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Review Article

Regulating immunity to malaria

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SUMMARY

The optimal outcome of a malaria infection is that parasitized cells are killed and degraded without inducing significant pathology. Since much of the pathology of malaria infection can be immune-mediated, this implies that immune responses have to be carefully regulated. The mechanisms by which anti-malarial immune responses are believed to be regulated were discussed at the recent Malaria Immunology Workshop (Bloomberg School of Public Health, Johns Hopkins University, Baltimore, Maryland, USA; February 2005). Potential regulatory mechanisms include regulatory T cells, which have been shown to significantly modify cellular immune responses to various protozoan infections, including leishmania and malaria; neutralising antibodies to pro-inflammatory malarial toxins such as glycosylphosphatidylinositol and haemozoin; and self-regulating networks of effector molecules. Innate and adaptive immune responses are further moderated by the broader immunological environment, which is influenced by both the genetic background of the host and by co-infection with other pathogens. A detailed understanding of the interplay between these different immunoregulatory processes may facilitate the rationale design of vaccines and novel therapeutics.

Keywords *anti-toxic immunity, glycosylphosphatidylinositol, IL-10, nitric oxide, regulatory T cell, TGF-beta*

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INTRODUCTION

Malaria infection has a very variable clinical phenotype, ranging from a mild febrile illness to life-threatening severe anaemia, acidosis and end-organ failure, even among individuals with little or no acquired anti-malarial immunity. In part this is explained by heritable differences in susceptibility to malaria infection or parasite proliferation known to be governed, amongst others, by erythrocyte and haemoglobin polymorphisms (1). Recent data have also begun to suggest that there may be differences in virulence between parasite genotypes which manifest as variable *in vivo* growth rates (2,3), with those that grow most rapidly being associated with severe disease outcomes. However, it is also apparent that quantitative and qualitative differences in the nature of the anti-malarial immune response have profound effects on disease progression and eventual outcome. Some of these differences may turn out also to have a heritable basis (polymorphisms in a number of immune response genes have already been tentatively linked to susceptibility to malaria in humans and in rodent models (4)), but it is also the case that environmental effects – related to the frequency and intensity of malaria infection; co-infection with viruses, bacteria and other parasites; nutritional status and so on – may also substantially modify the immune response and thus the clinical outcome of malaria infection.

The unifying theme that is emerging from studies of all these potential immune modifiers is that the optimal immune response to malaria infection is characterized by early, intense, pro-inflammatory cytokine-mediated, effector mechanisms that kill or clear parasite-infected cells and which are then equally rapidly suppressed by anti-inflammatory effectors once parasite replication has been brought under control (reviewed by (5)); clearance of remaining parasites and prevention of recrudescence or reinfection appears to be primarily antibody-mediated (6). Some pro-inflammatory

responses, however, fail to clear parasites, and when excessive can cause pathology and fatalities. The successful resolution of a malaria infection may thus require a coordinated progression from one type of immune response to another, and anything that upsets either the timing or the balance of this progression can lead to chronic and/or severe infection. In other words, the regulation of anti-malarial immune responses is as important as their induction in determining the final outcome of infection. This article reviews some of the current ideas about how immune responses to infection might be regulated, and how this might lead to establishment of optimal host–parasite interactions, and summarizes the presentations and discussions on the topic at the Malaria Immunology Workshop, Bloomberg School of Public Health, Baltimore, Maryland (February 8–11, 2005).

REGULATORY T CELLS AS AGENTS OF IMMUNE TOLERANCE

Malaria is similar to many – or even most – other infections in that the immune effector mechanisms that are required to eliminate it are also inherently damaging to host tissues. The immune system has evolved a variety of mechanisms – collectively known as immune tolerance – to allow potentially pathogenic microorganisms to be controlled while avoiding or minimizing host damage. The term tolerance, although bedevilled with many different and sometimes very restrictive definitions over the years, should properly be applied to any endogenous mechanism by which a potentially injurious immune response is prevented, suppressed, or shifted to a non-injurious class of immune response. Tolerance is no longer viewed as simple deletion or absence of autoreactive cells, although thymic negative selection is clearly an essential tool for reducing the peripheral population of potentially autoreactive T cells; it is now appreciated that functional tolerance can involve ignorance, anergy, deletion of antigen-specific T cells, immune deviation and active cellular regulation (suppression) (reviewed by (7,8)). Local micro-environments are now known to play a key role in maintaining tolerance, such that immune responses can differ between tissues in intensity and character. Importantly, understanding how immune responses are regulated by various tolerogenic mechanisms may reveal strategies of immune manipulation for alleviating autoimmune diseases, cancer and transplant rejection as well as infectious diseases.

Immune suppression can be viewed as one mechanism for maintenance of tolerance and it is now recognized that populations of regulatory T lymphocytes (the modern reincarnation of 'suppressor' cells) are key agents of immuno-regulation (9). Regulatory T cells may be either naturally produced and widely dispersed at sites of antigenic challenge (endogenous or natural regulatory cells; Treg) (10,11), or induced in response to a specific insult (Tr1, Th3 and CD8+ Treg cells) (12). The potential of T cells to regulate each other is clear from the demonstration that when T cells from a normal, healthy mouse are depleted of a subpopulation of CD4+ T cells, that are CD25+ (IL-2Rα) in the resting state and constitutively express the transcription factor Foxp3, and transferred into athymic mice, the recipients rapidly develop autoimmune diseases including inflammatory bowel disease, gastritis, inflammation of the salivary glands (Sjogren's disease), thyroiditis, diabetes and others (8). These endogenous Treg cells, which develop in the thymus and continue to be released into the periphery, are believed to be a central component of peripheral tolerance, limiting the damage caused by autoreactive T cells which evade thymic selection. Treg comprise 5–10% of CD4 T cells and although they are in some respects anergic to TCR engagement – in many cases, failing to proliferate for example – signalling via the TCR is required for them to manifest their suppressive effects.

