobin-haptoglobin complexes and heme-hemopexin complexes would enter the macrophage via binding to their respective receptors on the macrophage surface, heme would be liberated in the intracellular environment, and HO-1 would be induced.³⁰⁴ At the same time increasing intracellular heme would increase expression of ferroportin and ferritin. Ferroportin is the sole iron exporter in mammalian cells and transfers ferrous (Fe^{2+}) iron extracellularly where it is converted to Fe^{3+} and binds to plasma transferrin before being trafficked to the bone marrow for re-utilization.³⁰⁴ Ferroportin is dominantly regulated by plasma hepcidin, a hormone synthesised predominantly in the liver, which binds to and causes internalization and degradation of ferroportin, thus preventing iron efflux.³⁰⁶ Hepcidin is induced as part of the innate immune response to infection,³⁰⁷ and is upregulated in malaria,³⁰⁸⁻³⁰⁹ where it may limit superinfection by additional *Plasmodium* strains or species by depriving them of iron that they require for growth in the liver prior to blood stage infection.³¹⁰ One obvious consequence of increased hepcidin expression in malaria is that heme-derived iron will remain sequestered in macrophages,³⁰⁵ and if HO-1 is induced by the acquisition of heme in these cells, its activity will result in increasing intracellular Fe^{2+} and ferritin. Presumably this iron would be available to siderophilic intracellular pathogens such as NTS. However, downregulation of cell surface CD163 expression on monocytes and macrophages, as observed in the study of Gambian children presented in this thesis, may to some extent limit this increase in intracellular Fe^{2+} , until the stage that haptoglobin is saturated with hemoglobin and free hemoglobin begins to release heme. If hemolysis is sufficiently severe to reach this stage, then heme will bind to hemopexin and can enter cells through CD91 binding, which appears unimpaired by malaria, and will induce HO-1 and be catabolised as normal. Thus one level of protection against the damaging effects of hemolysis may be sacrificed in order to limit intracellular iron accumulation. If this is so, then what happens to the circulating hemoglobin-haptoglobin complexes that are not being removed by their normal pathway? In addition to loss of surface CD163 expression as described in Chapter 6, others have described increased soluble CD163 levels in acute malaria,³¹¹ and soluble CD163 may bind the circulating hemoglobin-haptoglobin complexes, but how might this alter the fate of these complexes? At present the

answers to these questions are uncertain, but pinocytosis of the hemoglobin-haptoglobin complexes by hepatocytes is one possibility,³¹² and urinary excretion is another possible explanation, which might contribute to iron deficiency in children suffering frequent episodes of malaria.

Another reason why changes in the host responses to hemolysis and regulation of iron metabolism may be important, is because of the association between hemolysis and severe malaria. Although somewhat different mechanisms have been proposed, hemolysis appears to contribute to both endothelial dysfunction in *P. falciparum* malaria³¹³ and cytotoxicity in experimental severe malaria in mice,¹¹² as discussed in Chapter 8. It is conceivable that administration of haptoglobin,³¹⁴ hemopexin,¹¹⁵ or both might be considered as potential adjunctive therapeutic strategies, to limit these deleterious effects. Understanding the potential benefits and risks of these therapies will require further exploration of the fates of intravascular haptoglobin-hemoglobin and heme-hemopexin complexes during malaria.

Conclusions

The aim of this thesis was to investigate the mechanism of susceptibility to bacterial infection caused by malaria and its relationship with hemolysis. Initially I reviewed the evidence for immunosuppression by malaria and found that in humans it appears to be limited to a few specific situations, with the term immunosuppression probably being misleading. Based on existing literature describing the susceptibility to NTS infection in mice and humans, and recent literature describing the crucial role of HO-1 in tolerance to malaria, I hypothesised that HO-1 induction might also mediate the susceptibility to NTS. In a mouse model I have demonstrated that hemolysis-derived heme causes HO-1 dependent dysfunction of neutrophils, characterised by impaired oxidative burst activity, failure to kill *S. typhimurium*, and consequently the creation of a new niche for replication. In a proof-of-principle study in Gambian children I found evidence of similar neutrophil dysfunction, and that this was associated with hemolysis and HO-1 induction, supporting the applicability of the findings in the mouse model. The implications of these findings may extend to bacterial infections associated with other

hemolytic diseases, and may offer a potential for reversal of the susceptibility to NTS following malaria. I also found evidence that malaria infection causes changes in the host pathways involved in scavenging and recycling heme, which may have implications for the pathogenesis and treatment of severe malaria.

In a study which began as a separate project alongside the investigations on HO-1 in malaria, I found that severe malaria in Gambian children was not associated with extensive parasite sequestration, questioning the current dogma that severe malaria is caused by sequestration of parasitized red blood cells. This prompted me to review the evidence for sequestration and other mechanisms in the pathogenesis of severe malaria, and my conclusions were that endothelial dysfunction is more likely to be the proximal cause of severe disease than is sequestration. The reasons for endothelial dysfunction are multiple, but prominent amongst them is the role of hemolysis in impairing nitric oxide bioavailability and endothelial activation and injury. Thus the destruction of infected red blood cells and release of their contents as a consequence of the intraerythrocytic life cycle of *Plasmodium* species appears to be the primary cause of much of both the direct and indirect burden of malaria.

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Appendices

**1. *Scientific coordinating committee and ethics committee
common application form.***

Application to Undertake a Research Project

A General Information on the Project

A1 Pertinent section

| | |
|---|---------------------------------------|
| Principal Investigator: <i>(Please insert name, address, email, phone, fax)</i> | |
| Dr Aubrey Cunnington, From July 2010 MRC laboratories, PO Box 273, Atlantic Road Fajara, The Gambia Until July 2010 Immunology Unit, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, UK aubrey.cunnington@lshtm.ac.uk Tel: 44 20 7927 2294 Fax: 44 20 7927 2807 | |
| Project title: Studying the effect of Plasmodium falciparum malaria and heme oxygenase-1 induction on neutrophil function | |
| Date of submission: | Short title (if applicable) |
| 26/04/2010 | Malaria, HO-1 and neutrophil function |
| Type of Project: Interventional study* <input type="checkbox"/> Observational study <input checked="" type="checkbox"/> In vitro/animal study <input type="checkbox"/> NA <input type="checkbox"/> | |
| Nature of submission: Letter <input type="checkbox"/> SCC Proposal <input checked="" type="checkbox"/> Report: Annual <input type="checkbox"/> Interim <input type="checkbox"/> Final <input type="checkbox"/> | |
| Sponsor: MRC Core <input checked="" type="checkbox"/> MRC External <input checked="" type="checkbox"/> Non MRC Project <input type="checkbox"/> | |
| SCC Number: 1207 | Version 1.0 |
| | |

Summary:

Malaria predisposes to bacterial infections, most notably non-Typhoid Salmonella (NTS) bacteraemia. Studies in mice suggest that haemolysis causes this susceptibility, and our own work suggests that this leads to a defect in neutrophil oxidative burst, which prevents killing of Salmonella by neutrophils. This defect in oxidative burst may be related to increased plasma heme and heme oxygenase-1(HO-1) induction. We propose to investigate whether the same findings are observed in children with *Plasmodium falciparum* malaria. We will use the existing longitudinal study of severe and mild malaria to collect acute and convalescent samples from children with malaria and healthy controls. We will assess neutrophil function: oxidative burst, degranulation, and ex-vivo phagocytosis and killing of Salmonella. We will assess whether neutrophil function correlates with HO-1 expression and with simple plasma biomarkers of haemolysis (such as carboxyhaemoglobin, plasma heme, haptoglobin, hemopexin).

Planned end date of this project for review:
End of March 2011

- Investigation on a medicinal product not licensed in The Gambia
- Investigation on a medicinal product licensed in The Gambia, but used different from recommended routine use
- Other kind of intervention (e.g. licensed medicinal product within recommended use, food, food additive, medical device, other)

A2 Proposal Identifier

Resubmission

Yes No

A3 Confidentiality

Restrict access to SCC members

Yes No

A4 Responsible parties or bodies of project

a) Sponsor and/or Funding body

External MRC Both

The ongoing longitudinal severe and mild malaria study platform is funded by the MRC core programme. Contributions to these costs and the laboratory research project is funded externally by the MRC (Clinical Research Training Fellowship awarded to A Cunnington)

b) Investigators (Principal Investigator first) and main collaborators

| Name | Institution | Position and Key role |
|-------------------------|--|---|
| Dr Aubrey Cunnington | MRC Laboratories, The Gambia / LSHTM, UK | PI; MRC Clinical Research Training Fellow |
| Dr. Michael Walther | MRC Laboratories, The Gambia | Sen. Immunologist |
| Sarah Nogaro | MRC Laboratories, The Gambia | MRC PhD Student |
| Dr. David Conway | MRC Laboratories, The Gambia | Head of Programme |
| Dr. David Jeffries | MRC Laboratories, The Gambia | Sen. Statistician |
| Bankole Ahadzie | MRC Laboratories, The Gambia | LSHTM PhD Student |
| Ismaela Abubakar | MRC Laboratories, The Gambia | Data Manager |
| Professor Eleanor Riley | LSHTM, UK | Prof of Immunology |

Who will introduce the proposal at SCC?

Dr Michael Walther

c) How will the safety oversight be done (if applicable)?

Safety Monitor TSC DMC/DSMB Other NA

A5 Location(s) of research

Samples from malaria cases will be collected from the Jammeh Foundation for Peace Hospital, the MRC Outpatient Department and the Brikama Health Centre.

Samples from healthy volunteers will be collected from Brefet Village.

The laboratory work will be carried out at the Laboratories, Fajara, The Gambia

Some of the subsequent analysis may be done at the London School of Hygiene & Tropical Medicine

A6 Are adjunct studies planned?

Yes No

Depending on feasibility we may wish to study the same questions in children with sickle cell disease attending clinic – this would be the subject of an additional application if this appears feasible based on preliminary findings in the study set out in this application form.

A7 Checklist for Administration

- a) **Has the project been discussed and cleared with the institutions in which research will be carried out including health services to which the study will need access?**

Yes No Recruitment for this study forms an integral part of the ongoing study initially described in SCC 1003 and SCC 1002, with recent modifications described and approved for SCC 1180. The new scientific questions will be presented to the CEO of the Health Centre prior to start of recruitment.

- b) **Have all investigators and collaborators given their agreement to take part in the study as described?**

Yes No

- c) **Have ethical issues been addressed?**

Yes No

- d) **Does the project require laboratory work, new laboratory procedures, or the riding of motorcycles?**

Yes No Yes, the project does require laboratory procedures to be carried out on the samples by the PI. Sample transport will be in an MRC Landrover or on MRC motorbikes.

If yes, have the safety issues been addressed, if any?

(The Health and Safety Manager will advise on risk assessment)

Yes No The PI will be made aware of local health and safety regulations during his introduction.

- e) **Will the project require data taken out of The Gambia?**

Yes No Since the PI is registered as a PhD student at LSHTM and will be required to return to London in April 2011, he will be required to take data with him. Data will be transported on an encrypted device; a safety copy of the data will remain in the Gambia.

If it is an MRC project, the Principal Investigator ensures that a copy of the complete data set remains with the MRC Data Management Office in The Gambia at the following two time points:

1. After data entry and verification ("raw data sets")

Yes No

2. At the point of submission for publication of final report (“analysis data sets”)

Yes No

At the end of the study and after publication, a master file containing all raw data in an anonymous format will be made available to the Unit.

Note: Before export of any data, separate approval by SCC and Ethics Committee is required and for MRC studies the signature of the Unit Director or designee

f) Will the project require biological materials taken out of The Gambia?

