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Malaria – how this parasitic infection aids and abets EBV-associated Burkitt lymphomagenesis

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Abstract

Burkitt lymphoma (BL) is >90% EBV-associated when this pediatric cancer is diagnosed in regions heavily burden by endemic *Plasmodium falciparum* malaria and thus has been geographically classified as endemic BL. The incidence of endemic BL is 10-fold higher compared to BL diagnosed in non-malarious regions of the world. The other forms of BL have been classified as sporadic BL which contain EBV in ~30% of cases and immunodeficiency BL which occurs in HIV-infected adults with ~40% of tumors containing EBV. Within malaria endemic regions, epidemiologic studies replicating Denis Burkitt's seminal observation continue to show differences in endemic BL incidence linked to intensity of malaria transmission. However, the mechanisms by which malaria contributes to B cell tumorigenesis have not been resolved to the point of designing cancer prevention strategies. The focus of this review is to summarize our current knowledge regarding the influence of prolonged, chronic malaria exposure on defects in immunosurveillance that would otherwise control persistent EBV infections. And thus, set the stage for ensuing mechanisms by which malaria could instigate B cell activation and aberrant activation-induced cytidine deaminase expression initiating somatic hypermutation and thereby increasing the likelihood of an *Ig/Myc* translocation, the hallmark of all BL tumors. Malaria appears to play multiple, sequential and simultaneous roles in endemic BL etiology; the complexity of these interactions are being revealed by applying computational methods to human immunology. Remaining questions yet to be addressed and prevention strategies will also be discussed.

Epstein-Barr virus and endemic Burkitt lymphoma

Since the discovery of Epstein-Barr virus (EBV) within a B cell tumor in 1964 [1], how EBV promotes B cell tumorigenesis has been extensively studied and are reviewed in detail

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elsewhere (reviewed in [2]). As a scientific community we are generally in agreement that EBV is a causative agent for several cancers, in that viral proteins, mircoRNA and epigenetics play a role in driving cell proliferation and rescue from apoptosis (reviewed in [3,4]). It also appears that EBV takes advantage of rare compensatory host cell mutations that disable apoptotic pathways in conjunction with the oncogenic *c-myc* translocation that drives unrestrained cell growth and proliferation [5••]. In immunocompetent individuals, EBV alone is insufficient for malignant transformation since this gamma-herpesvirus is a life-long and typically asymptomatic infection in most adults around the world [6]. How EBV promotes cancer is not the focus of this review, however, in the interest of the ensuing discussion it should be noted that healthy children residing in areas with a high incidence of endemic Burkitt lymphoma (eBL) experience their primary EBV infection, asymptomatically before 3 years of age if not within the first year of life [7••,8, 9], and holoendemic *Plasmodium falciparum* malaria exposure has been associated with higher frequencies of EBV reactivation and more episodes of viremia compared to early-age EBV-infected children not repeatedly co-infected with malaria [7,10–13•].

The postulated roles for falciparum malaria in the etiology of endemic Burkitt lymphoma

There are two synergistic mechanisms by which malaria is hypothesized to contribute to eBL etiology: 1) by inducing defects in immune surveillance to EBV antigens and thereby failing to limit the number of latently EBV-infected B cells during episodes of lytic reactivation; and 2) by inducing B cell activation that leads to EBV lytic reactivation as well as aberrant expression of activation-induced cytidine deaminase (AID) and thereby increasing the likelihood of a successful *c-myc* translocation triggering oncogenesis. Given this background, we will review the evidence gathered to date to address the long-standing question, ‘How *precisely* does malaria contribute to eBL tumorigenesis?” and highlight as yet unresolved questions.

Epidemiologic overlay of endemic Burkitt lymphoma on *Plasmodium falciparum* malaria

Plasmodium falciparum malaria being named as a co-factor in eBL etiology was suggested after Denis Burkitt’s famous ‘tumor safari’ which first described rainfall and altitude as being geographically associated with eBL incidence in Africa [14]. The suggestion that malaria played a role in eBL pathogenesis [15] was also made during the first global push for malaria eradication (1950-1970) and when hemoglobin (Hb) AS heterozygosity was discovered to protect individuals against severe malaria but resulted in sickle cell disease when people where HbAA homozygous [16]. It was subsequently postulated that severe malaria equated to a higher risk of eBL and an initial study appeared to show that HbAS may protect against eBL [17]. However, a series replication studies conducted over the course of 30 years and in three different African countries concluded that children diagnosed with eBL had the same frequency of HbAS heterozygosity as appropriately matched population-based controls [18–20]. In addition, the lack of documented family clusters of eBL, especially in families with many siblings, also argues against a strong, simple host-

genetic predisposition. This lack of a link between severe malaria and eBL is also supported by considering the age-dependent epidemiology of both of these diseases. Age-structured modeling consistently demonstrates that severe malaria susceptibility decreases as a child reaches 5 years of age when residing in malaria holoendemic areas (reviewed in [21]). Of note, this is the age at which the incidence of eBL precipitously increases until it tapers off by 9 years of age (reviewed in [22]). Thus, the clinical and epidemiologic evidence points toward syndemic mechanisms that require prolonged malaria-induced perturbations of EBV homeostasis and immune surveillance in order to culminate in eBL tumorigenesis.