Treg-mediated suppression of T cell activity – which includes inhibition of lymphoproliferation by inhibition of IL-2 mRNA expression and arresting cells at the G1-S phase of the cell cycle, cytotoxicity and cytokine release – requires direct contact with the target cell (at least, this is the case *in vitro*) and is not antigen specific (i.e. once the Treg cell has been activated by TCR ligation it can suppress cells of any TCR specificity) (13). A number of potential mediators of Treg activity have been identified, including glucocorticoid-induced TNF-R (GITR), cytotoxic T lymphocyte Ag-4 (CTLA-4), Foxp3, TLR4, CD25 and the anti-inflammatory cytokines IL-10 and TGF-β (8,9,11,14–18). Although soluble cytokines are, at first sight, unlikely mediators of contact-dependent suppression, absence of TGF-β or disruption of TGF-β signalling leads to chronic inflammation and autoimmune disease; TGF-β null, dnTβRll transgenic and SMAD3 null mice all exhibit constitutive T cell and macrophage activation, dysregulated apoptosis, multifocal organ inflammation and lethal autoimmune-like disease (19). These findings can now be reconciled, following the demonstration that CD4+ CD25+ Treg express both TGF-β and TβRII on their cell surface which is up-regulated after TCR ligation and that CD3/TCR stimulation induces TβRII expression on CD4+ CD25– responder T cells which then become responsive to TGF-β signalling (19). Furthermore, CD4+ CD25+ Treg induce phosphorylation of SMAD2/3 in responder T cells (indicative of TGF-β signalling via TβRII) and anti-TGF-β reverses Treg-mediated immuno-suppression (20). Thus, a model has been developed in which responder cells become susceptible to inhibition via up-regulation of cell surface TβRII, which binds TGF-β on the Treg surface; this model is consistent with Treg being anergic, mediating suppression by cell–cell contact, requiring TCR stimulation (to increase TGF-β and TβRII expression)

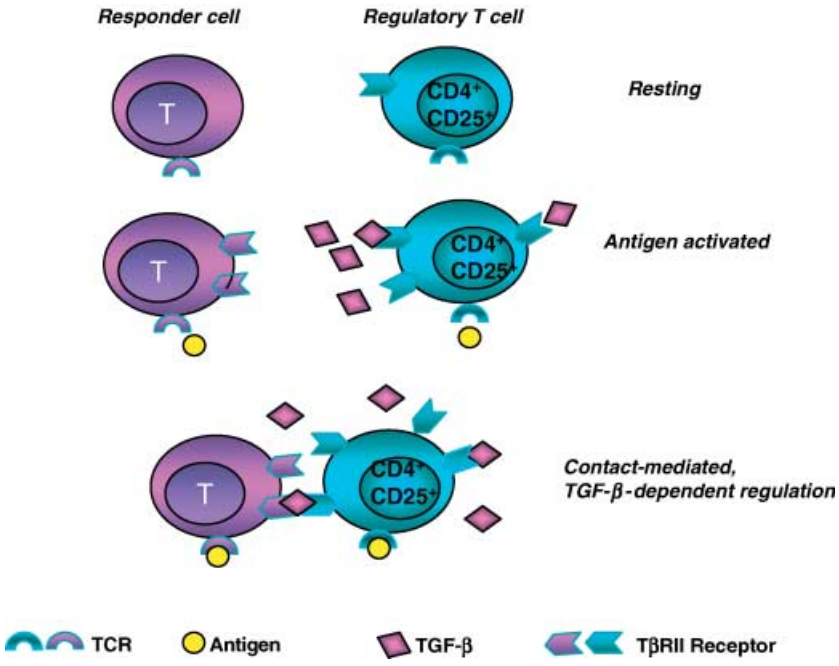

Figure 1 Theoretical model for contact-mediated and TGF-β-dependent regulation of T cell function by regulatory T cells. Engagement of the TCR by antigen leads to expression of TGF-β receptors on CD4+ and CD8+ effector T cells and also up-regulates TGF-β receptor expression on regulatory T cells (Treg). TGF-β, either secreted by the Treg, or available from other cellular sources, binds to the Treg and forms a bridge between it and the effector cell, sending a negative signal into the effector cell which shuts down effector functions..

and being antigen non-specific in their effects (Figure 1). This has subsequently revealed potential roles for GITR and CTLA-4 as 'regulators of the regulators', with GITR inhibiting the TGF-β signal, leading to loss of suppression, increased inflammation and autoimmunity, whilst CTLA-4 induces TGF-β expression in Treg, boosting suppression, increasing tolerance and reducing autoimmunity (21). It is not clear however, that TGF-β is an essential mediator of Treg activity in all situations, as in some systems TGF-β is not required for Treg suppressor function (22,23). Furthermore, inhibition of IL-2 production can be a major mechanism underlying the suppressor activity of Tregs (24).

The finding that mutations in the Foxp3 (scurfin; Forkhead/winged helix transcription factor) gene lead to autoimmune and inflammatory syndromes in mice (the lethal Scurfy phenotype (25)) and in humans (IPEX/XLAAD Syndrome (26,27)) raised the suspicion that this gene might also be involved in immune regulation. The role of Foxp3 as the key regulatory gene for development and function of Treg was demonstrated by showing that it is uniquely expressed in CD4+ CD25+ Treg and not in CD4+ CD25– responder T cells nor CD4+ CD25+ activated T cells, and that gene transfer of Foxp3 converts naïve T cells into Treg (16,18,28). Importantly, it has recently been shown that inducible regulatory T cells also express Foxp3 and that co-culture of CD4+ CD25– responder cells with cognate antigen (or anti-CD3 and anti-CD28) in the presence of TGF-β1 leads to up-regulation of Foxp3 and acquisition of a typical regulatory cell phenotype, including anergy and the ability to suppress responder T cells (29). Inducible regulatory cells differentiate from antigen-specific, CD25– and Foxp3– cells during an immune response, acquiring Foxp3 expression in the process, and mediate their effects by release of IL-10 (Tr1 and CD8+ Treg) or TGF-β (Th3 and CD8+ Treg) (8,9,11). The notion that TGF-β converts peripheral T cells to CD4+ CD25+ regulatory T cells is consistent with previous evidence that TGF-β induces anergy in peripheral CD4+ Th1 and Th2 cells and that TGF-β is a physiological inducer of Foxp3 (29).

