

# Interferon- $\gamma$ —central mediator of protective immune responses against the pre-erythrocytic and blood stage of malaria

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## ABSTRACT

Immune responses against *Plasmodium* parasites, the causative organisms of malaria, are traditionally dichotomized into pre-erythrocytic and blood-stage components. Whereas the central role of cellular responses in pre-erythrocytic immunity is well established, protection against blood-stage parasites has generally been ascribed to humoral responses. A number of recent studies, however, have highlighted the existence of cellular immunity against blood-stage parasites, in particular, the prominence of IFN- $\gamma$  production. Here, we have undertaken to chart the contribution of this prototypical cellular cytokine to immunity against pre-erythrocytic and blood-stage parasites. We summarize the various antiparasitic effector functions that IFN- $\gamma$  serves to induce, review an array of data about its protective effects, and scrutinize evidence for any deleterious, immunopathological outcome in malaria patients. We discuss the activation and contribution of different cellular sources of IFN- $\gamma$  production during malaria infection and its regulation in relation to exposure. We conclude that IFN- $\gamma$  forms a central mediator of protective immune responses against pre-erythrocytic and blood-stage malaria parasites and identify a number of implications for rational malaria vaccine development. *J. Leukoc. Biol.* **88**: 1131–1143; 2010.

## Introduction

IFN- $\gamma$  is the only type-II IFN and is the prototypical Th1 cytokine, inducing cell-mediated immunity by promoting Th1 over Th2 differentiation of T cells, inducing IgG class-switching to cytophilic isotypes, and activating phagocytes (reviewed in ref. [1]). It is produced predominantly by lymphocytes, including NK, NKT cells,  $\gamma\delta$ T, and  $\alpha\beta$ T cells, but may also be produced by cells of the myeloid lineage [2, 3]. Its induction is largely dependent on IL-12 and IL-18 production by activated myeloid APCs [4, 5], in addition to signals directly activating lymphocytes themselves.

IFN- $\gamma$  is an important mediator of the immune response against intracellular (myco)bacteria and some viruses [6–8] but is also involved in protection against intra- and extracellular protozoan parasites such as *Leishmania* spp. [9], *Trypanosoma cruzi* [10], and *Toxoplasma gondii* [11]. In this review, we will focus on the involvement of IFN- $\gamma$  in immune responses against malaria.

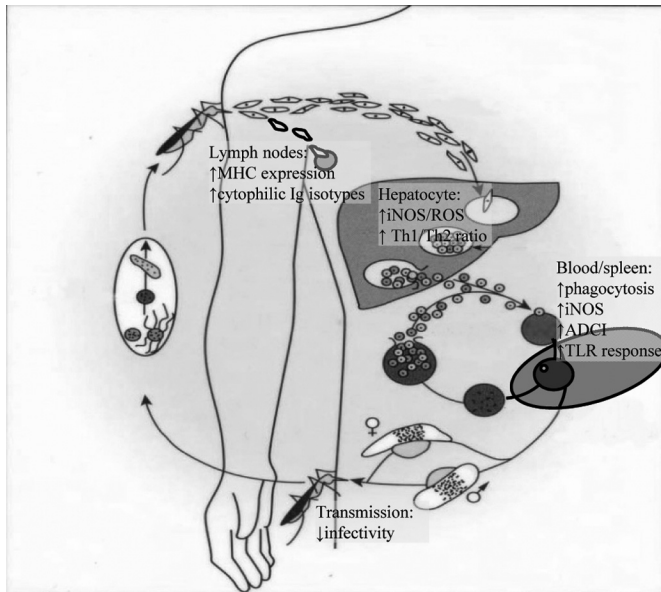
Malaria is caused by protozoan parasites of the genus *Plasmodium*, and *Plasmodium falciparum* in particular has major clinical importance for human disease at a global scale. *Plasmodium* parasites have a complicated, multistage lifecycle involving intra- and extracellular stadia in an Anopheline mosquito vector and a vertebrate host (Fig. 1).

By convention, immune responses in malaria are dichotomized into pre-erythrocytic responses (directed against sporozoites and liver-stage parasites) and blood-stage responses (directed against merozoites and intraerythrocytic parasites). Whereas humoral (antibody) responses against extracellular sporozoite stages are well documented [13, 14], pre-erythrocytic immunity is generally considered to consist largely of cellular responses against infected hepatocytes, which inhibit intracellular parasite development through the induction of reactive nitrogen intermediates (reviewed in ref. [15]). In contrast, humoral responses against extracellular merozoites and intraerythrocytic parasites (pRBC) have traditionally been considered the most important component of blood-stage immunity [16–18]. T cell responses against pRBC remain less well understood, partly because erythrocytes lack MHC class I or II presentation capacity. Nevertheless, cellular responses against pRBC have been suggested to contribute to protection in humans in the absence of antibodies [19, 20]. Finally, monocyte/macrophage-mediated responses, in particular phagocytosis (e.g., ref. [21]), and ADCI (e.g., ref. [22]), also form an important component of blood-stage immunity.