Yes No

Although it is planned to carry out all lab work related to the project at the MRC laboratories, The Gambia, depending on the results it may become of interest to examine for instance the proportion of free and protein bound heme in plasma. This would be done by high performance liquid chromatography in collaboration with Dr Harparkash Kaur at LSHTM. In that case, separate permission will be sought from the SCC / EC to transfer aliquots of the samples collected during this study to another laboratory outside of The Gambia.

The Principal Investigator will ensure that appropriate aliquots of the biological material being taken out of the country remain with MRC Laboratories The Gambia

If it is an MRC project, the Principal Investigator ensures that appropriate aliquots of the biological material being taken out of the country remains with MRC Laboratories The Gambia

Yes No

Note: Before export of any biological material, separate approval by SCC and Ethics Committee is required and for MRC studies the signature of the Unit Director or designee

g) For projects to be carried out at MRC Laboratories

Has the project been discussed with the following support staff as appropriate and resource requests agreed?

Yes No

This project takes advantage of the ongoing longitudinal study of severe and mild malaria cases established here at the coast since 2005 (SCC 1002 SCC 1003). The work suggested in this project should be seen as a continuation of SCC1077 (Role of HO-1 in *P. falciparum* infection). In addition, two further research projects will be based on this platform during the forthcoming season: SCC 670 and SCC 1180. All issues re safety, transport etc. have been discussed and agreed previously and have not changed. No new staff

are required. The laboratory work will require mainly standard techniques that are well established in our lab. A copy of this proposal has been sent to Ousman Secka. Risk assessments for the use and culture of genetically modified (fluorescent protein-expressing) *Salmonella* Typhimurium have been undertaken.

| Support staff | Date discussed | Comment |
|--|----------------|---------------|
| Health and Safety Manager Pknight@mrc.gm | April 26, 2010 | Sent by email |
| Head of IT/Senior Data Manager Dcotsell@mrc.gm ; Dparker@mrc.gm | April 26, 2010 | Sent by email |
| Director of Clinical Services tcorra@mrc.gm | April 26, 2010 | Sent by email |
| Scientific Administrator Imanneh@mrc.gm | April 26, 2010 | Sent by email |
| Transport Manager pknight@mrc.gm | April 26, 2010 | Sent by email |
| Finance Manager abarry@mrc.gm | April 26, 2010 | Sent by email |
| Personnel Manager khill@mrc.gm | April 26, 2010 | Sent by email |
| Director of Operations mkilpatrick@mrc.gm | April 26, 2010 | Sent by email |
| Other services – specify | | |

Signature of Principal Investigator and date:

Signature

Date

(Please sign the hard copy before the project commences and provide a copy to the SCC secretary for filing)

A8 For interventional studies on human participants (Clinical Trial)

Synopsis

| | |
|--|--|
| Population studied: | |
| Number of sites: | |
| Study duration: | |
| Subject participation duration: | |
| Investigational products or Intervention: | |
| Objectives: | |
| Endpoints or Outcome parameters: | |
| Study Design: | |

B Scientific Description of the Project

B1 Background and Rationale

Malaria causes a huge global burden of ill-health: an estimated 243 million cases of malaria and 863,000 deaths in 2008 [1]. However, the direct burden of disease may underestimate the overall effect of *P. falciparum* on the health of a population. Epidemiological and ecological studies suggest that infection with *P. falciparum* is a stronger risk factor for death than can be directly attributed to malaria itself^{3,5-6}, and it has been estimated that up to half of the variation in child mortality in Africa may be accounted for by differences in parasite prevalence³. One reason for this may be that *Plasmodium falciparum* malaria is associated with increased susceptibility to bacterial infection (particularly non-Typhoidal Salmonella, NTS)[5-8]. Susceptibility to NTS bacteraemia is most strongly associated with severe malarial anaemia [5,6] and is more closely related to the incidence of malaria than to stool carriage of Salmonella[5]. These data are supported by increased susceptibility to Salmonella infection in experimental malaria-Salmonella co-infections in mice [10,11]. Susceptibility to NTS also occurs in humans with sickle cell disease [12], and mice with pyruvate kinase deficiency [13] or experimentally induced immune-mediated or chemical haemolysis [11,14]. This strongly suggests that haemolysis, rather than “malaria” itself is central to the pathogenesis. Most investigators have assumed that haemolysis due to malaria or other factors has an adverse effect on macrophage function [5,10,11,14], since macrophages are the main host cell type in which Salmonella Typhimurium replicates in otherwise healthy mice [15]. However, there is no direct evidence that the malaria-induced susceptibility to infection involves impairment of macrophage function, and the mechanism is unknown.

Heme oxygenase-1 (HO-1) is the stress inducible, cytoprotective isoform of the enzyme responsible for heme degradation [reviewed in 16]. It is induced by its substrate heme (hence it is induced during haemolysis), as well as a huge number of potentially harmful stimuli. Induction of HO-1 has many immunomodulatory effects [reviewed in 17], including suppression of the generation of reactive oxygen species important for intracellular signaling and the oxidative burst [18-20]. In general these effects reduce inflammation and protect host tissues from damage. Induction of HO-1 has been demonstrated in human and rodent malaria infections [21-24], and its timely induction can prevent severe manifestations of malaria in mice [25]. Although induction of HO-1 has been shown to be beneficial to survival in other models of sepsis [26], we hypothesized that its induction by malaria or other causes of haemolysis might inhibit killing of intracellular Salmonella, which requires generation of reactive oxygen and reactive nitrogen species [27].

We have reassessed malaria-Salmonella co-infection in mice using Green Fluorescent Protein (GFP) expressing Salmonella (allowing cellular localization to be determined) and the non-lethal rodent malaria parasite *P. yoelii* 17X (Py17X) (Cunnington et al. unpublished observations). Co-infected mice died much more rapidly than those infected with Salmonella alone, and showed massively increased bacterial loads in blood, livers and spleen. At this time point most bacteria were found in neutrophils and monocytes, with a marked neutrophil predominance in co-infected mice. These findings are consistent with recent observations that neutrophils are the first host cells for *Salmonella* Typhimurium early in infection, and that Salmonella activate transcription of genes to allow survival in neutrophils [28]. We found that neutrophils from Py17X infected mice did not show increased phagocytosis of Salmonella when challenged *ex-vivo*, suggesting that there may be a defect in their ability to kill Salmonella. We found that the neutrophil oxidative burst was markedly reduced by Py17X infection, whereas neutrophils retained normal degranulation. This defect was progressive and correlated with the rise in parasitaemia, fall in erythrocyte count (ie haemolysis), plasma heme levels and systemic and neutrophil specific induction of HO-1. These data suggested that a defect of neutrophil function may be responsible for the susceptibility of malaria infected mice to systemic Salmonellosis, and the induction of HO-1 by hemolysis may suppress the neutrophil oxidative burst.

Work undertaken in The Gambia (M. Walther et al, unpublished observations), has already examined HO-1 expression in malaria. This work has clearly identified that HO-1 is induced by *P. falciparum* malaria and that neutrophils are the major cell type expressing HO-1. These findings in humans helped to shape the mechanistic studies we have been performing in the animal model, which in turn has provided insight into a potential functional role of HO-1, and we now aim to translate this back into a study in humans.

We wish to investigate whether a defect in neutrophil function occurs in Gambian children with malaria, and whether there are any simple clinical, haematological or biochemical indicators, which correlate with the severity of this defect, which might allow us to determine which children might be most susceptible to bacterial co-infection. We will employ the existing longitudinal study of severe and mild malaria, so that we have a spectrum of clinical malaria cases, each with acute (day 0) and convalescent (days 7 and 28 and week 8 post-presentation) samples. We will compare neutrophil function on day 0 with convalescent values for the same subject, enabling us to attribute any change to malaria. We will also determine the relationship between

HO-1 expression and neutrophil function, to evaluate whether HO-1 may be contributing to any suppression of the oxidative burst. Methods for detection of HO-1 expression by flow cytometry, rt-PCR and ELISA, as well as neutrophil isolation from human blood samples, have been developed and validated in the MRC laboratories in The Gambia (M. Walther et al, unpublished results) and greatly enhance the chances of completing this study successfully.

B2 References

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B3 Project description

a) What is the hypothesis of the project?

Malaria causes a defect in neutrophil function through the induction of heme oxygenase-1.

b) What are the primary and secondary objectives?

Primary Objective

To determine whether neutrophil function (neutrophil oxidative burst and ability to kill *Salmonella enteritidis* serovar Typhimurium, *in vitro*) is suppressed by *P. falciparum* infection, and inversely correlated with HO-1 protein expression in neutrophils.

Secondary Objectives

1. To determine factors correlated with HO-1 induction (eg plasma heme)
2. To determine the duration of any effect of acute malaria on neutrophil function.
3. To determine whether neutrophil dysfunction is associated with heme oxygenase-1 gene expression / enzyme activity in peripheral blood neutrophils.
4. To determine whether neutrophil function differs between different clinical manifestations of malaria (severe vs. mild; correlation with degree of anaemia).
5. To assess whether there are any simple haematological or biochemical markers (eg. carboxyhaemoglobin saturation, bilirubin, total plasma heme, methemoglobin, haptoglobin or hemopexin) that predict impaired neutrophil function.

c) What type of study design is proposed?

Longitudinal study, using the severe and mild malaria longitudinal study platform.

This study platform will also be used to recruit subjects for studies SCC 670 and SCC 1180, although blood samples from each individual subject will only be used for one of the three studies.

In addition, one-off blood samples will be collected from healthy control children (to determine a normal range for neutrophil function).

d) What are the subjects and/or samples studied and their number?

Study subjects will be paediatric patients enrolled in the ongoing longitudinal study, and healthy control children from Brefet Village.

Patients will be recruited from the following sites: Jammeh Foundation for Peace Hospital, MRC Outpatient Department and the Brikama Health Centre, during the 2010 transmission season.

Patients who are enrolled in the ongoing longitudinal study suffer from either severe or mild malaria and so these patients are mainly young children. In the last three years between 120 and 180 children have been recruited per malaria season.

In total, 58 children will be required for the longitudinal study outlined in this application. Another 20 healthy control children will be required.

Blood samples will be collected on days 0, 7 and 28, and week 8, from those patients enrolled in the longitudinal study.

In addition 20 healthy control children (10 children 1-4 years old, 10 children 5-12 years old) will be recruited from Brefet Village (the purpose of these samples is to estimate the normal range for neutrophil function tests in Gambian children, not for formal comparison with acute malaria samples).

e) What are the endpoints or outcome parameters?

The main outcome is neutrophil function (oxidative burst, degranulation and *ex-vivo* killing of Salmonella). This will be compared between acute and convalescent samples for each malaria case.

f) Which methods (laboratory and/or field) will be used, what samples/specimens, if any, will be taken and what investigations will be conducted?

Initially, when patients enroll in the ongoing longitudinal study during the 2010 transmission season, the nurse will explain the study to the parent/guardian. The nurse will make clear to them that a total of up to four bleeds will be requested from their child for this study (days 0, 7, 28, and week 8). For those who are willing to participate, individual written informed consent will be obtained. Participants will be made aware that they can withdraw at any time during the study without this affecting the health care provided by the MRC clinic. For

healthy children enrolled from Brefet Village, the study will be explained as above, but it will be made clear that only a single blood sample is required.

In line with the Ethics Committee's guidelines, a volume of venous blood appropriate for the respective age group will be collected from all study participants at each visit. The blood will be used for the following tests:

-A thick film will be prepared for slide microscopy to test for presence or absence of parasitaemia.

- 2 EDTA microtainer tubes will be filled with not more than 250µl (for children less than 5 years) or not more than 500µl (for children > 5years) of blood each, to perform i) a full blood count that will be used for patient management and research purposes, ii) to test for sickle cell status (day 0), iii) to perform a qualitative PCR for *Plasmodium* DNA, and /or to perform a PCR for bacterial DNA to determine the presence of co-infection.