The potential of TGF-β as a tool to induce regulatory T cells for therapeutic intervention has been demonstrated in a murine allergy model where transfer of TGF-β-converted regulatory T cells inhibited house-dust mite-induced lung pathology, suppressing inflammation, Th2 cytokine (IL-13, IL-4) production, leucocyte infiltration, mucus metaplasia, airway obstruction and airway hyper-reactivity (29). But, this raises another important question – in the context of infection, are regulatory cells good for the host (preventing pathology) and/or can they be exploited as a mechanism for immune escape by the pathogen?

A ROLE FOR REGULATORY T CELLS IN MODULATING IMMUNE RESPONSES TO INFECTION: LESSONS FROM LEISHMANIASIS

Evidence is emerging from a number of infectious disease models that regulatory T cells can suppress pathogen-specific T cells *in vivo*; the net result of this interaction appears to be reduction of immune pathology at the expense of failure of pathogen elimination. That is, by down-regulating the effector

T cell response, regulatory T cells facilitate chronic infection. For example, Treg suppress CD8 T cell activation by *Listeria monocytogenes* (30) and CD8 T cell responses to *Herpes simplex* virus (31); they also suppress effector responses to *Helicobacter hepaticus* (32). Of particular relevance in terms of immunity to protozoa, regulatory T cells suppress the CD4+ CD25– effector T cell response to *Leishmania major*.

Parasite persistence following clinical cure is typical of Leishmania infection and can give rise to recrudescent infection in immunocompromised individuals (33). Acquired resistance involves induction of a polarized Th1 response which is IL-12 driven, CD40/CD40L dependent, and requires activation of CD4+ and CD8+ T cells to secrete high levels of IFN-γ (34). Parasite killing can be accompanied by significant tissue damage and scarring but healed lesions contain significant numbers of infectious organisms, serving as a reservoir of infection for the sand fly vector (35). CD4+ T cells in the chronic infection site comprise both IFN-γ secreting and IL-10 secreting populations and sterile immunity – demonstrated by absence of parasite reactivation in mice receiving neutralizing antibody to IFN-γ (36) – can be obtained in IL-10 deficient (IL-10–/–) mice and in mice treated with a neutralizing antibody to IL-10R (37), demonstrating an essential role for IL-10 in facilitating parasite persistence. IL-10 producing cells in the chronic lesion were shown to be CD4+ CD25+, whereas IFN-γ-producing cells were CD4+ CD25– (38). When the two populations of CD4+ cells isolated from a chronic Leishmania lesion were sorted and adoptively transferred to rag1–/– mice, the CD25+ population was not only unable to confer anti-parasite immunity but, when transferred in a 1 : 10 ratio with CD25– cells, was able to reverse the protection conferred by the CD25– population (38). The effect of the CD25+ cells was IL-10-dependent, since CD25+ cells derived from lesions in IL-10–/– mice were unable to inhibit the protective effect of transferred CD25– cells.

These data from *L. major* infections in mice suggest that an imbalance in the numbers or activities of Leishmania-specific regulatory and effector cells might account for the more severe forms of human leishmaniasis, such as non-healing cutaneous leishmaniasis, visceral leishmaniasis and reactivation of latent infections. The murine model of non-healing cutaneous leishmaniasis (*L. major* in BALB/c mice) was originally explained as an imbalance of induction of Th1 to Th2 cells, characterized by a lack of IFN-γ and sustained production of IL-4 (35), but there is little evidence to link non-healing lesions in humans with Th2 immune responses: IFN-γ producing cells and IFN-γ mRNA remain readily detectable in patients with kala-azar, post-kala-azar dermal leishmaniasis (PKDL) and chronic forms of cutaneous leishmaniasis, and the opposing cytokine most commonly found in these clinical settings is not IL-4, but IL-10 (39,40). Interestingly, a strain of *L. major* (Sd) isolated from a patient with non-healing lesions is able to induce non-healing cutaneous lesions in typically resistant C57BL/6 mice; despite the presence of large numbers of IFN-γ-secreting CD4+ and CD8+ cells in the lesion, parasite growth is not contained and tissue damage is severe. Significantly, mRNA for both IL-10 and Foxp3 is highly expressed in these non-healing lesions and IL-10–/– mice or mice treated with anti-IL-10R or anti-CD25 antibody are able to resolve Sd infections as effectively as less virulent strains of *L. major* (41).

Taking all these data together it is clear that naturally occurring regulatory T cells can facilitate persistence of *Leishmania major* in healed lesions, at least in mice. Interestingly, a strong Treg/IL-10 response induced by primary infection does not prevent the expression of immunity in a secondary challenge site, offering a very plausible explanation for the phenomenon of concomitant immunity. Furthermore, immunity to rechallenge cannot be sustained in IL-10–/– or anti-IL-10R treated mice, indicating that parasite persistence may be required to maintain protective immunity, the phenomenon known as premunition (42). This appears to indicate a highly evolved trade-off which benefits both the host (preventing reinfection and limiting immune-mediated tissue damage) and the parasite (allowing establishment of reservoirs of infection for onward transmission to naïve hosts). The implications of these findings for immunotherapy of human leishmaniasis are now beginning to be explored. Of immediate relevance is the possibility that regulatory T cells facilitate immunization against *L. major* by the process of leishmanization, in which low dose immunization with viable *L. major* promastigotes confers protection against subsequent natural infection (35).

EVIDENCE FOR A ROLE FOR REGULATORY T CELLS IN MALARIA INFECTION

Infection with malaria parasites has long been known to be persistent and associated with 'suppressed' immune responses to the parasite and to unrelated antigens (43). Furthermore, maintenance of acquired immunity to malaria depends upon periodic reinfection or chronic, low level parasitism, i.e. premunition (44). Given the delicate balancing act to be achieved between control of overwhelming infection and prevention of immunopathology (5), it is very tempting to invoke a role for regulatory T cells. Indeed, evidence has been accumulating for more than a decade to suggest that T cells, or at least T cell-derived cytokines, play an important role in regulating immunity to malaria; re-examination of these data in the context of regulatory T cells suggests that these cells are central to regulation of immune-mediated pathology.