Given the above, IFN- $\gamma$  likely forms an important component of immunity against both stages of malaria infection. Indeed, IFN- $\gamma$  plays a plethora of roles in malaria, functioning equally as an inducer and effector of innate and adaptive im-

Abbreviations: ADCI=antibody-dependent cellular inhibition, mDC=myeloid DC, pRBC=parasitized RBC, SM=severe malaria, TRAP=thrombospondin-related anonymous protein, UM=uncomplicated malaria

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**Figure 1. Lifecycle of malaria parasites and effector functions of IFN- $\gamma$ .** Infective sporozoite-stage parasites are unwittingly injected into the skin of human hosts by blood-feeding mosquitoes, where a proportion is trapped in draining lymph nodes, but the remainder migrates quickly to the liver. These sporozoites invade hepatocytes, in which they mature and multiply intracellularly over 6–7 days without causing symptoms. Once fully ripe, these liver-stage schizonts rupture, releasing merozoites into the bloodstream, which invade erythrocytes (RBCs) immediately. These intraerythrocytic parasites multiply asexually over roughly 48 h into blood-stage schizonts, which again rupture, releasing a second generation of merozoites that in turn invade new RBCs. It is this exponential multiplication cycle that is responsible for the clinical symptoms and potentially severe complications of malaria. A small number of blood-stage parasites develop into male and female gametocyte forms, which can be taken up by a second blood-feeding mosquito. Within the mosquito, these gametes undergo sexual replication, eventually resulting in a new generation of infective sporozoites. Various effector functions of IFN- $\gamma$ , enhancing immunological recognition and elimination of the parasite's different lifecycle stages, are indicated. This figure is modified from ref. [12] with permission.

mune responses (**Table 1**) throughout the parasite lifecycle (Fig. 1). It could even be argued that IFN- $\gamma$  forms the central determinant of all immunological pathways involved in protection against malaria.

However, what then is the hard evidence for a protective role of IFN- $\gamma$  responses? What host and parasite factors determine those responses, and how can we exploit those factors to design an effective malaria vaccine rationally? In this review, we first focus on the wealth of evidence for the protective role of IFN- $\gamma$  responses and critically discuss any evidence for their possible immunopathological (side-)effects. Next, we examine the various potential cellular sources of IFN- $\gamma$ , their differing requirements for activation, and their relative contribution to total IFN- $\gamma$  production during pre-erythrocytic and blood-stage infection. Finally, we consider the relationship between exposure and IFN- $\gamma$  responses and discuss immunoregulation by the malaria parasite. We conclude by identifying a number of implications arising from (gaps in) our current understanding

of IFN- $\gamma$  responses, with particular regard to rational malaria vaccine development. Where possible, we present direct evidence from human studies of *P. falciparum* infection, which we supplement with data from in vitro and murine malaria models.

## EVIDENCE FOR PROTECTION

As a result of distinct biological, immunological, and pathological characteristics for each stage (Fig. 1), most research into immunological correlates of protection in malaria has focused on pre-erythrocytic or erythrocytic stages separately. A wealth of data about both stages exists from murine malaria models and human studies. Here, we will briefly discuss the consensus derived from murine immunological studies and focus primarily on human data, including experimental malaria infections, cross-sectional and longitudinal field studies, and efficacy data from vaccine studies.

### Pre-erythrocytic stages

Pre-erythrocytic protection can be induced in murine malaria models by various immunization strategies, including irradiated sporozoites, recombinant peptide, or nucleic-acid vaccines. Although immunological effector mechanisms vary slightly between approaches and between inbred mouse strains (e.g., refs. [24, 43–48]), the core elements tend to consist of CD8<sup>+</sup> and/or CD4<sup>+</sup> T cells and IFN- $\gamma$ -mediated responses against infected hepatocytes (reviewed in ref. [15]). Indeed, protection against pre-erythrocytic malaria in naïve mice, rats, and monkeys can also be induced by simply injecting exogenous IFN- $\gamma$  [23, 49] or IL-12 [50, 51].

Some of the most compelling evidence for the protective role of IFN- $\gamma$  responses in humans comes from experimental malaria infections. The strength of these studies lies in the tight control exercised over previous exposure, timing, and measure of infection, factors that often confound field-based studies. Following a primary infection, high IFN- $\gamma$  responses are associated with reduced asexual parasite multiplication rates, although eventually, all volunteers do develop patent parasitemia [52].

Since the days of malaria therapy, however, scientists have known that sterile immunity against malaria can be induced in humans unexposed previously. The best-studied approach has been through inoculation of irradiated sporozoites by repeated mosquito bites [53]. These radiation-attenuated sporozoites arrest during the liver stage and induce humoral (e.g., ref. [54]) and CD8<sup>+</sup> [55] and CD4<sup>+</sup> [56] cytotoxic T cell and IFN- $\gamma$  responses against sporozoites and liver-stage antigens [57, 58]. Nevertheless, multiple rounds of immunization, equating to at least 1000 infected mosquito bites, are required to generate sterile protection in >90% of the volunteers.