- 0.5 ml blood will be collected into a PAX tube to preserve the mRNA.

- 4.0ml-13.0ml of blood (depending on age of the child) will be collected into heparinized tubes and transported in an insulated box to the MRC within two hours of collection.

For all samples, when blood arrives in the MRC laboratory it will be processed in small batches as follows:

Heparinised Blood

400ul whole blood for oxidative burst and degranulation assay, performed immediately.

Up to 500ul aliquot centrifuged to separate plasma (aliquot of plasma frozen at -80C), red cells lysed, leukocytes stored on ice for later flow cytometry assay for HO-1.

5ul aliquot for COHb assay.

Remainder: plasma separated, erythrocytes lysed and neutrophils isolated from remaining blood for *ex-vivo* Salmonella killing assay.

EDTA Blood: processed as indicated above

PAX gene tubes: frozen at -80C until January 2011.

1. Oxidative burst in response to phorbol myristate acetate (PMA) or *Salmonella* Typhimurium. This is measured using a whole blood flow cytometric assay with conversion of dihydrorhodamine 123 to rhodamine providing a fluorescent indicator of intracellular hydrogen peroxide generation (Richardson et al, Journal of Immunological Methods 1998 219: 187-93). The strength of the oxidative burst can be determined from the median fluorescence intensity of rhodamine. Neutrophils and monocytes can be distinguished based on their forward scatter and side-scatter properties, and / or by the addition of fluorochrome conjugated antibodies, their expression of different surface markers eg CD16b, CD14.

2. Degranulation in response to phorbol myristate acetate (PMA). Upregulation of surface CD11b is measured in the same whole blood flow cytometric assay by addition of a fluorescent labeled anti-CD11b antibody. CD11b is contained in primary granules of neutrophils and this can be used as an indicator of degranulation.

3. *Ex vivo* killing of *Salmonella*. Neutrophils will be isolated from the remaining blood using positive selection with magnetic beads. Isolated neutrophils will be incubated with serum opsonised[†] *Salmonella* Typhimurium which has been genetically modified to express green fluorescence constitutively and red fluorescence when cultured in the presence of L-arabinose ([Helaine et al. PNAS 2010], provided by Prof D. Holden, Imperial College, UK). Invasion of neutrophils by *Salmonella*, and killing of *Salmonella* will be determined by culture and flow cytometry at various time-points in a gentamicin protection assay. Intracellular replication of *Salmonella*, if any, can be determined by dilution of red fluorescence when cultured in the absence of L-arabinose. If necessary, intracellular *Salmonella* can also be quantified by fluorescent microscopy. Neutrophils in excess of numbers required for the assay will be stored in RNAlater for subsequent rtPCR or appropriate buffer for HO-1 activity analysis.

4. Heme oxygenase-1 expression by flow cytometry. Using a whole blood staining procedure for flow cytometry established by the programme, the level of HO-1 expression by different leucocyte subsets will be determined.

For secondary objectives:

1. Heme oxygenase-1 expression will be determined by rtPCR in whole blood collected into PAX tubes, and in isolated neutrophils. Heme oxygenase activity will be measured using a standard assay for bilirubin production. Neutrophil HO-1 expression and activity will be correlated with neutrophil function.

2. Carboxyhaemoglobin saturation and plasma methaemoglobin and heme will be determined using spectrophotometric assays with a nanodrop spectrophotometer. Bilirubin will be determined using a Quantichrom bilirubin kit, and plasma HO-1, haptoglobin and hemopexin will be measured by ELISA. These parameters will be correlated with neutrophil function in univariate analyses.

Detection / exclusion of bacterial co-infection by whole blood RT-PCR (using protocol developed by G Morris and adapted by Matt Edwards at MRC laboratories, The Gambia)

[†]In order to perform optimal bacterial killing assays it is helpful to use serum which already contains antibodies against Salmonella. This will be achieved using pooled donor serum collected from adults in the village of Brefet.

Sample Processing Overview

| Assay/Time Point | Day 0 (presentation) | Day 7 | Day 28 | Week 8* |
|------------------|-------------------------|-------|--------|---------|
| Sickle Screen | X | | | |
| Blood Film | X | X | X | X |
| Full Blood Count | X | X | X | X |
| Oxidative Burst | X | X | X | X |
| Degranulation | X | X | X | X |
| Killing Assay | X | X | X | X |
| HO-1 Expression | X | X | X | X |
| Biomarkers | X | X | X | X |
| Bacterial RT-PCR | X | | | |

* Week 8 time point will be used at the beginning of the study but its necessity will be determined in an interim analysis. Please see section B5 for further details.

Blood tests will be performed on healthy control child samples as for day 0 above.

g) What are the anticipated time-scales?

Recruitment will run from the start of September 2010 to end of December 2010. The last blood samples may be taken up to 8 weeks after close of recruitment.

All assays requiring live cells (ie those for the primary outcome measures) will be performed on fresh samples on the day of collection, thus all data collection for these assays will be completed by the end of February 2011. Assays on stored samples will be performed in batches between December and March 2011.

Data will be analysed, reports written and manuscript(s) submitted for peer-reviewed publication between March and August 2011.

B4 Projects involving human subjects

a) How and where will the study participants be selected?

Patients will be recruited from the three different study sites namely, from the Jammeh Foundation for Peace Hospital, the MRC Out-Patient Department and the Brikama Health Centre.

Healthy control children will be recruited from Brefet Village, based on demographic records. 10 children aged 1-4 years and 10 children aged 5-12 years old will be recruited, to allow stratification for age. Parents of children of appropriate age will be identified and invited to bring their children for a single blood test.

Will it be confirmed that they are not already involved in other studies?

Yes No as in the past, they will be part of the longitudinal study of severe and uncomplicated malaria cases and as such, samples may also be used for parasitological projects that make use of the red blood cell pellets not

used by the immunological projects. However, neither the frequency of bleeds nor the amount of blood taken at any time is affected by this.

b) What inclusion/exclusion criteria will be applied?

Eligibility criteria:

- African child living within the study area

Mild malaria:

- clinical symptoms compatible with malaria (e.g. fever > 37.5°C) and
- more than 5000 asexual *P. falciparum* parasites/μl, and
- no other obvious cause of fever, and no complications

Severe malaria:

- Blood film positive more than 5000 asexual *P. falciparum* parasites/μl, plus 1 of the following:
- Hb < 6mg/dl
- Blood glucose < 2.2mmol/L
- Repeated convulsions (\geq 3/24hours)
- Unrousable coma as defined by a Blantyre coma score of 2 or below
- Lactate concentration on whole blood or capillary blood > 7mmol/L
- Difficulties in breathing defined as the presence of one or more of these features: abnormalities in the respiratory rate (according to the age), lower chest in-drawing or deep breathing
- Inability to drink or suck (observed)
- Inability to sit unsupported (observed)

Healthy Control Children:

- Parents of children aged 1-4, and 5-12 years will be identified and invited for their children to participate in the study.

Exclusion Criteria

Children with clinical or microbiological evidence of bacterial co-infection will be excluded from the primary analysis of the effect of malaria on neutrophil function. However these results will probably only be available after the initial assays have been performed, and if there are sufficient children with evidence of bacterial co-infection they may be analysed as a sub-group.

c) Will treatment be given?

Yes No

Nature of treatment(s):

Treatment will be performed according to the national Gambian Treatment Guidelines

For drugs: Formulation, dosage, dosage regimen, route of administration and duration of treatment:

As indicated in treatment guidelines

Person(s) responsible for administering treatment:

Treatment will be provided by Health Centre staff, supported by MRC research nurses.

d) Will questionnaires be used?

Yes No

e) Will interviews been conducted?

Yes No

Who will be conducting these?

A trained nurse will be involved in conducting interviews, transcribing answers from questionnaires, checking for eligibility and obtaining informed consent.

B5 Data management and statistical analysis

a) What are the statistical considerations and sample size calculations involved in determining the size of the study?

The sample size calculation for the study is based on the primary outcome measure: neutrophil function. We assume that there is a consistent linear relationship between the magnitude of the neutrophil oxidative burst and the

ability of neutrophils to kill *Salmonella* in an *ex-vivo* assay (as has been demonstrated for *Staphylococcus aureus* [Ellson et al. *Journal of Experimental Medicine* 2006]), and hence base the sample size calculation on the neutrophil oxidative burst in response to PMA stimulation.

In ethnically mixed healthy subjects, the oxidative burst assay performed by this technique results in a stimulated mean rhodamine fluorescent intensity of approximately 1200 units (standard deviation 490 units) [Siddiqi et al *Cytometry* 2000 46:243-6] with a relatively normal distribution.

It is not known whether there is a threshold for the reduction of oxidative burst and susceptibility to infection. We expect differences in humans to be smaller than in the mouse model, particularly since the incidence of NTS infections and of severe malarial anaemia have both decreased dramatically in The Gambia in recent years. In animal studies the oxidative burst must be reduced to $\leq 80\%$ of normal before a significant effect is seen on bacterial killing. Therefore we regard a 20% reduction in oxidative burst in acute malaria, compared with convalescent samples, as clinically significant and worth detecting

Since we do not know the duration of any effect of malaria on neutrophil function, we plan to obtain convalescent samples at intervals up to 2 months after presentation (days 7 and 28, and week 8). Due to the short life span of neutrophils in the circulation (no more than a few days) it is expected that by day 28 after infection, neutrophil function will have returned to normal, whereas at day 7 there may be some residual effect from parasite products such as hemozoin. However it is conceivable that neutrophil function may be suppressed for longer periods, depending, for example, on what happens to progenitor cells in the bone marrow. For this reason we will begin by obtaining samples at days 7 and 28, and week 8 but will undertake an interim analysis after the first 2 months of the study, to determine if the samples at week 8 are necessary for the remainder of the recruitment period.

It should be noted that the primary objective of this study is not to assess differences in neutrophil function between individuals with severe and mild malaria, but we do wish to have a spectrum of clinical cases which might result in a greater range of HO-1 expression and possibly oxidative burst values.

Sample size calculation: (Using Stata Sampsi)

Paired sample t-test: Acute vs Convalescent oxidative burst (likely to be D28)

Two-sided p value = 0.025 (to allow for multiple testing caused by planned interim analysis)

Power = 80%

Estimated means: Acute = 960 units, Convalescent = 1200 units

Standard deviation: 490 units for both acute and convalescent samples

Sample size = 40

Practical Considerations for Sample Size:

In previous years approximately 85% of subjects have provided both acute and convalescent (day 28) samples. We anticipate that loss to follow-up may be as high as 25% by 8 weeks after presentation, and therefore increase the sample size to reflect this.

It is likely that the distribution of oxidative burst values may not be normally distributed in this population, particularly in the acute samples, so we will adjust the power calculation to take into account the fact that non-parametric testing may be necessary. The maximum increase in sample size requirement if a non-parametric test is required is 15%, therefore this will be added to the sample size calculated above.

The combination of loss to follow-up and contingency for non-normal distribution increases the sample size required to 58 subjects.

Final recruitment target in longitudinal study

58 children with acute malaria

Healthy Control Subjects

20 children, (10 children aged 1-4 years, 10 children aged 5-12 years). Note, these children are not included in formal statistical analysis, but allow us to estimate the normal range for Gambian children.

b) Who is responsible for the statistical design and analysis of the study?

The study was designed by the PI, his MRC supervisor Dr M. Walther, and his LSHTM supervisor Prof E. Riley. The study design and analysis plan has been revised following advice from Dr David Jeffries, MRC Senior Statistician. Initial analysis will be performed by the PI, and subsequent analysis will be performed with assistance from Dr Jeffries and Bankole Ahadzie.

c) What is the plan of analysis?