In a recent series of studies, essential roles have been demonstrated for both IL-10 and TGF-β in the regulation

of type-1 responses during rodent malaria infections. IL-10 was first shown to protect against fatal Th1-driven pathogenesis in *Plasmodium berghei* infections, because *in vivo* neutralization of IL-10 promotes pathogenesis and fatalities in disease resistant mice and administration of recombinant IL-10 prevented fatalities in otherwise susceptible animals (45). Similarly, IL-10–/– mice show enhanced pathology as a result of infection with *P. chabaudi chabaudi* (46), and this was subsequently shown to be mediated by excess production of pro-inflammatory cytokines, including TNF-α (47). Similarly, normally non-lethal *P. chabaudi* infections are universally fatal in BALB/c mice receiving a neutralizing antibody to TGF-β, whilst administration of recombinant TGF-γ reduces the mortality from a normally lethal *P. berghei* infection; manipulation of TGF-β is accompanied by reciprocal changes in circulating IFN-γ and TNF-α concentrations (48). In C57BL/6 *P. chabaudi*-infected mice, neutralization of TGF-β in IL-10–/– mice is universally fatal (47). Importantly, the source of TGF-β and the timing of its production seem to be crucial. Thus, rapid (< 24 h pi) induction of bioactive TGF-β is associated with failure to develop a type-1 response and failure to control parasite replication in C57BL/6 mice infected with lethal *P. yoelii* (YM/17XL); neutralization of TGF-β and IL-10 in this model significantly increases type-1 cytokine production and enhances survival (49). Conversely, the closely related non-lethal parasite (*P. yoelii* 17X) fails to induce an early TGF-β response and parasitaemia is effectively controlled by a type-1 immune response; TGF-β is up-regulated only once parasitaemia has been brought under control and at this time CD25+ T cells from spleens of *P. yoelii* 17X-infected mice secrete TGF-β in response to parasite antigens *in vitro* (49).

Taken together, these observations suggest that activation of regulatory T cells may profoundly influence the outcome of malaria infection. Indirect evidence for a role for regulatory cells came from a study in which blockade of CTLA-4, which on binding to CD28 delivers a negative signal to the T cell and is a marker for some populations of regulatory T cells, exacerbated *P. berghei*-mediated immunopathology (50,51). Direct support for this hypothesis comes from two studies in which mice were depleted of CD4+ CD25+ T cells prior to lethal *P. berghei* (52) or *P. yoelii* 17XL infection (53). In naïve mice infected with *P. berghei* NK65, depletion of CD4+ CD25+ T cells led to a modest but statistically significant delay in parasite growth (suggesting that effector responses are enhanced in the absence of T reg) but had no reported effect on mortality (52). By contrast, after depletion of CD4+ CD25+ T cells in mice infected with *P. yoelii* 17XL, T cell responses to parasite antigens were potentiated and the normally lethal infection was controlled.

It is not clear why *P. yoelii* 17XL triggers such potent regulatory T cell activity, but one intriguing explanation is that it is directly linked to induction and activation of TGF-β. Thrombospondin-like molecules and metalloproteases present in extracts of *P. yoelii* 17XL are able to activate latent TGF-β to its bioactive form (54), raising the possibility that – as described above – priming of malaria-specific T cells in the presence of TGF-β might up-regulate Foxp3, leading to the development of regulatory T cells. Activation of constitutively produced latent TGF-β by malaria parasites thus represents a potential immune evasion mechanism and may be a component of parasite virulence. Importantly, immunization of mice against lethal *P. yoelii* 17XL is associated with reduced early TGF-β activation (49), indicating that the parasite-derived molecules responsible for TGF-β activation may be suitable targets for anti-malarial vaccines.

The divergent observations in different rodent malaria infections point to the complex role of pro-inflammatory and counter-regulatory responses in protective immunity and immuno-pathogenesis. However, the data are highly consistent with the notion that the outcome of malaria infection depends on a delicate balance between production of pro-inflammatory and anti-inflammatory cytokines, with the timing, source (and site?) of anti-inflammatory cytokine secretion being crucial. Optimally, it seems, IL-10 and/or TGF-β production needs to be triggered in an antigen-specific manner at the time of peak parasitaemia, allowing the effects of type-1 cytokines to be controlled once they have themselves brought parasite replication under control. Emerging evidence from clinical studies suggests that this model holds true in human malaria infections. Thus, clinical immunity to malaria is accompanied by marked down-regulation of IFN-γ responses to iRBC (55), disease severity is affected by the balance of pro- and anti-inflammatory cytokines (56–59), plasma TGF-β is low during acute *P. falciparum* infection (57) and a relative deficiency of plasma IL-10 to TNF-α predisposes to severe anaemia (60,61). In a prospective study, high ratios of PHA-induced IL-12, IFN-γ or TNF-α to TGF-β and IL-10 were associated with reduced risk of malaria infection but increased risk of non-malarial fever (62), indicative of an antagonistic relationship between pro-inflammatory and anti-inflammatory cytokines. Most recently, in human volunteers experimentally infected with *P. falciparum* via the bites of infected mosquitoes in the context of malaria vaccine trials, systemic activation of TGF-β at the time of emergence of blood-stage parasites from the liver has been correlated with up-regulation of Foxp3 and more rapid parasite growth. Furthermore, depletion of CD25+ cells from peripheral blood mononuclear cells of infected individuals specifically enhanced proliferative and IFN-γ responses to *P. falciparum* antigens (Walther *et al.* submitted for publication). As in lethal *P. yoelii* infections in mice, this is consistent with a model in which parasite-mediated activation of latent TGF-β induces differentiation

of malaria-specific regulatory T cells, which then modulate the developing Th1 response. In summary, as for Leishmania infection, it seems that regulatory T cells might compromise clearance of malaria-infected red blood cells, but also limit infection-induced pathology. Whether this is ultimately of benefit to the host, the parasite or both remains to be seen.