Early-age primary Epstein-Barr virus infection: implications for immune surveillance within the context of malaria co-infections

The prolonged time interval between primary EBV infection in African children and the induction of pediatric eBL could in part, be explained by the natural progression of acquired immunity to malaria and how this changes malaria from an acute to chronic infection. Anti-malarial immunity is intrinsically complex and depends on age at time of infection, cumulative exposure and genetic variation of the parasite [23]. Therefore, a simple Th1 versus Th2 immune response dichotomy does not necessarily apply to human malaria (reviewed in [24]). In addition, infants born to mothers residing in malaria endemic areas benefit from some degree of transplacentally transferred antibody-mediated immune protection (reviewed in [25]). As maternal antibodies naturally wane by around 6 months of age, the majority of infants and young children experience repeated, acute uncomplicated malaria infections, as opposed to manifestations of severe malaria (reviewed in [26]). With cumulative malaria exposure and immunologic maturation, that tends to occur around the age of 5 years, children develop premunition. This is also known as anti-disease immunity which allows children to tolerate chronic, asymptomatic parasitemias [21].

In keeping with the concept of dynamic human immune heterogeneity, recent studies demonstrate a reduced transfer of maternal antibodies against EBV and signs of increased viral reactivation when some mothers are infected with malaria during pregnancy [27,28]; which could result in earlier-age, higher viral load infections during infancy [10]. Acute, uncomplicated malaria has been associated with EBV lytic reactivation and a higher frequency of episodes of measurable viremia [7,10,12]. The apparent immune defects determined from infant cohort studies conducted in Kenya include decreased IFN-γ responses to EBV lytic and EBV latent antigens, in addition to skewed EBV-specific CD8+ T cell immune profiles that may not be as efficient at limiting expansion of EBV latency [13,29–31••]. The role of malaria in altering EBV-specific immunity appears to be an indirect effect whereby high EBV antigen load over time results in a degradation of EBV-specific immune surveillance and signals that prevent the development of immunologic effector-memory.

Effective control over EBV has been shown to be mediated by both innate and adaptive immunity when this infection occurs during adolescence or adulthood and has been shown to undergo temporal changes during the course of infection (reviewed in [32]). Preliminary studies in the Moormann lab suggest that natural killer (NK) cell subsets [33–35•] of

children co-infected with malaria are significantly different in phenotype and function compared to age-matched, non-malaria exposed children, with additional defects apparent in children who are diagnosed with eBL (unpublished, Moormann). In addition, longitudinal infant cohort studies suggest that cytotoxic T cell mediators [32] differ in phenotype and function after cumulative, high burden exposure to malaria which may also prevent proper immune control over EBV (unpublished, Moormann). The delayed age of onset for eBL could possibly be explained by NK cells being more important in controlling EBV [34•,36] primary EBV infection during infancy, prior to the maturation of antigen presentation signaling pathways that promote the development of effector-memory T cells. Transcriptome and computational analyses of human immune cell subsets are starting to shed new light into which transcription factors regulate the selection and depletion of T cell subsets (reviewed in [37]) and can be applied within the context of EBV and malaria co-infections in children.

EBV-infected memory B cell susceptibility to malaria-induced aberrant activation-induced cytidine deaminase

If EBV-specific immune surveillance is sufficiently impaired and malaria has become a chronic infection, the stage is set for a prolonged assault on EBV-infected B cells. At this point, EBV latency within memory B cells has been established (reviewed in [4]) and viral proteins, LMP1 and LMP2 are mimicking host CD40 and B cell receptors (BCR), respectively providing signals for cell growth and down regulation of pro-apoptotic signals (reviewed in [3]). In addition, there is evidence that AID, which is the key to somatic hypermutation and class switch recombination for antibody generation in memory B cells (reviewed in [38]), can be expressed outside the germinal center environment as demonstrated by peripheral blood AID expression of children co-infected with malaria [39••] and EBNA3C directly inducing AID in B cells [40]. Malaria has also been shown to induce AID in a p53 deleted Eμ-mouse model and *in vitro* studies using human tonsillar cells [41•,42•]. A caveat to the Robbiani et al. study is that *Plasmodium chabaudi* mouse malaria appears not have a functional homologue to *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) which is also involved in parasite sequestration [24,43]. The cysteine-rich interdomain region 1 alpha (CIDR1a) of PfEMP1 has been identified as the T cell-independent polyclonal B cell activator, Ig binding protein, and inducer of EBV lytic reactivation [44••,45••]. Therefore, induction of AID within this model system could be mediated by inflammatory signals not specific to malaria [46,47]. Along this line, the more benign forms of human malaria *P. vivax, P. ovale, P. malariae* and *P. knowlesi* have not been implicated in eBL etiology. Further studies of AID induction across plasmodia species would be required to determine the antigen-specificity of malaria-derived red blood cell variant surface proteins [43]. In the study by Torbor et al, it appears that malaria induces AID expression by also engaging the innate toll-like receptor, TLR9 [48] and CD40 receptors as would be provided by cognate antigen-specific CD4+ follicular helper T cell signaling pathways. Malaria has been recently described to induce less functional Th1-polarized CXCR3+ follicular helper CD3 T cells in children [49]. If this defect influences the fate of T cell help for EBV-infected B cells within children remains speculative. In addition, the impact of activation dynamics or AID thresholds needed to achieve somatic

mutations within a co-infection model remain to be explored, leaving these biologically relevant questions open to further investigation.