In an attempt to improve upon this protocol, we recently immunized volunteers with patent sporozoites under cover of the blood schizonticidal drug chloroquine, which leaves development of liver stages unaffected [20]. This approach exposes volunteers' immune systems to the full course of intrahepatic development, in addition to the first cycle of intraerythrocytic

Table 1. Effects of IFN- $\gamma$  on Innate and Adaptive Immune Responses against Malaria

Function	References
Innate immune responses	
Increased production of reactive nitrogen species (iNOS) by liver cells against intrahepatic parasites	[23–25]
Increased production of reactive oxygen ( $H_2O_2$ ) and nitrogen species (iNOS) by monocytes against blood-stage parasites	[26–29]
Enhanced phagocytosis of merozoites and pRBC	[27, 30–32]
Inhibition of gametocyte infectivity to mosquitoes	[33]
Interplay between innate and adaptive systems	
Proinflammatory priming of TLR responses	[34–37]
Up-regulation of MHC class I and II expression	[38]
Enhanced ADCC against blood-stage parasites	[22]
Adaptive immune responses	
Increased Th1/Th2 ratio amongst T cells	[39]
Class-switching by B cells to cytophilic antibody isotypes	[40]
Enhanced induction of cellular (central) memory responses	[41, 42]

development. Following 3 rounds of immunization (totalling 36–45 infectious bites), sterile protection against subsequent patent sporozoite challenge was achieved in all volunteers, none of whom developed detectable blood-stage parasitemia. Interestingly, robust cellular responses were detected in all immunised volunteers, consisting of pluripotent effector memory T cells that produced IFN- $\gamma$ , in addition to TNF- $\alpha$  and IL-2 in response to pRBC in vitro, suggesting an important role for stage-transcending cellular immunity. In contrast, antibody responses against pre-erythrocytic and blood-stage antigens were detectable at low titers in only 8 of 10 and 3 of 10 volunteers, respectively [20].

Many field studies in malaria-endemic areas have sought to correlate (cellular) immune responses with protection against malaria. The most robust of these included multiple assay points and a prospective follow-up, whereas cross-sectional surveys and other single-point measurements can suffer from the commonly observed temporal variability in individual immune responses to malaria [59–61]. Furthermore, it can prove difficult sometimes to differentiate between pre-erythrocytic and blood-stage protection, particularly in clinical studies.

Initial studies measuring only plasma IFN- $\gamma$  levels in malaria patients found a correlation with protection against reinfection [62], but results from subsequent studies remained equivocal, failing [63–65] or succeeding [66–69] to demonstrate an association between clinical protection and IFN- $\gamma$  responses to selected pre-erythrocytic antigens. The explanation for these discrepancies may be related to obvious differences in setting, endemicity, age, and assay techniques. Alternatively, this outcome may represent an insufficiently measurable effect of such individual responses amidst the full spectrum of antiparasite immune reactivity. Simple ex vivo assays, representing effector (memory) responses, may be less representative of in vivo protection than cultured assays representing central memory responses, as has been shown for the vaccine candidate TRAP (ref. [70] vs. ref. [71], respectively) and circumsporozoite protein [69].

Associations between IFN- $\gamma$  responses to individual pre-erythrocytic antigens and clinical protection have also been studied in phase IIa and phase IIb malaria vaccine trials, in

particular in the context of RTS,S, a pre-erythrocytic vaccine candidate containing epitopes from the circumsporozoite protein coupled to hepatitis B surface antigen particles in a proprietary oil-in-water adjuvant [72]. In an initial phase IIa trial, prolonged IFN- $\gamma$  responses by CD4<sup>+</sup> and CD8<sup>+</sup> T cells against the circumsporozoite protein associated with protection upon experimental challenge [73]. Although in another study only a trend was seen for higher circumsporozoite protein-specific ex vivo and cultured ELISPOT IFN- $\gamma$  responses in PBMC from protected volunteers [74], a review of subsequent phase IIa trials confirmed that higher IFN- $\gamma$  ELISPOT counts were observed consistently in protected volunteers [75]. Results from phase IIb field studies have been slightly less forthcoming, but nevertheless, PBMC-cultured IFN- $\gamma$  ELISPOT responses to 1 circumsporozoite protein epitope were associated with protection in Gambian adults [69], and a trend was seen for higher CD8<sup>+</sup> IFN- $\gamma$  responses to circumsporozoite protein in protected Mozambiquan infants [76].