ALTERNATIVE MODELS FOR REGULATION OF PRO-INFLAMMATORY IMMUNE RESPONSES TO MALARIA: ANTI-TOXIC IMMUNITY TO *P. FALCIPARUM* MALARIA

As discussed above, many of the well-known manifestations of severe malaria are side-effects of the powerful inflammatory cytokine response that contributes to killing parasites. Parasite exoantigens are strongly implicated in the initiation of this cytokine cascade (of which TNF-α is a signature cytokine) and it has long been proposed that these antigens may be suitable targets for disease-attenuating vaccines (63,64). The most well-defined of these parasite toxins is glycosylphosphatidylinositol (GPI) (65–69) and anti-GPI immunization in a mouse model of severe malaria clearly demonstrated proof-of-principle for such an approach (70). However, functional aspects of naturally acquired anti-GPI antibodies still need to be investigated further.

Anti-GPI antibodies develop in humans following natural infection with *Plasmodium falciparum* and in a study in children in Western Kenya a significant association was found between the presence of these antibodies and reduced risk of severe anaemia (71). Subsequent cross-sectional studies reported no association between presence of anti-GPI antibodies and protection against mild clinical malaria (72), severe malaria (73) or maternal immunity (74); similarly, a prospective study also found no association between anti-GPI antibodies and resistance to clinical malaria (73). Conversely, in a recent study from Senegal, anti-GPI antibody concentrations were significantly lower in cerebral malaria patients than in those with mild malaria (although antibody levels did not differ between those who died and those who survived) (75), and anti-GPI antibodies were associated with markedly reduced risk (Odds Ratio of 4·0) of symptomatic infection in children of Javanese migrants to Papua (76). Plausible explanations for the variable results of epidemiological studies are several. Firstly, anti-GPI antibodies are slow to develop in infants and young children, and the short-lived nature of the antibody response suggests that frequent boosting is required (77), therefore antibody titres at the time of infection may not be sufficiently high to protect the most vulnerable individuals, especially in areas of sporadic or seasonal malaria transmission, as discussed previously (73). Secondly, cross-sectional designs limit the power of these studies, because rapid boosting of the antibody response during infection may mask the fact that antibody levels were in fact much lower at the time of infection. Longitudinal study designs with repeated antibody measurements are required to properly address many relevant questions. Alternatively, anti-GPI antibodies may comprise a heterogeneous population with varying capacity to mediate anti-toxic immunity. To investigate the latter possibility, de Souza *et al*. have used a toxin-neutralization assay to assess the ability of sera from *P. falciparum*-immune or acutely infected individuals to block parasite-induced TNF-α secretion from macrophages *in vitro*. Preliminary data indicate that only sera with very high anti-GPI titres were able to block parasite-induced TNF-α induction *in vitro*, indicating that antibody concentration and antibody avidity are crucial determinants of effector function. Interestingly, some sera with little or no anti-GPI activity very efficiently inhibited TNF-α induction, suggesting that GPI may not be the only toxic component of malaria parasites and that other parasite-derived toxins may also be involved in the induction of TNF-α (de Souza *et al*. submitted for publication). Thirdly, some of the protocols used to measure anti-GPI antibody levels may be suboptimal. For example, the use of detergents in ELISA wash buffers – which is necessary to remove non-specifically bound proteins – may lead to uncontrolled loss of unconjugated free GPI from 96-well plates, with resulting loss of sensitivity and specificity of the assays. There is also continued debate in the field about the most appropriate antigen to use for antibody detection. Most studies have used as their detection antigen native GPI prepared in a one-step HPLC protocol (71); using this protocol, lipids have been identified as the dominant epitope within the GPI (71). However, using synthetic GPI material as the detection antigen, the carbohydrate domain is immunodominant, providing the basis for a vaccine development strategy (70). Thus more extensive assay validation is required to clarify this area.

Numerous studies suggest that haemozoin (an insoluble pigment derived from parasite degradation of haemoglobin) may also cause TNF-α release from macrophages (78). This suggests that there may be more than one 'toxin' in malaria. *In vitro* studies in both human and murine mononuclear cells illustrate that haemozoin ingestion augments pro-inflammatory production of TNF-α (79–82) and nitric oxide (NO) (83,84). However, haemozoin is also reported to inhibit DC maturation (85), to induce differentiation of IL-10-producing T cells, causing inhibition of general proliferative responses in human leucocytes (86), and to promote monocyte/macrophage dysfunction, impairing phagocytosis and the expression of MHC Class II, CD54 and CD11c (87,88). Overall, haemozoin appears highly immunosuppressive (89,90), but because there are no standardized protocols, and few examples of detailed

compositional analysis (for example by GC/MS), preparations of haemozoin may vary widely among laboratories and may contain non-covalently associated lipids, proteins and carbohydrates as well as the polyporphorin rings (91). Removal of non-covalently associated lipids from haemozoin has been shown to abolish haemozoin bioactivity (91), indicating that contaminating lipids or glycolipids mediate some or all of the activities ascribed to haemozoin. However, experiments using synthetic haemozoin, β-haematin, have yielded similar results to those of non-purified haemozoin. A number of laboratories have shown that haemozoin and β-haematin promote similar patterns of pro- and anti-inflammatory cytokines, effector molecules, and chemokines, such as IL-1β, TNF-α, IL-10, NO, macrophage inflammatory protein (MIP)-1α, and MIP-1β from cultured human PBMCs (79,80,84). Recent findings demonstrate that injection of β-haematin into BALB/c mice induces expression of IL-1β, IL-6, MIP-1α, MIP-1β, MIP-2, and monocyte chemoattractant protein, MCP-1 (83). Since β-haematin is identical, by infrared spectroscopy, to highly purified preparations of haemozoin (92) which do not contain host- and parasite-derived lipids, proteins, and carbohydrates, these results suggest that phagocytosis of the polymerized core iron moiety, ferriprotoporphyrin IX, may promote immune dysregulation similar to that of haemozoin. The relative contributions of haemozoin and GPI to the inflammatory cascade that is so characteristic of acute malaria are currently unclear but may be revealed by future epidemiological studies of anti-toxic immunity.