All laboratory data will be stored in the database.

All data collected onto a CRF will be reviewed by the PI prior to double entry and cleaning.

Eventually both datasets (from the CRF and the laboratory) will be merged according to the study identifier.

Pre-specified analyses comparing the primary outcomes between acute and convalescent samples will be performed by the PI. For secondary exploratory analyses, the PI will perform multivariable analyses with assistance from Dr Jeffries.

Primary analysis:

Flow cytometry data for the neutrophil oxidative burst will be subjected to objective clustering analysis to allow unbiased quantification of the magnitude of the unstimulated and stimulated neutrophil oxidative burst for acute and convalescent samples. Data at each time point will be examined to determine the possible duration of any suppression of neutrophil function compared to the subsequent time points (ie. based on when the oxidative burst seems to stop increasing with time, expected to be day 28). In the simplest analysis, acute and convalescent stimulated oxidative bursts will be compared using a paired sample test appropriate for the distribution of the data. A similar analysis will be performed for the bacterial killing assay at the same time points. The relationship of the bacterial killing to oxidative burst will be assessed using a model to fit the best curve for this relationship accounting for multiple observations on each individual. We will also use the model to define if there is a threshold for oxidative burst to influence bacterial killing. Heme oxygenase expression in neutrophils will be

quantified from flow cytometry fluorescence intensity data (again using objective clustering analysis) and will be correlated with oxidative burst and bacterial killing using a similar modeling approach. If necessary parametric or rank-based longitudinal methods taking account of intra subject correlation will be used if more than two time points are necessary.

Subsequent analysis:

We do not expect that the magnitude of the convalescent oxidative burst will be substantially influenced by age, sex or ethnic group, however it is possible that this may be the case. We will explore the associations between these variables and oxidative burst, neutrophil function and HO-1 induction in a multivariable analysis. If necessary, we will repeat the primary analyses with adjustment for these factors.

The associations of clinical, haematological and biochemical indices with neutrophil function and HO-1 expression will be assessed and the strongest candidates for diagnostic tests will be identified. We will calculate threshold values and their sensitivity and specificity for predicting impaired bacterial killing in vitro.

d) Who will be primarily responsible for database design and data management?

Ismaela Abubakar

e) Will Microsoft Access be used as database?

Yes No

Laboratory data will be stored in an Access Database.

The OpenClinica database will be used for clinical data, to allow for monitoring the data as it is being entered.

B6 Expected outputs and Dissemination of results

a) What are the expected outputs (publications) from this project?

We expect to be able to publish the results from this study, whether or not an association can be demonstrated between malaria and suppression of the oxidative burst.

If this study demonstrates suppression of oxidative burst and Salmonella killing by neutrophils from patients with malaria we would aim to prepare 2-3 manuscripts:

1. An immunology paper showing the magnitude and nature of this effect, and possible mechanism
2. A clinical-immunological correlation paper showing the relationship between clinical / haematological / biochemical indices and neutrophil dysfunction
- (3. A review / opinion article about the implications of malaria control activities for reducing susceptibility to Salmonella infection and additional reductions in child mortality)

b) What other arrangements will there be to disseminate the findings?

We would also aim to present the findings at an appropriate international conference, as well as seminars at the MRC laboratories, LSHTM and other institutions (if invited).

C Ethical Issues

C1 Outline how the study will contribute to improving the health of people of The Gambia

Apart from the health care provided to the patients at the study site, there is no direct immediate benefit to the health of the people of the Gambia. However, if we identify mechanisms and correlates of susceptibility to NTS infection in children with malaria, this may ultimately lead to specific therapy to reduce the risk, or to risk stratification and improved use of empirical antibiotic treatment. This could reduce morbidity and mortality due to NTS infection.

C2 Summarise the potential risks and benefits to individuals, communities or country

Participants may experience some discomfort such as local bruising or fainting when blood is being drawn by venepuncture. The field worker will explain this to them during the consenting process. EMLA cream will be applied before taking blood samples to minimize discomfort.

Referrals to MRC or RVTH will be facilitated where necessary.

Findings from this study will contribute to our understanding of how malaria influences susceptibility to other infections, and may provide means of protecting individuals from infection.

C3 How will informed consent be obtained?

A trained nurse will explain the study to the subjects, or in the case of children to their parent/guardian. The field worker will answer any questions the participants may have regarding the study or will refer them to another investigator if they are unable to answer them.

C4 How will confidentiality of the data gathered be ensured?

Study participants will be issued a unique three digit number as study ID. Only the first page of the CRF will allow us to link the participant's name and address to the ID and will be stored separately in a locked filing cabinet, accessible only by the PI. The CRFs, once entered, will be locked in a filing cabinet accessible only by the PI and senior fieldworkers involved in the conduct of the study.

C5 Will HIV Testing be required?

Yes No

C6 Please ensure:

Is a consent form attached?

Yes No

Is a subject information sheet attached?

Yes No

Is the questionnaire (if applicable) attached?

Yes No

D Resources Requested

D1 Timeline

First samples will be collected from September 2010 until end December 2010. Follow-up samples will be collected up until the end of February 2011. Initially, samples will be collected at day 0, 7 and 28, and week 8 for each participant.

D2 Resource Request Spreadsheet

(available in Excel on request)

Existing staff

| Who(names needed) | Grade | % time | Source of funding |
|-------------------|----------|--------|---|
| Aubrey Cunnington | | 100 | MRC Clinical Research Training Fellowship |
| Michael Walther | Band 3 | 10% | Sen Immunologist |
| Madi Njie | Grade B3 | 25% | Lab technician |
| Simon Correa | Grade C2 | 25% | Sen Lab technician |

| Who(names needed) | Grade | Training needed | Source of funding |
|-------------------|-------|-----------------|-------------------|
| | | | |
| | | | |

New staff

| Grade/Band | Where recruited from | % time on project | Source of funding |
|------------|----------------------|-------------------|-------------------|
| | | | |
| | | | |

Consumables

| | What | Cost | Source of funds |
|-------------------|---------------------------|------|--|
| LAB | | | |
| | Oxidative burst assay | 354 | External, MRC CRTF (G0701427) AJ Cunningham |
| | Degranulation assay | 477 | G0701427 |
| | Bacterial Killing Assay | 3824 | G0701427 |
| | Neutrophil RNA extraction | 176 | G0701427 / MRC Core |
| | Flow cytometry | 4218 | G0701427 / MRC Core |
| | Biochemical Assays | 863 | G0701427 |
| | Whole blood rtPCR | 2366 | G0701427 / MRC Core |
| | Bacterial Culture | 130 | G0701427 |
| | Disposable plastics | 1300 | G0701427 / MRC Core |
| | Other Consumables | 1300 | G0701427 / MRC Core |
| | | | |
| | | | |
| DRUGS | | 500 | MRC Core |
| | | | |
| | | | |
| STATIONERY | | 125 | G0701427 |
| | | | |
| | | | |

| | | | |
|-----------------------|--------------|------|----------|
| COMMUNICATIONS | | 50 | G0701427 |
| | | | |
| OTHER | | | |
| | Shipment | 750 | G0701427 |
| | Publications | 2000 | |
| | Petty cash | 150 | |
| | | | |

Existing equipment to which access is needed

| What | How often |
|---------------------------|-------------------------|
| ELISA reader | Several times per month |
| CyAN | Daily |
| PCR machine | Several times per month |
| Incubator | Daily |
| Biological Safety Cabinet | Daily |

New minor equipment required

| List: | Cost | Source of funds |
|-------|------|-----------------|
| | | |
| | | |

Use of laboratory services

| What: | No of specimens | Cost | Source |
|------------------------------|---|------|----------|
| HLA Typing | | | |
| Clinical Microbiology | | | |
| Haematology/ Biochemistry | Thick film 252 FBC 252 Sickle Screen 78 | 1455 | MRC Core |
| Serology | | | |
| HIV testing | | | |

Transport and local travel

| What: | Km | Cost | Source |
|------------------|-------|--------|----------|
| Land Rover usage | 3200 | 1120 | MRC Core |
| Motorcycle usage | 13860 | 1965.5 | MRC Core |
| Allowances | | | |
| Fares | | | |

Space (Office, Lab, Fridges/Freezer)

| What | Where | % use |
|--------|--|-------|
| Office | Whittle Building | 100 |
| Lab | Whittle Building (malaria tissue culture room) | 50 |

Others

| What | Where | % use |
|------|-------|-------|
| | | |

| | | |
|-----------------|------------------|----|
| Freezer (-80°C) | Whittle Building | 5 |
| Freezer (-20°C) | Freezer room | 5 |
| Fridge (4°C) | Whittle Building | 20 |

Signature of Data Manager and date:

Signature

Date

Signature of Laboratory Manager and date:

Signature

Date

(Please sign the hard copy as appropriate before the project commences and provide a copy to the SCC secretary for filing)

D3 Sources of funds

Dr AJ Cunnington holds a MRC Clinical Research Training Fellowship (G0701427), administered through the London School of Hygiene and Tropical Medicine. This was intended to provide approximately £10,000 of research costs for work undertaken at MRC laboratories, The Gambia.

2. Scientific coordinating committee approval

Scientific Coordinating Committee

MRC (UK) The Gambia, Fajara
PO Box 273 Banjul, The Gambia
West Africa

Switchboard (+220) 4495442/6 Ext 2308

Fax (+220) 4494154/4494498

E-mail: scc@mrc.gm

Website (intranet): \\open.mrc.gm\home.asp



Leading Research For Better Health

10th May 2010

Dr Aubrey Cunnington
Immunology Unit
London School of Hygiene and Tropical Medicine
Keppel Street, London
WC1E 7HT
UK

Dear Dr Cunnington

SCC 1207, studying the effect of *Plasmodium falciparum* malaria and heme oxygenase-1 induction on neutrophil function

Thank you for submitting your proposal dated 26th April 2010 for consideration by the SCC at its meeting held on 8th May 2010.

The committee considered this to be an interesting proposal. A number of aspects were discussed in the meeting that were responded to and clarified well, and we are pleased to approve the proposal to be forwarded to the Ethics Committee for consideration at its meeting on 28th May 2010.

With best wishes,

Yours sincerely,

A handwritten signature in blue ink, which appears to read 'David Conway', is written over a light blue horizontal line.

Dr David Conway
Chair, Scientific Coordinating Committee

Cc: Dr Michael Walther

Additional documents submitted for review:

- Information Sheet & Consent Form (Control), Version 1 – 26 April 2010
- Information Sheet & Consent Form (Mild), Version 9 – 26 April 2010
- Information Sheet & Consent Form (Severe), Version 9 – 26 April 2010
- Questionnaire
- CV – Aubrey Cunnington

3. Ethical approval

The Gambia Government / MRC Laboratories Joint
ETHICS COMMITTEE

C/o MRC Laboratories Fajara
P. O. Box 273, Banjul
The Gambia, West Africa
Fax: +220 – 4495919 or 4496 513
Tel: +220 – 4495442-6 ext. 2308

16th June 2010

Dr Aubrey Cunnington
Immunology Unit
London School of Hygiene and Tropical Medicine
Keppel Street, London
WC1E 7HT
UK

Dear Dr Cunnington

SCC 1207, studying the effect of *Plasmodium falciparum* malaria and heme oxygenase-1 induction on neutrophil function

Thank you for your response letter dated 4th June 2010 addressing the queries raised by The Gambia Government/MRC Joint Ethics Committee at its meeting held on 28th May 2010.

I am pleased to give Chair's approval for this study to start.