A phase IIa trial of liver-stage antigen-1 [77] in a similar adjuvant system induced robust IFN- $\gamma$  recall responses but unfortunately failed to induce protection against challenge in malaria-naïve volunteers. More success has been achieved with the vaccine candidate malaria epitope-TRAP, administered in the form of prime-boost regimens with attenuated vaccinia and adenoviral vectors. This vaccine has been shown to induce protection against challenge in a proportion of naïve volunteers, which correlates with IFN- $\gamma$  responses by cultured, but not ex vivo, ELISPOT [78, 79].

Thus, sufficiently strong IFN- $\gamma$  responses against selected, pre-erythrocytic antigens are associated with protection against (clinical) malaria episodes.

### Blood stage

Cellular responses, including IFN- $\gamma$ , are also important in controlling blood-stage parasites in murine models (reviewed in refs. [80, 81]). Perhaps the most clear-cut evidence for this arises from infections in IFN- $\gamma$ <sup>−/−</sup> [28, 82, 83] and IFN- $\gamma$ R<sup>−/−</sup> mice [84], who fail to control the initial wave of parasite multiplication following blood-stage challenge and succumb rapidly to hyperparasitemia. A similar failure to control parasitemia is



observed in immunocompetent animals, in which IFN- $\gamma$  is depleted during infection [85–87].

To assess blood stage-specific protection in human volunteers, Pombo and colleagues [19] infected volunteers repeatedly with submicroscopic inocula of blood-stage parasites, which were subsequently drug-cleared before they became patent. These inoculations induced strong CD4<sup>+</sup> T cell-mediated responses against pRBC, including proliferation and IFN- $\gamma$  production, but no measurable antibody responses. The volunteers were subsequently found to be protected against a similar blood-stage challenge. This landmark study was thereby the first to demonstrate a protective effect of cell-mediated immune responses against blood-stage malaria parasites in humans [19], although a concomitant effect of residual circulating antimalarial drug concentrations could not be excluded [88].

Several field studies have also assessed cellular correlates of protection against blood-stage malaria by measuring IFN- $\gamma$  production to whole parasites (i.e., live pRBC or pRBC lysate) in whole blood or PBMC assays. With the exception of 1 small retrospective study [89], other prospective studies have consistently found pRBC-induced IFN- $\gamma$  responses to be associated with reduced risk of fever and clinical malaria episodes [90–92]. Similarly, we recently found pRBC-specific IFN- $\gamma$  responses to correlate with protection against parasitemia at an ethnic and individual level [93]. Associations between protection and IFN- $\gamma$  responses to individual (vaccine candidate) blood-stage antigens are not conclusive: although most studies failed [94–99], two other studies did show such associations [66, 100].

In conclusion, broad, antiparasite IFN- $\gamma$  responses, but not necessarily responses to individual blood-stage antigens, are associated with protection against (clinical) malaria episodes.

### Evidence for IFN- $\gamma$ in inflammation and immunopathology

Clinical malaria is characterized by strong, proinflammatory responses, in particular the production of IL-1 $\beta$ , IL-6, and TNF- $\alpha$  endogenous pyrogens, which induce the disease's characteristic of high fever. Overproduction of these cytokines has also been implicated in the immunopathology underlying various forms of SM, in particular cerebral malaria [101–105]. IFN- $\gamma$  in itself is not a pyrogen but can induce downstream pyrogenic cytokines, in addition to its many other immunomodulatory functions (Table 1). IFN- $\gamma$  has been shown to be involved in many [83, 106–109] but not all [110–113] murine models of cerebral malaria, and similar discrepancies exist for other forms of severe disease in rodent models [114–116]. What then is the evidence that IFN- $\gamma$  contributes to pyrexia and more importantly, to immunopathology in human malaria? Studies in malaria-naïve volunteers have indeed indicated (temporal) correlations between IFN- $\gamma$  responses and fever during experimental infection [52, 117]. Most (but not all [118]) case-control studies have similarly measured higher plasma IFN- $\gamma$  levels in symptomatic malaria patients compared with healthy or uninfected controls [119–121]. Furthermore, ex vivo IFN- $\gamma$  responses to blood-stage exoantigens [95] but not whole

pRBC [90] have prospectively been associated with susceptibility to pyretic malaria episodes. Thus, it seems likely that IFN- $\gamma$  does indeed contribute to inflammation and fever in malaria.

However, the evidence is less clear-cut for an association between IFN- $\gamma$  responses per se (as opposed to TNF- $\alpha$  and other proinflammatory cytokines) and manifestations of SM. Whereas several case-control studies found higher plasma IFN- $\gamma$  levels in SM compared with UM patients [122–126], various similar studies have found higher plasma levels of TNF- $\alpha$  [120, 127–130], IL-2R [131], IL-6 [127, 132], IL-1 $\alpha$  [128], or IL-1 $\beta$  [130] but never IFN- $\gamma$  in SM compared with UM patients. Furthermore, plasma levels of IL-12 [130, 133–136] and IL-18 [134–136], which induce IFN- $\gamma$ , have been found to be lower in SM than UM patients. Finally, a higher proportion of children with UM registered ex vivo IFN- $\gamma$  responses to malaria antigens, although absolute cytokine concentrations did not differ between UM and SM groups [66].