One potential outcome of vaccination against malaria 'toxins' is that inhibition of the host's inflammatory response may impede parasite elimination such that vaccinated individuals are protected against toxicity at the expense of being unable to contain parasite multiplication, with ensuing risks of severe anaemia and death. In the mouse model anti-GPI vaccination neither promoted nor inhibited parasite replication; after challenge infection, vaccinated mice developed parasitaemias that were not significantly different from unvaccinated mice up until the point where unvaccinated mice died of acute pathology (70). However, anti-GPI vaccinated mice were not able to eliminate infections and eventually succumbed to the typical haemolytic anaemia syndrome resulting from overwhelming parasitaemias in rodent malarias. Thus, although the preclinical data indicate a role for GPI in severe pathogenesis, it still remains to be determined whether anti-toxic vaccines can be deployed alone (where they may prevent or delay severe pathology) or whether the vaccine needs to be given in combination with an effective anti-parasite vaccine (in order to prevent the possibility that, if acute clinical symptoms are effectively suppressed, cases may take longer to present for treatment).

THE ROLE OF EFFECTOR MOLECULES IN MODULATING THE CELLULAR IMMUNE RESPONSE TO MALARIA

Although pro- and anti-inflammatory cytokines have many direct actions on the cellular immune response, one means by which they limit parasitaemia is through the generation of toxic free radicals such as NO. Generation of NO and L-citrulline from the L-arginine precursor is catalysed by NO synthases (NOS) (93). During an inflammatory state such as malaria, NO production is derived primarily from a cytokine-inducible isoform, NOS2 (or iNOS), present in macrophages, and numerous other cells. Pro-inflammatory cytokines (including IL-12, IFN-γ, and TNF-α) increase NOS2 gene expression, while anti-inflammatory cytokines (e.g. IL-10 and transforming growth factor-β, TGF-β) decrease NOS2 expression [for review see (94)]. IFN-γ stimulates monocytes to secrete TNF-α that can act in an autocrine and paracrine fashion to generate NO which has potent anti-plasmodial properties *in vivo* and *in vitro* (95–100). The relative balance of pro- and anti-inflammatory cytokines therefore regulates disease pathogenesis in malaria, at least in part, through modulation of NOS2-promoted generation of NO.

Although much of the research relating to the regulation of NO has been performed in murine model systems, numerous examples demonstrate that human monocyte-macrophages produce NO, particularly during disease states [for review see (101)]. The role of NO in the pathogenesis of malaria, however, remains controversial. Studies in Tanzanian children infected with *P. falciparum* show that PBMC NOS2 expression and peripheral levels of NO_2^- and NO_3^- (NO_x) are inversely related to disease severity (102). Additional investigations in Gabonese adults and children illustrate that elevated plasma levels of NO_x are associated with increased parasitological clearance and improved clinical outcomes (103) and that elevated baseline levels of PBMC NOS2 gene expression and elevated circulating NO_x are associated with mild vs. severe forms of malaria (104). However, a number of important studies in falciparum malaria have shown that NO is either not associated with disease severity or is correlated with enhanced disease severity (105–108). The apparent discrepancy in these data probably reflects, as for other inflammatory mediators, the importance of the timing and quantity of its release, i.e. in appropriate amounts at appropriate times during the inflammatory response NO can be protective, whereas a sustained NO response that fails to eliminate the pathogen can result in host tissue damage. This appears to be well illustrated in the context of malarial anaemia. Although high baseline levels of NO appear to be protective against the development of severe malaria (104,109), high levels of NOS2-derived NO are associated with malarial anaemia in children with acute falciparum

malaria (84). Thus, although NO can provide protective immunity by inhibiting the growth of malaria parasites (97,98), high levels of NO can also promote suppression of erythropoiesis (110) and induce apoptosis of erythropoietic stem cells (111) and thereby enhance pathogenesis. The pleiotropic role of NO in the regulation of malarial immunity is further underscored by studies demonstrating that genetic variation in the gene encoding NOS2 is associated with different phenotypic outcomes of malaria disease severity (see below).

THE INFLUENCE OF HOST GENETICS ON REGULATION OF CELLULAR IMMUNITY TO MALARIA

Human genetic diversity has been shaped in part by infectious diseases and one of the best examples of this is the impact of selective pressures exerted by malaria. The high mortality rates associated with endemic malaria, and particularly deaths of children, lead to strong selection in favour of genes that confer a degree of resistance. The best defined examples of genetic polymorphisms that have been selected by malaria are in genes expressed in erythrocytes (1), but there is now a significant body of evidence to indicate that the genes affecting the immune response can also influence the outcome of malaria infection (4). The completion of the human genome sequence and the advent of new methods for high throughput, high resolution genotyping are leading to an explosion in genetic epidemiology, i.e. studies that look for associations between clinical and epidemiological data and host genotype. Numerous studies reporting apparent associations between polymorphisms in immune system genes and outcome of malaria infection have been published in the last 10 years, although there are concerns that many of these associations may be spurious because of the small size of the studies and low levels of population matching (112). Definitively linking a particular polymorphism to a clinical or epidemiological phenotype requires large, carefully designed studies in which both the affected and unaffected individuals are drawn from the same environment (and thus are at equal risk of becoming infected and are exposed to the same environmental risk factors that might predispose to severe disease) and from the same gene pool, in order to avoid inadvertent associations caused by the population structure.

As mentioned above, one gene that has come under intense scrutiny in a number of studies is the NOS2A gene encoding NOS2. Reports from The Gambia demonstrate that shorter forms of a CCTTT microsatellite repeat in the NOS2A promoter are associated with fatal cerebral malaria (113). Other reports in children with non-cerebral malaria shown that a single nucleotide polymorphism in the NOS2A promoter (G-954C) is associated with less severe forms of malaria (i.e. high-density parasitaemia and malarial anaemia), and increased baseline NO in children with the heterozygous genotype (109,114). Additional studies in Ugandan children revealed that heterozygotes for the G-954C polymorphism had a significantly lower incidence of malaria compared to children with the wild-type alleles (115). However, studies in Tanzanian children with cerebral malaria failed to show any association of the G-954C with either disease severity or altered levels of systemic NO production (116). Recent studies show that a single nucleotide polymorphism in the NOS2A promoter (C-1173T) is associated with protection against severe malarial anaemia in a cohort of Kenyan children in whom cerebral malaria is exceedingly rare, and protection against symptomatic malaria in Tanzanian children (117). The recent discovery of a single haplotype defined by the NOS2A-1659T allele has also shown to be protective against cerebral malaria in Gambian children (118). Taken together, these results suggest that NO can have both a protective and pathogenic role in malaria, and that much of the variability observed in NO production in African populations is the result of genetic variation in the NOS2 promoter.