Best wishes,

Yours sincerely,


Mr Malcolm Clarke
Chairman, Gambia Government/MRC Joint Ethics Committee

Cc: Dr Michael Walther

Additional documents submitted for review:

- Information Sheet & Consent Form (Control), Version 1 – 26 April 2010
- Information Sheet & Consent Form (Mild), Version 9 – 26 April 2010
- Information Sheet & Consent Form (Severe), Version 9 – 26 April 2010
- Questionnaire
- CV – Aubrey Cunnington

The Gambia Government / MRC Laboratories Joint Ethics Committee:

Mr Malcolm Clarke, Chairman
Mrs Kathy Hill, Secretary
Mrs Naffie Jobe, 2nd Secretary
Professor Ousman Nyan, Scientific Advisor
Mr Dawda Jagne
Mrs Bertha Mboge
Mr Modou Phall

Professor Tuman Corrah
Professor Hilton Whittle
Dr Stephen Howie
Dr Bekai Camara
Dr Lamin Sidibeh
Mr Malamin Sonko

4. Subject information and consent forms

A study to help understand the causes of severe malaria and how malaria makes people susceptible to other infections

The following information sheet should be explained in the appropriate local language:

You and your child are being invited to take part in a research study that aims to explore various aspects as to why some children suffer from severe disease, while others “only” suffer a relatively mild illness when they are infected with malaria. Before you decide, it is important for you to understand why the research is being done and what it would involve. Please take time to read the following information carefully, and ask us if there is anything that is not clear, or if you would like more information.

Reason for study

Your child has malaria, which is one of the most common diseases in African children, and a major health problem in the Gambia too, although it can be prevented by sleeping under an insecticide treated bednet. Malaria is caused by parasites (small germs) that are carried by some types of mosquitoes, and which enter the blood when we are bitten by those mosquitoes. The parasite makes its way into the red blood cells (important for the transport of oxygen) and destroys them. Most cases of malaria are usually mild. However, severe cases of malaria do also occur and some patients are seriously ill and are at risk of dying, especially children. It is not known why some children are able to fight malaria strongly while others get severe malaria. We know that part of the reason is that some people are born with the ability to fight malaria better than others, and these differences are called “genetic”. Differences in the ability of the child’s immune system (defense mechanism of the body against infections) to fight germs and parasites such as malaria, may contribute too. We know that the structure of the surface of the parasites varies, and it might be that some forms are more dangerous than others.

We hope that by comparing mild with severe cases of malaria, we can get a better understanding of these genetic and immunological differences, and may be able to identify structures on the parasite that make it a dangerous one. This may allow us to find new treatments or a vaccine that can prevent severe forms of malaria in the future. We also want to study how the body learns to protect itself against malaria. One way how the body fights malaria is by antibodies (tiny molecules produced by so called B cells in the blood) that destroy the parasites. Previously, we have examined healthy children of different age groups and found that younger children have less B cells that can fight malaria than older children. Now, we want to find out more how these cells develop after a known infection with malaria. Therefore, we would like to follow up your child on regular intervals for 1.5 years after s(he) got malaria, and to compare his/her B cell responses to those of other children of different ages.

We also know that having malaria makes you more likely to get certain other infections, particularly germs entering your blood. It seems that malaria can weaken certain parts of your child’s immune system. We want to find out why this is, and whether we can identify who is at most risk, so that we can prevent these additional infections from happening.

How to take part?

It is up to you to decide whether or not you and your child take part. If you do decide to take part you will be asked to sign or thumbprint a consent form on behalf of your child, and the field worker will ask you a number of questions to see if your child is eligible for this study. You are free to withdraw your child from the study at any time without giving a reason, and whether or not your child takes part will not affect the health care he/she will receive in any way.

What would happen to my child if he/she takes part?

If you agree that your child will join the study and the child is eligible, the study nurse will take a blood sample. In agreement with the recommendations of the Gambian Ethics Committee, not more than 5mls (about 1 teaspoon) will be taken out of your child’s vein if it is less than 5 years old. If your child is 5 to

Malaria Study SCC 670, 1077, 1143, 1178, 1179, 1180, 1207. Information Sheet / Consent Form
(mild cases)

Version 9.0, April 26, 2010

9 years old, we would like to request 10mls (2 teaspoons) of blood. From children that are older than 9, we would like to request 15mls (3 teaspoons). A mouth swab would be taken to collect some cells from the inner site of your child's cheek. These samples will be used to carry out immunological, parasitological and genetic research, described above. Furthermore, a stool sample will be collected at all the visits to assess your child's worm carriage. Your child will receive the recommended treatment for malaria. We would also like to review the case notes when your child leaves the hospital so that we can write down his/her final diagnosis, clinical features of the illnesses and the outcome. We would ask you to bring back your child one and four weeks after your child presented at the out patient department or the ward, to ensure that he/she is in good health. At both visits, we would collect another blood sample from your child as before, for the studies described above. Thereafter, we would like to arrange for a follow up visit to see your child 2, 4, 6, 8, 10, 12, 14, 16 and 18 months after s(he) had been to hospital. On these visits, the child's health will be checked and a further blood sample will be requested to allow us to do the work on B cells mentioned earlier.

What are the side effects and possible risks and benefits of taking part?

The mouth swab or collecting blood from your child will not harm him/her. The venepuncture may cause mild tenderness or bruising. For the duration of the study, the nurse will be happy to treat minor ailments free of charge and facilitate referral to the MRC clinic when necessary. Transport costs for the study visits will be reimbursed to you.

What tests would be done on my blood?

Comparing blood samples from cases of mild malaria with cases of severe malaria aims to answer three questions: 1) By examining the surface of the malaria parasites of severe and mild cases of malaria, we hope to identify those forms that are more dangerous than others. 2) The blood will also be examined to see in what ways the body's immune response (defense mechanism) to the malaria parasite differs between mild cases and severe cases. 3) The molecules that carry the genetic information (called genes) your child is born with may be responsible for how well he/she can fight malaria. From the collected blood genetic material will be extracted and analysed in the Gambia and also by our collaborators in the UK or elsewhere. The aim is to identify genes that are particularly common in children with severe malaria. This may help to identify new targets for vaccines.

For the study that wants to find out how malaria makes you vulnerable to bacterial infection, we will look at how well cells of the immune system produce factors that kill bacteria (germs), and how well the cells kill bacteria (germs) when they are mixed together in the laboratory. We will also look at how these factors are related to the severity of the malaria infection your child had, and to a variety of molecules that can be measured in the blood, to try to find which ones might be the best test to determine who is at risk of bacterial infection.

Future research in malaria is likely to increase our knowledge about the genetic mechanisms of the disease. Thus, it would be particularly useful to be able to use some of the blood samples from this study to examine them again in the future in the light of such new findings. With your consent we would therefore like to store some of the leftover blood samples for up to 25 years. No studies concerning diseases other than malaria will be done.

Would my taking part in this study be kept confidential?

All information that is collected about you or your child during the course of the research will be kept strictly confidential. The data will be available only to the study team, the Ethics Committee and Government regulatory authorities who can ask to audit or monitor the study. No unauthorized person will have access to who your child is or the information that you give us.

Who has reviewed the study?

A panel of scientists at the Medical Research Council has reviewed the study and has approved its scientific value. The Gambian Government/MRC Joint Ethics Committee has also approved the study and has granted permission for this study to take place.

Whom can I contact?

If you have any questions at any time during the trial, please ask Dr Michael Walther at the MRC Laboratories, Fajara (Tel: 4495442/6 Ext: 4009) or Dr Aubrey Cunnington (Ext: 4008). They will be happy to talk about any worries or queries.

Thank you very much for your time.

Consent form for study: A study to help understand the causes of severe malaria and malaria makes people susceptible to other infections

Dear patient, parent or guardian,

Please circle answer

1. Have you read or has the Information Sheet Version _____, dated _____ been read to you? **Yes/No**
2. Did you have a chance to ask questions and did you receive satisfactory answers? **Yes/No**
3. Do you understand what participation in the study means to you / your child? **Yes/No**
4. Do you understand that the participation of you /your child is voluntary and that you are free to withdraw at any time, without giving any reason, without your medical care or legal rights being affected? **Yes/No**
5. Do you understand that the information regarding you / your child that is collected in the course of this study will remain confidential? **Yes/No**
6. Do you understand that laboratory tests will be done on the blood samples you / your child provide, and do you agree that some of the leftover blood samples will be stored and may be used for further studies of the body's response to malaria? . **Yes/No**
7. Do you agree that we could collect blood from your child, one of the following amounts depending on his/her age as described today and on another 11 visits over the next 1.5 years?
-5mls (1 teaspoon) of blood if your child is less than 5 years old
-10mls (2 teaspoons) of blood if your child is aged between 5 and 9 years of age
-15mls (3 teaspoons) of blood if your child is older than 9 years old **Yes/No**
8. In case you are the mother or father of the child: Do you agree to donate 5mls of blood today? **Yes/No**
9. Do you understand that if you / your child gets sick during the study period, you can go to the clinic where study staff are providing care, and be examined and treated for free? **Yes/No**
10. Do you agree that we could perform a mouth swab from your child? **Yes/No**
11. Do you agree for your child to take part in this study? **Yes/No**
12. In case you are the mother / father: Do you agree to take part in this study? **Yes/No**

Name of child (in block letters) _____

Name of parent or guardian (in block letters) _____

Signature or thumbprint of parent or guardian of the child

I have read / explained the above to _____ (name of parent or guardian of the child) in a language he/she understands.

I am confident that he/she has understood what I explained and that he/she freely agreed that his/her child can take part in this study.

Signature of Field worker supervisor (or designate): _____

Date: ___/___/___

Name (in block letters): _____

Malaria Study SCC 670, 1077, 1143, 1178, 1179, 1180, 1207. Information Sheet / Consent Form (mild cases)

Version 9.0, April 26, 2010

A study to help understand the causes of severe malaria and how malaria makes people susceptible to other infections

The following information sheet should be explained in the appropriate local language:

You and your child are being invited to take part in a research study that aims to explore various aspects as to why some children suffer from severe disease, while others “only” suffer a relatively mild illness when they are infected with malaria. Before you decide, it is important for you to understand why the research is being done and what it would involve. Please take time to read the following information carefully, and ask us if there is anything that is not clear, or if you would like more information.

Reason for study

Your child has malaria, which is one of the most common diseases in African children, and a major health problem in the Gambia too, although it can be prevented by sleeping under an insecticide treated bednet. Malaria is caused by parasites (small germs) that are carried by some types of mosquitoes, and which enter the blood when we are bitten by those mosquitoes. The parasite makes its way into the red blood cells (important for the transport of oxygen) and destroys them. Most cases of malaria are usually mild. However, severe cases of malaria do also occur and some patients are seriously ill and are at risk of dying, especially children. It is not known why some children are able to fight malaria strongly while others get severe malaria. We know that part of the reason is that some people are born with the ability to fight malaria better than others, and these differences are called “genetic”. Differences in the ability of the child’s immune system (defense mechanism of the body against infections) to fight germs and parasites such as malaria, may contribute too. We know that the structure of the surface of the parasites varies, and it might be that some forms are more dangerous than others.

We hope that by comparing mild with severe cases of malaria, we can get a better understanding of these genetic and immunological differences, and may be able to identify structures on the parasite that make it a dangerous one. This may allow us to find new treatments or a vaccine that can prevent severe forms of malaria in the future.

We also want to study how the body learns to protect itself against malaria. One way how the body fights malaria is by antibodies (tiny molecules produced by so called B cells in the blood) that destroy the parasites. Previously, we have examined healthy children of different age groups and found that younger children have less B cells that can fight malaria than older children. Now, we want to find out more how these cells develop after a known infection with malaria. Therefore, we would like to follow up your child on regular intervals for 1.5 years after s(he) got malaria, and to compare his/her B cell responses to those of other children of different ages.