Any association between IFN- $\gamma$  and specific outcomes of SM remains similarly unclear: whereas plasma levels of IFN- $\gamma$  were lower in Indian patients with cerebral malaria than in patients with other forms of SM or UM [130], no such difference was seen in Burundi [137]. Postmortem studies have identified [138] and failed to identify [139] elevated IFN- $\gamma$  in the brains of cerebral malaria victims. The largest study to date in 287 Vietnamese patients with severe disease found plasma levels of TNF- $\alpha$  and IL-6 and IL-10 but not IFN- $\gamma$  to be positively correlated with the risk of death. Amongst patients with various manifestations of severe disease, high plasma IFN- $\gamma$  levels were particularly associated with hyperparasitemia and to a lesser extent with jaundice and shock, but not with renal failure and were negatively correlated with cerebral malaria [140]. Similarly, amongst Malian children with cerebral malaria, plasma IFN- $\gamma$  levels and the prevalence of the IFN- $\gamma$  promoter polymorphism 183G/T (which increases gene transcription [141, 142]) were lower than in matched UM controls [143].

Whereas some studies have found elevated plasma levels of IFN- $\gamma$  associated with (severe) malarial anemia [144, 145], other studies found no such association [90, 146] or even an inverse relationship between IFN- $\gamma$  responses and anemia [147]. Finally, IFN- $\gamma$  has been associated with adverse pregnancy outcome [148, 149], particularly in primigravidae, but again, this has not been a universal finding [150, 151].

Some caution must be exercised when interpreting these findings, as cross-sectional immunological measurements, in particular plasma cytokine levels during infection, may represent both cause and effect of clinical presentation. Thus, although IFN- $\gamma$  responses appear to be correlated with symptomatic infection, the relationship between IFN- $\gamma$  responses and manifestations of severe disease appears to be much more complex and will require further dissection. Nevertheless, the greater part of evidence from human studies would at least suggest a negative association between IFN- $\gamma$  responses and cerebral malaria.

## HOST AND PARASITE FACTORS DETERMINE THE MAGNITUDE OF IFN- $\gamma$ RESPONSES AGAINST MALARIA

### Cellular sources of IFN- $\gamma$ against different parasite life stages

$\alpha\beta$ T cells,  $\gamma\delta$ T cells, NKT cells, and NK cells have variously been shown to produce IFN- $\gamma$  in response to *Plasmodium* parasites, although mechanisms of activation differ amongst these lymphocyte subsets, and their relative magnitude varies between parasite stages. Delineating these various pathways and their potential contribution to the total magnitude of IFN- $\gamma$  production is an important step toward understanding protective cellular immunity against malaria.

Classical "adaptive" ( $\alpha\beta$ )T cell responses are dependent on presentation of cognate antigen in the context of MHC class I or II molecules. CD8<sup>+</sup> T cells recognize parasite-infected hepatocytes in the context of MHC class I presentation [44], leading to IFN- $\gamma$  production (e.g., ref. [152]), but first require priming by cross-presenting DCs in skin-draining lymph nodes [153, 154]. CD4<sup>+</sup> T cells also recognize pre-erythrocytic antigen in MHC class II context [56, 155, 156]. In addition to  $\alpha\beta$ T cells,  $\gamma\delta$ T responses have been shown to contribute to liver-stage protection [157], and NKT cells can inhibit intrahepatic parasite development through IFN- $\gamma$  production [158]. Finally, NK cell IFN- $\gamma$  responses have been demonstrated against sporozoites [159] and parasitized hepatocytes [47, 160]. Thus, considerable redundancy appears to exist between cellular sources of IFN- $\gamma$  against pre-erythrocytic parasite stages, but the relative importance of these various lymphocyte subsets for protection in humans remains unresolved (reviewed in ref. [15]).

The cellular source of IFN- $\gamma$  responses to intraerythrocytic parasites appears at first to form an immunological blind spot. Whereas CD4<sup>+</sup> T cells may recognize malaria antigen phagocytosed and presented on MHC II molecules by professional APCs, it was generally believed that CD8<sup>+</sup> T cells cannot respond to pRBC. However, it was demonstrated recently in murine malaria models that blood-stage infection can generate parasite-specific CD8<sup>+</sup> (cytotoxic) T cell responses, following cross-presentation by DCs [161, 162]. Intriguingly, it has been suggested that such blood-stage infection-induced CD8<sup>+</sup> T cells may be involved in protection against liver-stage but not blood-stage infection [48]. Furthermore, CD4<sup>+</sup> and CD8<sup>+</sup> pRBC-responding T cell clones have been isolated from human exposed previously [163].