A series of studies in one West African population, the Fulani who live in the Sahel in areas of intense but very seasonal malaria transmission in Senegal, Mali, Burkina Faso, northern Nigeria and The Gambia, has shown them to be much less susceptible to *Plasmodium falciparum* malaria (as reflected by lower parasite rates and fewer clinical episodes) than other sympatric ethnic groups (119,120). This relative resistance is believed to be due in part to the ability of Fulani to produce much higher levels of anti-malarial antibodies and this has, in turn, been linked to a polymorphism (C to T transition at position 580) in the IL-4 promoter (121). If this polymorphism is indeed causally associated with altered B cell responses, then individuals carrying this allele should make immune responses that are measurably different from those of individuals without this allele. In a recent study, parasitological and immunological parameters were compared between Fulani and the neighbouring ethnic group, the Dogon of Mali. The study was performed outside the malaria transmission season and all individuals included were healthy at the time. The study confirmed that the Fulani were less parasitized, had fewer circulating parasite clones in their blood and had significantly higher levels of anti-malarial IgG (IgG1 and IgG3) and IgE antibodies compared to the Dogon (122). Interestingly, the Fulani also had higher proportions of malaria-specific IL-4 and IFN-gamma-producing cells compared to the Dogon; no differences were seen in the numbers of IL-10- and IL-12-producing cells (123). In accordance with previous studies (124,125), despite the lower prevalence of malaria infection, splenomegaly was

more prevalent among the Fulani than the Dogon. The investigators suggest that their findings might suggest a role for CD1-restricted NKT cells, or other cells or molecules of importance in the early innate immune response, in protection against malaria, with these cells being more effective in the more resistant Fulani population.

CD1-restricted NKT cells are an 'intermediate' arm between innate and adaptive immunity, with specificity for glycolipid antigens. In addition to a role as a pro-inflammatory toxin, malaria GPI is a natural ligand (antigen) for NKT cells (126), as are closely related glycoconjugates such as mycobacterial phosphatidylinositolmannosides (127). As an important regulatory lineage capable of acting with accelerated kinetics to produce both pro-inflammatory and anti-inflammatory cytokines, and with a documented role in regulating downstream Th1/Th2 differentiation of the immune system (128), the CD1/NKT pathway could potentially play an important role in the regulation of malarial immunity and pathogenesis. In keeping with this role, CD1-restricted NKT cells have been shown to determine cytokine levels, the pro-inflammatory cascade, pathogenesis and fatality in the *P. berghei* murine severe disease model (129). Using the *P. berghei* infection model in wild-type and CD1d–/– mice from disease-resistant (BALB/c) and susceptible (C57BL/6) genetic backgrounds, CD1-restricted NKT cells were found to have a protective role against *P. berghei*-mediated cerebral malaria in BALB/c mice, since BALB/c CD1–/– animals were more susceptible to disease than resistant BALB/c wild-type controls. In contrast, NKT cells promote disease in the C57BL/6 susceptible background, since C57BL/6 CD1-deficient mice were partially protected against cerebral malaria (129).

The opposing activities of NKT cells in BALB/c and C57BL/6 backgrounds reflect a differential ability to secrete key regulatory cytokines, leading to divergent Th1/Th2 balances in response to infection. Natural killer (NK) and NKT cells from different mouse strains differ in their expression of genes encoded by the Natural Killer Complex (NKC). Therefore, to study whether the NKC genotype could influence the activity of NKT cells in infection, NKC homozygous congenic BALB.B6-Cmv1^r mice, in which the region of chromosome 6 containing the NKC from C57BL/6 has been introduced onto the BALB/c background (130,131), were challenged with *P. berghei*. Importantly, BALB.B6-Cmv1^r mice were found to be more susceptible to *P. berghei*-mediated cerebral malaria than BALB/c wild-type controls (129), showing an infection phenotype similar to that of the C57BL/6 strain. Moreover, NKT cells from malaria-infected BALB.B6-Cmv1^r mice produced substantially higher levels of IFN-γ than cells from BALB/c mice, indicating that expression of the C57BL/6 NKC markers favours Th1-driven responses by NKT cells. The findings therefore establish that the NKC (previously known only to regulate viral infections and cancers) is a genetic determinant of malarial pathogenesis (129) in this murine model, and has a major role also in controlling NKT cell function. The CD1/NKT pathway also regulates acute splenomegaly in mice and is an important determinant of B cell responses (132). Accordingly, in a recent study, the NKC was shown to regulate the severity of malarial anaemia in mice, to determine the isotype distribution of anti-malarial antibodies and the Th1/Th2 profile of T cells (133), and microarray analysis reveals that gene expression within the NKC locus is up-regulated in the brains of mice experiencing cerebral malaria (134).

Thus, the CD1/NKT pathway either prevents or promotes fatality, and determines downstream immunological differentiation endpoints, depending upon expression of alleles of the Natural Killer Complex located on mouse chromosome 6. These loci are differentially expressed on NKT cells in a strain-dependent manner and control the production of pro-inflammatory Th1 and counter-regulatory Th2 cytokines by NKT cells (135). Once mice are infected with malaria, their NKT cells produce significantly different levels of IFN-γ and IL-4, depending upon expression of polymorphic NKC alleles. Thus directly or indirectly, malaria imparts NKC-dependent signals to NKT cells that influence their Th1/Th2 profile. This body of evidence suggests the testable hypothesis that the syntenic human NKC encoded on human chromosome 12 and expressed on NK and NKT cells also influences Th1/Th2 polarization during malaria infection. To date there are few disease association studies for this lineage, although this is being addressed by Troye-Blomberg and colleagues. Of relevance however, individual variation has been reported in the propensity of human NK cells to produce IFN-γ in response to stimulation with *P. falciparum*-infected red blood cells (136), and this has been tentatively linked to genetic polymorphisms within the killer Ig-like receptor (KIR) locus on chromosome 19 (137).