We also know that having malaria makes you more likely to get certain other infections, particularly germs entering your blood. It seems that malaria can weaken certain parts of a child’s immune system. We want to find out why this is, and whether we can identify who is at most risk, so that we can prevent these additional infections from happening.

How to take part?

It is up to you to decide whether or not you and your child takes part. If you do decide to take part you will be asked to sign or thumbprint a consent form on behalf of your child, and the field worker will ask you a number of questions to see if your child is eligible for this study. You are free to withdraw your child from the study at any time without giving a reason, and whether or not your child takes part will not affect the health care he/she will receive in any way.

What would happen to my child if he/she takes part?

If you agree that your child will join the study and the child is eligible, the study nurse will take a blood sample. In agreement with the recommendations of the Gambian Ethics Committee, not more than 5mls (about 1 teaspoon) will be taken out of your child’s vein if it is less than 5 years old. If your child is 5 to 9 years old, we would like to request 10mls (2 teaspoons) of blood. From children that are older than 9, we would like to request 15mls (3 teaspoons). A mouth swab would be taken to collect some cells from the inside of your child’s cheek. We would also like to take 5mls of blood from you if you are the child’s biological mother or father. These samples will be used to carry out immunological, parasitological and genetic research, described above. Furthermore, a stool sample will be collected at all the visits to assess your child’s worm carriage. Your child will receive the recommended treatment for malaria. We would also like to review the case notes when your child leaves the hospital so that we can write down his/her final diagnosis, clinical features of the illnesses and the outcome.

We would ask you to bring back your child one and four weeks after your child presented at the out patient department or the ward, to ensure that he/she is in good health. At both visits, we would collect another blood sample from your child as before, for the studies described above. Thereafter, we would like to arrange for a follow up visit to see your child 2, 4, 6, 8, 10, 12, 14, 16 and 18 months after s(he) had been to hospital. On these visits, the child's health will be checked and a further blood sample will be requested to allow us to do the work on B cells mentioned earlier.

What are the side effects and possible risks and benefits of taking part?

The mouth swab or collecting blood from your child will not harm him/her. The venepuncture may cause mild tenderness or bruising. For the duration of the study, the nurse will be happy to treat minor ailments free of charge and facilitate referral to the MRC clinic when necessary. Transport costs for the study visits will be reimbursed to you.

What tests would be done on my blood?

Comparing blood samples from cases of mild malaria with cases of severe malaria aims to answer three questions: 1) By examining the surface of the malaria parasites of severe and mild cases of malaria, we hope to identify those forms that are more dangerous than others. 2) The blood will also be examined to see in what ways the body's immune response (defense mechanism) to the malaria parasite differs between mild cases and severe cases. 3) The molecules that carry the genetic information (called genes) your child is born with may be responsible for how well he/she can fight malaria. From the collected blood genetic material will be extracted and analysed in the Gambia and also by our collaborators in the UK or elsewhere. The aim is to identify genes that are particularly common in children with severe malaria. This may help to identify new targets for vaccines.

For the longitudinal study that wants to find out how B cells that can fight malaria develop in children at different ages, we will use assays that allow us i) to count the number of B cells that are specific to malaria, and ii) to characterize these cells further for instance by measuring the amount and type of antibodies they produce.

For the study that wants to find out how malaria makes you vulnerable to bacterial infection (germs), we will look at how well cells of the immune system produce factors that kill bacteria, and how well the cells kill bacteria when they are mixed together in the laboratory. We will also look at how these factors are related to the severity of the malaria infection your child had, and to a variety of molecules that can be measured in the blood, to try to find which ones might be the best test to determine who is at risk of bacterial infection.

Future research in malaria is likely to increase our knowledge about the genetic mechanisms of the disease. Thus, it would be particularly useful to be able to use some of the blood samples from this study to examine them again in the future in the light of such new findings. With your consent we would therefore like to store some of the leftover blood samples for up to 25 years. No studies concerning diseases other than malaria will be done.

Would my taking part in this study be kept confidential?

All information that is collected about you or your child during the course of the research will be kept strictly confidential. The data will be available only to the study team, the Ethics Committee and Government regulatory authorities who can ask to audit or monitor the study. No unauthorized person will have access to who your child is or the information that you give us.

Who has reviewed the study?

A panel of scientists at the Medical Research Council has reviewed the study and has approved its scientific value. The Gambian Government/MRC Joint Ethics Committee has also approved the study and has granted permission for this study to take place.

Whom can I contact?

If you have any questions at any time during the study, please ask Dr Michael Walther at the MRC Laboratories, Fajara (Tel: 4495442/6 Ext: 4009) or Aubrey Cunningham (Ext: 4008). They will be happy to talk about any worries or queries.

Thank you very much for your time.

Consent form for study: A study to help understand the causes of severe malaria and how malaria causes susceptibility to other infections

Dear patient, parent or guardian,

Please circle answer

1. Have you read or has the Information Sheet Version _____, dated _____ been read to you? **Yes/No**
2. Did you have a chance to ask questions and did you receive satisfactory answers? **Yes/No**
3. Do you understand what participation in the study means to you / your child? **Yes/No**
4. Do you understand that the participation of you / your child is voluntary and that you are free to withdraw at any time, without giving any reason, without your medical care or legal rights being affected? **Yes/No**
5. Do you understand that the information regarding you / your child that is collected in the course of this study will remain confidential? **Yes/No**
6. Do you understand that laboratory tests will be done on the blood samples you / your child provide, and do you agree that some of the leftover blood samples will be stored and may be used for further studies of the body's response to malaria? **Yes/No**
7. Do you agree that we could collect blood from your child, one of the following amounts depending on his/her age as described today and on another 11 visits over the next 1.5 years?
-5mls (1 teaspoon) of blood if your child is less than 5 years old
-10mls (2 teaspoons) of blood if your child is aged between 5 and 9 years of age
-15mls (3 teaspoons) of blood if your child is older than 9 years old **Yes/No**
8. In case you are the mother or father of the child: Do you agree to donate 5mls of blood today? **Yes/No**
9. Do you understand that if you / your child gets sick during the study period, you can go to the clinic where study staff are providing care, and be examined and treated for free? **Yes/No**
10. Do you agree that we could perform a mouth swab from your child? **Yes/No**
11. Do you agree for your child to take part in this study? **Yes/No**
12. In case you are the mother / father: Do you agree to take part in this study? **Yes/No**

Name of child (in block letters) _____

Name of parent or guardian (in block letters) _____

Signature or thumbprint of parent or guardian of the child

I have read / explained the above to _____ (name of parent or guardian of the child) in a language he/she understands.

I am confident that he/she has understood what I explained and that he/she freely agreed that he/she and his/her child can take part in this study.

Signature of Field worker supervisor (or designate): _____

Date: ___ / ___ / ___ Name (in block letters): _____

Malaria Study SCC 670, 1077, 1143, 1180, 1207 Information Sheet / Consent Form (severe cases) 3
Version 9.0; April 26, 2010

Information and Consent form for Healthy Control Subjects

A study to help understand how malaria causes susceptibility to other infections

The following information sheet should be explained in the appropriate local language:

You and your child are being invited to take part in a research study that aims to find out why malaria increases the risk of getting other infections. Before you decide, it is important for you to understand why the research is being done and what it would involve. Please take time to read the following information carefully, and ask us if there is anything that is not clear, or if you would like more information.

Reason for study

Your child is healthy at the moment, but many other children have malaria, which is a major health problem in the Gambia. Malaria can be prevented by sleeping under an insecticide treated bednet. Malaria is caused by parasites (small germs) that are carried by some types of mosquitoes, and which enter the blood when we are bitten by those mosquitoes. The parasite makes its way into the red blood cells (important for the transport of oxygen) and destroys them. Most cases of malaria are usually mild. However we know that having malaria makes you more likely to get certain other infections, particularly bacteria (germs) entering your blood. It seems that malaria can weaken certain parts of a child's immune system. We want to find out why this is, and whether we can identify who is at most risk, so that we can prevent these additional infections from happening.

How to take part?

It is up to you to decide whether or not you and your child take part. If you do decide to take part you will be asked to sign or thumbprint a consent form on behalf of your child, and the field worker will ask you a number of questions to see if your child is eligible for this study. You are free to withdraw your child from the study at any time without giving a reason, and whether or not your child takes part will not affect the health care he/she will receive in any way.

What would happen to my child if he/she takes part?

If you agree that your child will join the study and the child is eligible, the study nurse will take a blood sample. In agreement with the recommendations of the Gambian Ethics Committee, not more than 5mls (about 1 teaspoon) will be taken out of your child's vein if it is less than 5 years old. If your child is 5 to 9 years old, we would like to request 10mls (2 teaspoons) of blood. From children that are older than 9, we would like to request 15mls (3 teaspoons). These samples will be used to carry out the immunological research described above.

What are the side effects and possible risks and benefits of taking part?

Collecting blood from your child will not harm him/her. The venepuncture may cause mild tenderness or bruising. The MRC provides free healthcare for the Brefet community and you and your child are encouraged to take advantage of this.

What tests would be done on my blood?

We want to look at how well blood cells of the immune system produce factors that kill bacteria (germs), and how well the cells kill bacteria when they are mixed together in the laboratory. We will compare these factors between samples from healthy children and cases of mild malaria and severe malaria. This will tell us whether malaria makes people susceptible to bacterial infection by reducing the ability to kill bacteria. We will also look at how these factors are related to a variety of molecules that can be measured in the blood to help to determine who is at risk of bacterial infection.

Would my taking part in this study be kept confidential?

All information that is collected about you or your child during the course of the research will be kept strictly confidential. The data will be available only to the study team, the Ethics Committee and Government regulatory authorities who can ask to audit or monitor the study. No unauthorized person will have access to who your child is or the information that you give us.

Who has reviewed the study?

A panel of scientists at the Medical Research Council has reviewed the study and has approved its scientific value. The Gambian Government/MRC Joint Ethics Committee has also approved the study and has granted permission for this study to take place.

Whom can I contact?

If you have any questions at any time during the trial, please ask Dr Michael Walther at the MRC Laboratories, Fajara (Tel: 4495442/6 Ext: 4009) or Dr Aubrey Cunningham (Ext: To be confirmed). They will be happy to talk about any worries or queries.

Thank you very much for your time.

Consent form for study: A study to help understand how malaria causes susceptibility to other infections

Dear patient, parent or guardian,

Please circle answer

1. Have you read or has the Information Sheet Version _____, dated _____ been read to you? **Yes/No**
2. Did you have a chance to ask questions and did you receive satisfactory answers? **Yes/No**
3. Do you understand what participation in the study means to you / your child? **Yes/No**
4. Do you understand that the participation of your child is voluntary and that you are free to withdraw at any time, without giving any reason, without your medical care or legal rights being affected? **Yes/No**
5. Do you understand that the information regarding your child that is collected in the course of this study will remain confidential? **Yes/No**
6. Do you understand that laboratory tests will be done on the blood samples you provide? **Yes/No**
7. Do you agree that we could collect blood from your child one of the following amounts depending on his/her age as described today
-5mls (1 teaspoon) of blood if your child is less than 5 years old
-10mls (2 teaspoons) of blood if your child is aged between 5 and 9 years of age
-15mls (3 teaspoons) of blood if your child is older than 9 years old **Yes/No**
8. Do you agree for your child to take part in this study? **Yes/No**

Name of child (in block letters) _____

Name of parent or guardian (in block letters) _____

Signature or thumbprint of parent or guardian of the child

I have read / explained the above to _____ (name of parent or guardian of the child) in a language he/she understands.