$\gamma\delta$ T cells too can recognize malaria antigens in the MHC class II [164, 165] or I [166] context, inducing proliferation and IFN- $\gamma$  production [167–169]. Some studies have suggested this response to be IL-2-dependent [166, 170, 171], implying a crucial accessory role for CD4<sup>+</sup> T cells. However, the  $\gamma\delta$ TCR is also capable of recognizing nonpeptide antigens directly, particularly phosphoantigens, without MHC presentation. Thus, in contrast to  $\alpha\beta$ T cells and NK cells,  $\gamma\delta$ T cells can respond to pRBC in the absence of APCs [172, 173], although supplementation with APC-derived cytokines, e.g., IL-12, can augment  $\gamma\delta$ T cell IFN- $\gamma$  production further (reviewed in ref. [174]).

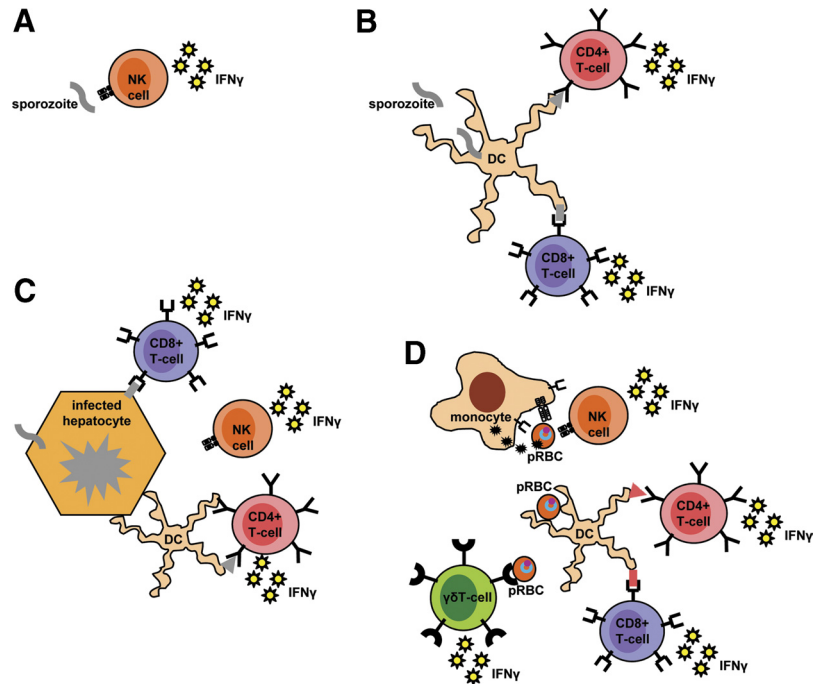
NK cells are considered innate lymphocytes as the first line of defense (reviewed in ref. [175]) and potent early producers of IFN- $\gamma$  in response to pRBC in vitro, for which they are dependent on IL-12 and IL-18 [176]. Myeloid APCs presumably form the source of the required IL-12 and IL-18, as the presence of these cells is required for IFN- $\gamma$  response by NK cells in vitro. Exactly which APC subsets are required remains somewhat unclear: some authors report that only monocytes suffice [177], whereas others also demonstrate this ability in mDCs [178]. The latter group further demonstrated the additional requirement of contact-dependent signals between APCs and NK cells [178]. Besides these accessory signals, NK cells must make direct cell contact with pRBC for an IFN- $\gamma$  response [179, 180]. Intriguingly, heterogeneity in killer cell Ig-like receptors appears to correlate with NK IFN- $\gamma$  responses to pRBC [179, 181], although this does not necessarily mean that these receptors interact directly with pRBC, which lack MHC class I molecules. More recently, it was also proposed that NK cells recognize the parasite surface protein *P. falciparum* erythrocyte membrane protein-1 via another NKR, Nkp30 [182]. Finally, IFN- $\gamma$  induction by pRBCs in NK cells is also IL-2-dependent [178] but needs additional helper signals from CD3<sup>+</sup> T cells (unpublished results). An overview of the various cellular pathways to IFN- $\gamma$  against different parasite stages is provided in Fig. 2.

The relative contribution of these different lymphocyte subsets to total IFN- $\gamma$  production in response to pRBC needs to be explored further, however. In in vitro stimulation experiments with PBMC from malaria-naïve donors, the majority of IFN- $\gamma$ -producing cells has, respectively, been identified as NK cells [176, 177],  $\alpha\beta$ T cells [171, 185], and  $\gamma\delta$ T cells [168], including intriguing NK-like  $\gamma\delta$ T cells [169]. Whether these inconsistencies represent differences between donors or in experimental setups is not fully clear, although it is evident that in most donors, all three subsets do contribute to the total response. Less still is known about in vivo sources of IFN- $\gamma$  during malaria infection in humans or in recall responses from previously exposed donors. Plasma samples taken from malaria-naïve volunteers undergoing experimental malaria infection revealed soluble granzyme induction in addition to IFN- $\gamma$  and other cytokines, suggesting an early role of NK cells [186], and during and following infection, not only  $\alpha\beta$ T cells but also  $\gamma\delta$ T cells and NK cells contributed to the overall increase in in vitro IFN- $\gamma$  responses against pRBC (unpublished results). In a naturally exposed, healthy pediatric population, the majority of IFN- $\gamma$ -producing lymphocytes was NK-like  $\gamma\delta$ T cells [91], and more data will be required to identify the different cell sources of IFN- $\gamma$  in (non-)immune malaria patients.