POLYPARASITISM AND ANTI-MALARIAL IMMUNITY

In endemic areas, malaria infections rarely if ever occur in isolation; malaria-infected individuals are almost inevitably already infected with other pathogens. These may be overt, as for example in the case of schistosomiasis, or covert, as in the case of subpatent infection with tuberculosis or herpes viruses. Surprisingly, studies to determine the nature, extent and significance of interactions between different pathogens inhabiting the same host have only recently been undertaken. Clinically, co-infection with schistosomiasis and malaria has been found to lead to much more severe

hepatosplenomegaly than is caused by either infection alone (138), and co-infection with HIV has been shown to lead to more frequent and moderately more severe malaria infections (139). However, data indicating that gastro-intestinal helminth infections might protect against severe malaria (140) have not been confirmed in other studies (141).

Clearly, the immunological interactions between different pathogens are likely to be governed by the timing of the infections in relation to each other, the intensity of duration of the infections, host polymorphisms and environmental factors. At this early stage of the work, exploration of co-infections under controlled conditions in model systems is likely to lead to robust hypotheses that can then be tested in epidemiological studies.

One potentially very important consequence of co-infection is modulation of the response to malaria vaccines. Helminth infections, which are highly prevalent in many parts of the world, including malaria-endemic areas, have been shown to modulate immune responses to unrelated pathogens (142,143) and have been implicated in poor efficacy of malaria vaccines in humans (144). In a direct test of the impact of gastrointestinal helminth infection on vaccination against malaria, Su, Stevenson *et al*. (submitted for publication) have compared the outcome of vaccination of mice against blood stages of *Plasmodium chabaudi* in mice infected or not with the gastrointestinal nematode, *Heligmosomoides polygyrus*. Immunization of C57BL/6 mice with crude blood-stage *P. chabaudi* antigen induced strong protection against malaria challenge. By contrast, *H. polygyrus*-infected mice were significantly less well protected by malaria vaccination, producing significantly lower levels of malaria-specific total Ig, IgG1 and IgG2a antibodies, significantly lower levels of IFN-γ and higher levels of IL-4, IL-13 and the immunoregulatory cytokine, IL-10, in comparison with helminth-uninfected mice. Interestingly, at the time of immunization, helminth-infected mice had high serum levels of TGF-β1 which, as discussed above, might favour the development of anti-inflammatory regulatory T cells during vaccine-induced priming of anti-malarial immune responses. Importantly, deworming of helminth-infected mice with an anthelminthic drug prior to immunization led to higher levels of protection against malaria in comparison with mice which were not dewormed. These data indicate that concurrent nematode infection has the potential to modulate development of the immune response to a malaria vaccine and suppress the efficacy of vaccine-induced protective immunity against malaria. Reassuringly however, this effect seems to be transient and can be overcome by deworming prior to vaccination, suggesting that clinical trials are warranted to determine whether routine deworming can enhance the efficacy of anti-malarial vaccines.

AN AGENDA FOR FUTURE RESEARCH?

The rational development of effective anti-malarial vaccines and novel therapies to alleviate or prevent the symptoms of severe malaria infection requires a better understanding of the various mechanisms by which immune responses to the parasite can be regulated. This is especially true for cell-mediated immune responses which give rise to effectors that are potentially damaging to the host as well as the parasite. As we have seen, the outcome of malaria infection can be profoundly affected by genetic and environmental variables, but these probably serve to moderate host–pathogen interactions rather than to completely alter them. The underlying immune processes that allow cellular immune responses to be induced and regulated are likely to be universal and generalizable from one disease to another. This is clearly demonstrated by the conservation of regulatory T cell mechanisms for regulation of inflammatory responses to allergens, self antigens and infections as diverse as viruses and protozoa.

There are clearly lessons to be learned from other protozoal infections regarding the relationship between immunoregulation, parasite persistence and premunition in malaria infections. Moreover, we still have only a very superficial understanding of the conditions which induce differentiation of regulatory T cells and of the signals required for their induction. Antigen presenting cells (APCs) are likely to be important regulators of this process which, in turn, may depend on very specific interactions between APCs and pathogens, such as induction of a tolerogenic/anergic/regulatory phenotype in dendritic cells following binding of malaria-infected blood cells to CD36 (145); (and Stevenson and Urban, this volume).

Whilst there is widespread agreement that much of the pathology of malaria is immune-mediated and might therefore be moderated by immunoprophylaxis, non-immunological factors also contribute to disease severity. Physiological maturity, i.e. age, independent of malaria exposure and acquired immunity, is a significant modifier of susceptibility (146) and it is now clear that there are physiological age limits below which severe malaria presents as anaemia and above which it presents as cerebral malaria (147). Whether these age effects are themselves manifestations of a changing immunological environment (148) remains an open question. Also unresolved is the relative importance of different lymphoid cell populations (αβ T cells, γδ T cells, NK or NK–T cells and macrophages) in mediating either protection or pathology. For example, do we need Th-1 effector cells to control blood stage malaria infection or would predominantly antibody-mediated effector mechanisms be as effective and less dangerous? If so, are the Th-1 cells that activate cellular effector mechanisms the same cell population that provide help to B cells, or can we identify subpopulations of cells

that promote antibody responses without causing pathology? Finally, to what extent do very early environmental effects (that might impact on the immune response prior to any immunological intervention) such as prenatal or perinatal exposure to malaria antigens, or other pathogens, modify any or all of these immune responses?

CONCLUSION

Type-1 immune effector mechanisms are essential to control malaria infection but need to be regulated in order to avoid induction of severe pathology. There appear to be several different mechanisms of regulation, which may act independently of each other or which may synergize for optimal fine control of the immune response. The notion that antitoxins might regulate the pro-inflammatory response has been around for more than 100 years and we are only now, gradually unravelling the molecular basis for acquired antitoxic immunity. However, the realization that the immune system is inherently self-balancing, and that there are endogenous (innate) as well as acquired mechanisms to achieve this, is much more recent and has led to the explosion of interest in regulatory cell populations and self-regulating networks of immune effector molecules. Intricately tied up in this concept of equilibrium is our gradual appreciation that immune responses to individual pathogens affect each other, and that the individual response to malaria infection represents a synthesis of their previous infection history with their current infection status.

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