I am confident that he/she has understood what I explained and that he/she freely agreed that his/her child can take part in this study.

Signature of Field worker supervisor (or designate): _____

Date: ___/___/___

Name (in block letters): _____



Malaria Programme

Medical Research Council Laboratories,

Fajara, P. O. Box 273 West Africa

Switchboard (+220)4495442/6 Ext: 4009

Email: mwalther@mrc.gm

Collection of human blood from healthy volunteers

Consent Form

You are asked to donate a venous blood sample for the malaria programme of the MRC laboratories, Fajara, PO Box 273, Banjul.

The experiments to be performed on your blood aim to further our understanding of the body's immune responses to *P. falciparum* malaria. All projects will have received ethical approval from the Gambian Government/MRC Joint Ethics Committee, and will be limited to research on malaria.

It is important that you understand that your participation is entirely voluntary. You are free to withdraw at any time, without giving any reason.

If you decide not to take part, this will not affect at all your legal rights as an MRC employee, or the medical care offered to you by MRC.

Blood will be collected only by clinically qualified staff using sterile procedures.

You are entitled to see all data arising from the use of your blood, on request.

To maintain confidentiality, your sample will be given a three digit study identification (ID) number derived from a list of random numbers. The Principal Investigator will keep a master list linking your name to a study ID. This list will be kept in a locked filing cabinet together with this consent form.

Details of the donor

| | |
|---|----------------------|
| Surname: | |
| First name: | |
| Tel number: | |
| Email address: | |
| Are you a regular blood donor in: The Gambia Elsewhere | YESNO YESNO |
| Country of origin: | |
| Have you lived in any countries apart from your country of origin and the Gambia? | YESNO |
| If YES, please list the countries and the dates when you lived there | |
| Blood group if known. | |

Blood volume to be taken: _____ Study ID: _____

How much blood have you donated within the last 8 weeks period? _____

(Note, within an 8 weeks period, not more than 500ml of blood should be taken)

I understand that my participation is entirely voluntary and does not affect my rights **Yes/No**

I understand that whether or not I participate does not affect the medical care offered by MRC **Yes/No**

I understand that I may withdraw at any time without giving a reason **Yes/No**

I have been given the opportunity to ask questions about the study and have received satisfactory answers **Yes/No**

I have received enough information about the research my blood would be used for **Yes/No**

I am happy to donate blood for the MRC malaria research programme **Yes/No**

Name (donor): _____ Signature of donor: _____

Date: _____

I have checked that including the blood volume taken today not more than 500ml have been taken within the past 8 weeks.

Name (phlebotomist): _____ Signature (phlebotomist): _____

5. Clinical record form

Hospital number _____

Hospital slide number: _____

TRIP in severe and mild malaria
MRC malaria Programme, Fajara The Gambia

This Case Record Form is to be used for children resident within a 40 km radius south of Banjul, excluding Banjul city, and the costal areas (Cape point, Bakau, Fajara, Kotu, Kololi, Bijilo), and for children enrolled at Brikama health centre that have agreed to participate in the study.

Subject's name

Father's name.....

Mother's Name.....

Compound Head.....

Telephone number (if available)

Address

.....

Sketch of the area where the participant lives

Referring Health Centre / facility

Form completed by

TRIP in severe and mild malaria

MRC malaria Programme, Fajara The Gambia

Enrolment at MRC Fajara (=1), at Brikama HC (=2), at JFfP-hospital (=3)

Date of Birth ___/___/___ Age: _____ (years)

Sex: male , female

Ethnic group: (1=Mandingo, 2=Wollof, 3=Fula, 4=Jola, 5=Serahuli,
6=Serere, 7=Manjago,8=Aku,9=Others, specify

Date of admission: _____
day / month / year

Inclusion criteria met? yes = 1, no = 2

Antimalarial treatment or other medication received in the last two weeks?
 yes = 1, no = 2 , if yes complete the table below

| Antimalarials | Yes = 1 No = 2 | Dates | Route | Comments |
|---------------|--------------------------|-------|-------|----------|
| Chloroquine | <input type="checkbox"/> | | | |
| Fansidar | <input type="checkbox"/> | | | |
| Quinine | <input type="checkbox"/> | | | |
| Septrin | <input type="checkbox"/> | | | |
| Coartem | <input type="checkbox"/> | | | |
| Unknown drug | <input type="checkbox"/> | | | |

Presenting symptoms

| Patient | | Duration |
|-----------------------|--------------------------|--|
| Presenting complaints | 1. | <input type="text"/> <input type="text"/> <input type="text"/> (Days) |
| | 2. | <input type="text"/> <input type="text"/> <input type="text"/> (Days) |
| | 3. | <input type="text"/> <input type="text"/> <input type="text"/> (Days) |
| HISTORY | Yes = 1 No = 2 | |
| Fever | <input type="checkbox"/> | <input type="text"/> <input type="text"/> <input type="text"/> (Days) |
| Convulsion | <input type="checkbox"/> | No of fits in last 24hrs <input type="text"/> <input type="text"/> <input type="text"/> |
| Unconscious | <input type="checkbox"/> | <input type="text"/> <input type="text"/> <input type="text"/> (Hours) |
| Diarrhoea | <input type="checkbox"/> | <input type="text"/> <input type="text"/> <input type="text"/> (Days) |
| Vomiting | <input type="checkbox"/> | <input type="text"/> <input type="text"/> <input type="text"/> (Days) |
| Unusually sleepy? | <input type="checkbox"/> | <input type="text"/> <input type="text"/> <input type="text"/> (Days) |

| | | |
|-----------------------|--------------------------|--|
| Restless/Irritable? | <input type="checkbox"/> | <input type="checkbox"/> <input type="checkbox"/> (Days) |
| Fast breathing? | <input type="checkbox"/> | <input type="checkbox"/> <input type="checkbox"/> (Days) |
| Breathing difficulty? | <input type="checkbox"/> | <input type="checkbox"/> <input type="checkbox"/> (Days) |
| Pallor/lethargy | <input type="checkbox"/> | <input type="checkbox"/> <input type="checkbox"/> (Days) |
| Cough? | <input type="checkbox"/> | <input type="checkbox"/> <input type="checkbox"/> (Days) |
| Reduced feeding? | <input type="checkbox"/> | <input type="checkbox"/> <input type="checkbox"/> (Days) |

EXAMINATION ON INITIAL PRESENTATION TO HOSPITAL/OPD

Physical examination performed? Yes = 1 No = 2

If yes, answer the following:

| | | |
|--|---|---|
| Pallor | 1=Conjunctival, 2=Palms, 3=Tongue, 4=All, 9=Nil | <input type="checkbox"/> |
| Jaundice | Yes = 1 No = 2 | <input type="checkbox"/> |
| Neck stiffness | Yes = 1 No = 2 | <input type="checkbox"/> |
| Respiratory Patterns | 1=Normal, 2=Deep breathing, 3=Irregular, 4=Gasping | <input type="checkbox"/> <input type="checkbox"/> |
| Prostration* | Yes = 1 No = 2 | <input type="checkbox"/> |
| Grunting | Yes = 1 No = 2 | <input type="checkbox"/> |
| Use of accessory muscles of respiration | Yes = 1 No = 2 | <input type="checkbox"/> |
| Auscultatory findings | 1=Normal, 2=Crackles, 3=Wheeze, 4=Bronchial | <input type="checkbox"/> |
| Dehydration | 1. Nil, 2. Mild, 3. Mod., 4. Severe | <input type="checkbox"/> |
| Axillary temp (°C) | <input type="text"/> <input type="text"/> <input type="text"/> . <input type="text"/> | |
| Heart/pulse rate/min | <input type="text"/> <input type="text"/> <input type="text"/> /min | |
| Respiratory rate/min | <input type="text"/> <input type="text"/> <input type="text"/> | |
| Weight (kg) | <input type="text"/> <input type="text"/> <input type="text"/> . <input type="text"/> | |

| | | |
|--------------------------|-------|--|
| | | |
| Height (cm) | _ _ _ | |
| Spleen size (cm) | _ _ | |
| Liver size (cm) | _ _ | |
| Other important findings | | |
| | | |

* –Prostrated children (inability to sit up in a child normally able to do so, or to drink in case of children too young to sit)

Blantyre Coma Score

| BEST MOTOR RESPONSE | Score | VERBAL RESPONSE | Score |
|---------------------------------------|-------|---------------------------|-------|
| Localizes painful stimulus* | 2 | Appropriate cry | 2 |
| Withdraws limb from pain** | 1 | Moan or inappropriate cry | 1 |
| Non specific or absent | 0 | None | 0 |
| EYE MOVEMENT | | | |
| Directed (e.g. follows mother's face) | 1 | | |
| Not directed | 0 | | |

*Localizes painful stimulus: rub knuckles on patient's sternum.

** Painful stimulus: firm pressure on thumbnail bed with horizontal pencil

Total Score |___|

Laboratory Investigations

HB on admission . gm/dl

Lactate on admission .
mmol/L

Blood sugar on admission .
mmol

Has malaria smear been taken? Yes = 1 No = 2 if yes complete the following table

Field stain: Yes = 1 No = 2 if yes enter results

Positive= 1, Negative =2 and 3= Not available

DENSITY: Trophozoites / HPF

Gametocytes / HPF

For subjects less than 5 years old:

Has a 4 mls blood sample been taken into a green heparinized tube?
 Yes=1, No=2

Has a 1 ml blood sample been taken into a PAX tube?
 Yes=1, No=2

Has a 0.25 ml blood sample been taken into a purple EDTA tube 1?
 Yes=1, No=2

Has a 0.25 ml blood sample been taken into purple EDTA tube 2?
 Yes=1, No=2

For subjects aged between 5-9 years old:

Has a 8 mls blood sample been taken into a green heparinized tube?
 Yes=1, No=2

Has a 1 ml blood sample been taken into a PAX tube?
 Yes=1, No=2

Has a **0.5 ml** blood sample been taken into a purple **EDTA tube 1?**

Yes=1, No=2

Has a **0.5 ml** blood sample been taken into a purple **EDTA tube 2?**

Yes=1, No=2

For subjects older than 9 years old:

Has a **13 mls** blood sample been taken into a green heparinized tube?

Yes=1, No=2

Has a **1 ml** blood sample been taken into a PAX tube?

Yes=1, No=2

Has a **0.5 ml** blood sample been taken into a purple **EDTA tube 1?**

Yes=1, No=2

Has a **0.5 ml** blood sample been taken into a purple **EDTA tube 2?**

Yes=1, No=2

Has a mouth swab been taken?

Yes=1, No=2

For all study subjects has a stool sample been collected? Yes = 1 No = 2

For patients with severe malaria only:

Has a 5mls blood sample been taken from: (Yes =1; No = 2)

the mother and / or

the father?