### Dynamics of IFN- $\gamma$ responses in relation to exposure

A second factor affecting the magnitude of IFN- $\gamma$  responses against malaria is their modulation in relation to exposure. Low-level IFN- $\gamma$  responses to pRBC have been demonstrated repeatedly in malaria-naïve donors [169, 176, 187–190] and have variously been ascribed to innate responses, nonspecific polyclonal responses to superantigens, or cross-reactive responses by T cells primed by environmental antigens (discussed in ref. [191]). In contrast, recall responses are mark-

**Figure 2. Induction and cellular sources of IFN- $\gamma$  against various malaria parasite stages.** (A) NK cells recognize free sporozoites directly [159]. (B) In skin-draining lymph nodes, sporozoites invade or are taken up by DCs, which in turn, prime CD4 $^{+}$  T cells (presentation on MHC-II) [156] and CD8 $^{+}$  T cells (cross-presentation on MHC-I) [153, 154]. (C) Primed CD8 $^{+}$  T cells recognize infected hepatocytes directly [44, 152]; primed CD4 $^{+}$  T cells respond to antigen presented by local APCs [56, 155, 156]. NK cells are activated in a bystander manner [47, 160]. (D) In blood or spleen,  $\gamma\delta$ T cells recognize pRBC-phosphorylated antigens directly without the need for APC presentation [172, 173]. APCs (monocytes and/or mDCs) recognize pRBC ligands through PRRs (e.g., hemozoin [183] and/or associated parasite DNA [184] via TLR-9). CD4 $^{+}$  and CD8 $^{+}$  recognize pRBC antigens presented by DCs in, respectively, MHC-II [163] and (cross-presented) MHC-I context [161, 162]. NK cells recognize pRBC directly [179] but require help from APCs and probably also T cells for full activation [177, 178].



edly increased in malaria patients following a first clinical episode [192–195]. Indeed, even subclinical infections are sufficient to induce robust IFN- $\gamma$  responses to pRBC in previously naïve donors [19, 20, 42].

In general, immune responses to malaria are commonly believed to be short-lived following exposure, based mainly on the short half-life of specific antibodies [196–198] (reviewed in ref. [199]), an explanation that is often offered for the slow development of immunity. It would appear that IFN- $\gamma$  responses to individual antigens are indeed relatively short-lived, i.e., declining within a few years of exposure [200, 201], or at least unstable [59–61]. However, even before the characterization of IFN- $\gamma$ , Wyler and Oppenheim [192] demonstrated that cellular, proliferative responses to a crude, whole parasite antigen could be detected in donors up to 15 years following a single malaria infection. More recently, Todryk et al. [42] found undiminished IFN- $\gamma$  effector responses at 3 months postinfection in previously naïve volunteers, and data from our own laboratory suggest such IFN- $\gamma$  recall responses to whole pRBC remain practically undiminished at least 14 months postinfection (unpublished results). Thus, although responses to individual antigens or epitopes may be unstable, possibly representing in vivo fluctuations in individual T cell clones, the total IFN- $\gamma$  response to pRBC can remain remarkably long-lived.

It comes somewhat of a surprise therefore to find that adult residents of highly endemic regions produce markedly lower IFN- $\gamma$  responses against pRBC than residents of low-endemic regions or indeed even nonexposed donors [194, 202] and that plasma IFN- $\gamma$  levels during clinical malaria episodes are relatively lower in semi-immune rather than in nonimmune patients [118, 119]. Depressed responses in highly exposed individuals can be rescued by supplementation of exogenous

IL-2 [203]. Furthermore, these defective responses appear to be antigen-specific [194, 202], suggesting clonal elimination or specific suppression by regulatory T cells [204, 205]. In either case, down-regulation of proinflammatory responses has been proposed to be a beneficial adaptation by the host to avoid immunopathology as a result of repeated or chronic malaria infections [206], although as we have seen that there is limited evidence to support this hypothesis with regard to IFN- $\gamma$  in humans.

### Modulation of IFN- $\gamma$ responses by the parasite

Various mechanisms by which malaria parasites may actively suppress cellular immune responses have been reviewed elsewhere [206, 207]. Suppression of (protective) proinflammatory responses may be an active strategy pursued by malaria parasites to prolong their own survival in the host. Indeed, suppression of proliferation (e.g., refs. [208, 209]) and IFN- $\gamma$  production [193, 210] during clinical malaria episodes is a common, although not universal [211], finding. Evidence that repeated or chronic parasitemia also suppress IFN- $\gamma$  responses has arisen from longitudinal field studies [59] and long-term chemoprophylaxis studies [212]. Thus, it appears that down-regulation of cellular responses in general and IFN- $\gamma$ , in particular, is not only an active strategy pursued by the parasite but also that this strategy is so central to its survival that it has evolved multiple mechanisms by which to achieve it.

### IMPLICATIONS FOR VACCINE DEVELOPMENT

Given this evidence for the protective effect of IFN- $\gamma$  against parasitemia, developing a malaria vaccine aimed at reproduc-



ing such IFN- $\gamma$  responses would appear to be desirable. Whether "the stronger, the better" in terms of IFN- $\gamma$  responses should be the ultimate goal of malaria vaccine development must eventually depend on a more precise understanding of the complex relationship between IFN- $\gamma$  and manifestations of severe disease. Nevertheless, in designing any such vaccine strategy, a number of lessons can be drawn from what we understand currently about the factors that determine the magnitude of IFN- $\gamma$  responses against malaria.

### Induce multiple cellular pathways and a broad IFN- $\gamma$ response

In addition to traditional  $\alpha\beta$ T cells, NK cells and  $\gamma\delta$ T cells form important tappable sources of IFN- $\gamma$ . Their IFN- $\gamma$  response against malaria parasites is induced through distinct pathways that can be exploited in vaccine design, i.e., by the inclusion of phosphoantigens to activate  $\gamma\delta$ T, or whole parasites to activate IFN- $\gamma$  production by NK cells (see Cellular sources of IFN- $\gamma$  against different parasite life stages above). Although generally considered "innate" lymphocytes, we (unpublished results) and others [213, 214] have clearly demonstrated "memory-like" patterns in the IFN- $\gamma$  responses of these cells, supporting their rational inclusion in vaccine design. Exploiting such alternative cellular pathways furthermore bypasses parasite-mediated inhibition of IFN- $\gamma$  responses in  $\alpha\beta$ T cells.

The problem of short-lived or IFN- $\gamma$  erratic responses to individual antigens can be overcome partially by inducing a broader response, e.g., by whole parasite-based vaccines. These expose the host's immune system to the full palette of parasite antigens, ideally also inducing IFN- $\gamma$  against multiple life stages. Furthermore, whole parasites contain a "built-in adjuvant", augmenting overall IFN- $\gamma$  responses further [183]. Indeed whole parasite-based vaccine approaches induce robust IFN- $\gamma$  responses in humans [19, 20, 53] and have generally proven more successful than subunit vaccines [215–218].

### Prevent exposure-mediated suppression of IFN- $\gamma$ responses

As suppression of IFN- $\gamma$  responses in relation to exposure does not seem to serve the host but rather appears solely a survival strategy by the parasite, the question arises whether/how such suppression can be avoided in the context of vaccine-induced IFN- $\gamma$  responses. In other words, does suppression of IFN- $\gamma$  responses automatically follow from repeated exposure, and can we design strategies to bypass it? Further field studies addressing the mechanism(s) underlying immune suppression in relation to exposure will be necessary but are complicated by the fact that in endemic settings, the effect of exposure cannot be distinguished readily from the effect of age [219]. Infants' and children's immune systems function differently from adults', quantitatively and qualitatively (e.g., refs. [220, 221]), and it could be hypothesized that the basis for life-long, suppressed IFN- $\gamma$  responses against malaria is laid in the immature immune systems of infants in highly endemic areas [222]. However, only little is understood about the effect of age on (cellular) immune responses to malaria from rodent models

[223–225], and although IFN- $\gamma$  responses against malaria in human children growing up in endemic areas tend to be weaker than in adults [61, 68, 226–228], the effect of prior exposure in these studies is again hard to distinguish from that of age per se.

One potential approach to answering these related questions would be to study IFN- $\gamma$  responses in people who become highly exposed to malaria only later in childhood or in adulthood, e.g., transmigrants, as has been performed for humoral responses in Javanese transmigrants to Irian Jaya [229–231] or in settings of epidemic or resurgent malaria such as Madagascar [232].

## CONCLUDING REMARKS

In this review, we have shown how IFN- $\gamma$  forms a critical component of immune responses against pre-erythrocytic and blood-stage malaria parasites. A wealth of evidence supports its protective efficacy against clinical malaria episodes, whereas the evidence associating IFN- $\gamma$  responses with immunopathology remains equivocal and will require further investigation. In the meantime, striving for strong and long-lasting IFN- $\gamma$  production appears justified as a malaria vaccine strategy, and to achieve this, such vaccines should be designed to induce a broad response via multiple cellular pathways. Additionally, the mechanism by which repeated exposure leads to suppression of such responses needs to be resolved.

## AUTHORSHIP

M.B.B.M. and R.W.S. wrote the paper.

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## KEY WORDS:

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