First OPD visit (VISIT 2, one week after admission)

Date of visit |__|__|/|__|__|__|/|__|__|

Did study participant come for this visit? |__| (Yes = 1; No = 2)

Temperature |__|__|. |__| 0C

Does the patient have any significant symptom Yes = 1 No = 2 |__|

If yes, record symptoms

SYMPTOMS

Duration in days (1 if started on day of visit)

| | | |
|----------------|----|----|
| Fever | __ | __ |
| Coughing | __ | __ |
| Headache | __ | __ |
| Dizziness | __ | __ |
| Vomiting | __ | __ |
| Diarrhoea | __ | __ |
| Nausea | __ | __ |
| Abdominal Pain | __ | __ |
| Chills/Rigors | __ | __ |
| Itching | __ | __ |

Jaundice

Others (specify)- _____ |__| |__|
_____ |__| |__|
_____ |__| |__|
_____ |__| |__|

Physical examination

Physical examination performed? Yes = 1 No = 2 |__|

If yes, answer the following:

Weight |__|__|. |__| Kg

| | Tick if normal, if not normal, record findings |
|-------|--|
| ENT | __ _____ |
| Chest | __ _____ |
| CVS | __ _____ |
| CNS | __ _____ |
| GIT | __ _____ |
| Skin | __ _____ |
| MSS | __ _____ |

Other abnormal findings or comments: _____

LABORATORY TESTS

Has malaria smear been taken? Yes = 1 No = 2 |__| if yes complete the following table

Field stain: Yes = 1 No = 2 |__| if yes enter results

Positive= 1, negative =2 and 3= not done |__|

DENSITY: Trophozoites |__|_|__|_|/|__|_|__|_| HPF

Gametocytes |__|_|__|_|/|__|_|__|_| HPF

HB |__|_|__| . |__|_| gm/dl

Antibiotic given at any time during the first week

(as documented in the notes) Yes = 1 No = 2 |__|

If yes please give details below

DRUGNAME _____ DOSE _____ DURATION _____

DRUGNAME _____ DOSE _____ DURATION _____

For subjects less than 5 years old:

Has a **4 mls** blood sample been taken into a green heparinized tube?

Yes=1, No=2

Has a **0.25 ml** blood sample been taken into a purple **EDTA tube 1?**

Yes=1, No=2

Has a **0.25 ml** blood sample been taken into purple **EDTA tube 2?**

Yes=1, No=2

For subjects aged between 5-9 years old:

Has a **8 mls** blood sample been taken into a green heparinized tube?

Yes=1, No=2

Has a **0.5 ml** blood sample been taken into a purple **EDTA tube 1?**

Yes=1, No=2

Has a **0.5ml** blood sample been taken into a purple **EDTA tube 2?**

Yes=1, No=2

For subjects older than 9 years old:

Has a **13 mls** blood sample been taken into a green heparinized tube?

Yes=1, No=2

Has a **0.5 ml** blood sample been taken into a purple **EDTA tube 1?**

Yes=1, No=2

Has a **0.5 ml** blood sample been taken into a purple **EDTA tube 2?**

Yes=1, No=2

Second OPD visit (VISIT 3, four weeks after admission)

Date of visit |__|__|/|__|__|__|/|__|__|

Did study participant come for this visit? |__| (Yes = 1; No = 2)

Temperature |__|__|. |__| 0C

Has participant been seen for the day 7 visit? Yes = 1 No = 2 |__|

Does the patient have any significant symptom Yes = 1 No = 2 |__|

If yes, record symptoms

SYMPTOMS

Duration in days (1 if started on day of visit)

| | | |
|----------------|----|----|
| Fever | __ | __ |
| Coughing | __ | __ |
| Headache | __ | __ |
| Dizziness | __ | __ |
| Vomiting | __ | __ |
| Diarrhoea | __ | __ |
| Nausea | __ | __ |
| Abdominal Pain | __ | __ |
| Chills/Rigors | __ | __ |
| Itching | __ | __ |

Jaundice

Others (specify)- _____ |__| |__|
_____ |__| |__|
_____ |__| |__|
_____ |__| |__|

Physical examination

Physical examination performed? Yes = 1 No = 2 |__|

If yes, answer the following:

Weight |__|__|. |__| Kg

| | Tick if normal, if not normal, record findings |
|-------|--|
| ENT | __ _____ |
| Chest | __ _____ |
| CVS | __ _____ |
| CNS | __ _____ |
| GIT | __ _____ |

Skin _____

MSS _____

Other abnormal findings or comments: _____

LABORATORY TESTS

Has malaria smear been taken? Yes = 1 No = 2 if yes complete the following table

Field stain: Yes = 1 No = 2 if yes enter results

Positive= 1, negative =2 and 3= not done

DENSITY: Trophozoites / HPF

Gametocytes / HPF

HB . gm/dl

For subjects less than 5 years old:

Has a 4 mls blood sample been taken into a green heparinized tube?
 Yes=1, No=2

Has a 1 ml blood sample been taken into a PAX tube?
 Yes=1, No=2

Has a 0.25 ml blood sample been taken into a purple EDTA tube 1?
 Yes=1, No=2

Has a 0.25 ml blood sample been taken into purple EDTA tube 2?
 Yes=1, No=2

For subjects aged between 5-9 years old:

Has a 8 mls blood sample been taken into a green heparinized tube?
 Yes=1, No=2

Has a 1 ml blood sample been taken into a PAX tube?
 Yes=1, No=2

Has a **0.5 ml** blood sample been taken into a purple **EDTA tube 1**?
 Yes=1, No=2

Has a **0.5 ml** blood sample been taken into a purple **EDTA tube 2**?
 Yes=1, No=2

For subjects older than 9 years old:

Has a **13 mls** blood sample been taken into a green heparinized tube?
 Yes=1, No=2

Has a **1 ml** blood sample been taken into a PAX tube?
 Yes=1, No=2

Has a **0.5 ml** blood sample been taken into a purple **EDTA tube 1**?
 Yes=1, No=2

Has a **0.5 ml** blood sample been taken into a purple **EDTA tube 2**?
 Yes=1, No=2

Has the possibility for VCT for HIV for both child and parents been offered to parents? Yes = 1 No = 2

Was a blood transfusion performed? Yes = 1 No = 2

If "yes", please indicate the date ___/___/___ and the

Blood volume given: _____ml

Diagnoses at enrolment, please tick the appropriate:

Mild malaria

Severe malaria

• Cerebral malaria

• Severe malaria anaemia

• Severe malaria respiratory distress

• Severe malaria, other entities

○ please describe: _____

Other diagnoses (to be assessed on day 28 with help of the notes)

1 _____

2 _____

3 _____

Outcome: 1=Alive without sequelae 2= Alive with neurological sequelae

3=Died

Date of discharge or death: ___/___/___

Interim Report on Study part 1

Date: ___/___/___

Study part 1 completed? Yes = 1 No = 2

If '**NO**' mark one reason.

1 Eligibility criteria not met

2 Protocol violation, specify one reason.

3 Lost to follow-up – give date of last contact: ___-/___/___

4 Subject died

Date of death: ___/___/___

5 Consent withdrawn

6 Physicians decision, specify.

INVESTIGATOR STATEMENT

I certify that I have carefully examined all the entries on the case report form and that all information entered on these pages by myself or associates is correct.

Signed:

Name & Date.

Third OPD visit (VISIT 4, eight weeks after admission)

Date of visit |__|__|/|__|__|/|__|__|

Has participant been seen for the day 28 visit? Yes = 1 No = 2

Since the last visit, has your child been ill with a fever at any time?
Yes = 1 No = 2

If yes, has the child's blood been examined for malaria?
Yes = 1 No = 2

If yes, what was the test result? Pos = 1, Neg = 2

Did you seek any advice or treatment for the illness from any source?
Yes = 1 No = 2

If yes, where have you been? _____

Did your child take any medicine for malaria?
Yes = 1 No = 2

Did your child sleep under a bednet the night before? Yes = 1 No = 2

Did the child have any other health problems since the last visit?
Yes = 1 No = 2

If yes, describe the nature of the problem and how it has been dealt with:

According to you, how is the child feeling today?

Please measure the temperature, weight and height of the child

Temperature |__|__|. |__| °C Weight |__|__|. |__| Kg Height |__|__|__|. |__| cm

LABORATORY TESTS

For subjects less than 5 years old:

Has a **4 mls** blood sample been taken into a green heparinized tube?
 Yes=1, No=2

Has a **0.5 ml** blood sample been taken into a PAX tube?
 Yes=1, No=2

Has a **0.25 ml** blood sample been taken into a purple **EDTA tube 1?**
 Yes=1, No=2

Has a **0.25 ml** blood sample been taken into purple **EDTA tube 2?**
 Yes=1, No=2

For subjects aged between 5-9 years old:

Has a **8 mls** blood sample been taken into **two** green heparinized tubes?
 Yes=1, No=2

Has a **0.5 ml** blood sample been taken into a PAX tube?
 Yes=1, No=2

Has a **0.5 ml** blood sample been taken into a purple **EDTA tube 1?**
 Yes=1, No=2

Has a **0.5 ml** blood sample been taken into a purple **EDTA tube 2?**
 Yes=1, No=2

For subjects older than 9 years old:

Has a **13 mls** blood sample been taken into **two** green heparinized tubes?
 Yes=1, No=2

Has a **0.5 ml** blood sample been taken into a PAX tube?
 Yes=1, No=2

Has a **0.5 ml** blood sample been taken into a purple **EDTA tube 1?**
 Yes=1, No=2

Has a **0.5 ml** blood sample been taken into a purple **EDTA tube 2?**
 Yes=1, No=2

For **all study subjects** has a **stool** sample been collected?
Yes = 1 No = 2

Fourth OPD visit (VISIT 5, four months after admission)

Date of visit |__|__|/|__|__|/|__|__|

Has participant been seen for the day 56 visit? Yes = 1 No = 2 |__|

Since the last visit, has your child been ill with a fever at any time?
Yes = 1 No = 2 |__|

If yes, has the child's blood been examined for malaria?
Yes = 1 No = 2 |__|

If yes, what was the test result? Pos = 1, Neg = 2 |__|

Did you seek any advice or treatment for the illness from any source?
Yes = 1 No = 2 |__|

If yes, where have you been? _____

Did your child take any medicine for malaria?
Yes = 1 No = 2 |__|

Did your child sleep under a bednet the night before? Yes = 1 No = 2 |__|

Did the child have any other health problems since the last visit?
Yes = 1 No = 2 |__|

If yes, describe the nature of the problem and how it has been dealt with:

According to you, how is the child feeling today?

Please measure the temperature, weight and height of the child

Temperature |__|__|. |__| °C Weight |__|__|. |__| Kg Height |__|__|__|. |__| cm

LABORATORY TESTS

For subjects less than 5 years old:

Has a **4 mls** blood sample been taken into a green heparinized tube?
 Yes=1, No=2

Has a **0.5 ml** blood sample been taken into a PAX tube?
 Yes=1, No=2

Has a **0.25 ml** blood sample been taken into a purple **EDTA tube 1?**
 Yes=1, No=2

Has a **0.25 ml** blood sample been taken into purple **EDTA tube 2?**
 Yes=1, No=2

For subjects aged between 5-9 years old:

Has a **8 mls** blood sample been taken into **two** green heparinized tubes?
 Yes=1, No=2

Has a **0.5 ml** blood sample been taken into a PAX tube?
 Yes=1, No=2

Has a **0.5 ml** blood sample been taken into a purple **EDTA tube 1?**
 Yes=1, No=2

Has a **0.5 ml** blood sample been taken into a purple **EDTA tube 2?**
 Yes=1, No=2

For subjects older than 9 years old:

Has a **13 mls** blood sample been taken into **two** green heparinized tubes?
 Yes=1, No=2

Has a **0.5 ml** blood sample been taken into a PAX tube?
 Yes=1, No=2

Has a **0.5 ml** blood sample been taken into a purple **EDTA tube 1?**
 Yes=1, No=2

Has a **0.5 ml** blood sample been taken into a purple **EDTA tube 2?**
 Yes=1, No=2

For **all study subjects** has a **stool** sample been collected?
Yes = 1 No = 2

Study Termination Page

Date: ___/___/___

Study completed? Yes = 1 No = 2

If '**NO**' mark one reason.

1 Eligibility criteria not met

2 Protocol violation, specify one reason.

3 Lost to follow-up – give date of last contact: ___/___/___

4 Subject died

Date of death: ___/___/___

5 Consent withdrawn

6 Physicians decision, specify.

INVESTIGATOR STATEMENT

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Signed:

Name & Date.

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Duc

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With my best wishes, Celia

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