It remains controversial if EBV is able to establish latency in naïve memory B cells by bypassing the germinal center (GC) reaction (antigen-independent) or if eBL pathogenesis requires GC transit and is antigen-dependent [50,51]. More recent studies of human B cell subsets altered by malaria [52,53] and the identification of two possible routes by which memory B cells can undergo class switch recombination, GC-dependent or independent [54], compel us to revisit the question of which B cell compartments harbor EBV in children who develop eBL. Therefore, the relevance of BCR antigen-specificity in eBL pathogenesis remains unanswered. However, it is tempting to speculate that if the surface IgM of EBV-infected memory B cells encounter their cognate antigen, triggering a 'secondary' GC reaction and affinity maturation, this could provide malaria another point at which to influence B cell activation. Combined with the non-specific mechanisms described above to induce AID this dual stimulation by both EBV and malaria could thus create a supercharged environment for genetic instability and oncogenesis.

Is malaria the only parasite that could modulate immune surveillance that abets EBV-infected B cell tumorigenesis?

No. Schistosomiasis is an equally common chronic childhood infection in lake regions of Africa [55,56] where we find eBL, and it induces Th1-cytokines during early infection that dramatically shifts to Th2-cytokines during chronic infection [57]. *S. mansoni* antigens are known to induce a robust type 2 cytokine response, including production of IL-4, IL-5, IL-10, and IL-13 [58]. A pre-existing polarizing cytokine milieu is likely to affect NK and CD8+ T cell function prior to or during their activation by a viral infection [59–61]. Strong type I interferon signaling drives NK cell maturation to the terminal effector stage, producing an NK cell population with an impaired ability to respond to herpesvirus infection [60,62], whereas strong IL-4 signaling by CD8+ T cells is characterized by poor cytotoxicity and reduced secretion of IFN-γ [59]. Studies of NK cell education and tolerance during chronic infections reveal a broad range of pathogen-specific ligand interactions and highlight differences between early and late differentiated NK cell subsets in protection from EBV which contrast those engaged to respond to CMV [34•,35•,63]. It remains to be determined if loss of immune control over EBV early in life is mediated by NK cell tolerance or T cell clonal deletion or exhaustion and if the cytokine milieu induced by other parasitic co-infections plays a role in eBL pathogenesis. However, the possibility that other chronic parasitic infections engender immune dysfunction or exhaustion is open for debate [64].

Clarifying malaria's role in endemic Burkitt lymphoma pathogenesis and questions remaining

In summary, malaria in not generally immunosuppressive but is a powerful driver of EBV-associated eBL pathogenesis. This review has gathered evidence in hopes of dispelling this lingering dogma. In fact, malaria is highly immunogenic (reviewed in [23]) and children diagnosed with eBL display robust immune responses to malaria antigens while deficient in

EBV-specific immunity [65••]. The impact of chronic malaria on EBV-specific immune surveillance appears to be due to its ability to drive EBV lytic reactivation in B cells that repeatedly triggers the cascade of EBV lytic and latent antigen expression that eventually leads to impaired immunosurveillance and subsequent, unhindered interactions between malaria-derived ligands/proteins and EBV-infected B cells that are already primed and poised for oncogenesis. Figure 1 illustrates the synergistic mechanisms by which malaria contributes to impaired EBV immune surveillance and aberrant AID expression within an EBV-infected B cell.

In vitro cell culture and animal models have been instrumental toward increasing our understanding of EBV biology and mechanistic pathways responsible for B cell transformation. Yet these models systems are limited in their ability to resolve many remaining question as to how malaria contributes to eBL pathology in children. The use of humanized mouse models show promise in addressing questions about human infectious diseases and cancer etiology [66,67]. Combined with descriptive natural co-infection studies of humans and computational approaches to human immunology and virology [68], the interwoven array of immune mediators are being identified and can now be interrogated within malaria and EBV co-infection studies. This new appreciation of human immunology will lead to designing appropriate interventions to prevent malaria and/or EBV infections in infants. Learning how to silence or divert signaling pathways that interfere with normal EBV immune surveillance and improving our understanding of how chronic malaria contributes to eBL etiology has implications for preventing this pediatric cancer in Africa.

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Abbreviations

AID	Activation-induced cytidine deaminase
BCR	B cell receptor
CD4 Tfh	CD4 follicular helper T cell
CSR	class switch recombination
eBL	endemic Burkitt lymphoma
EBV	Epstein Barr virus
LMP-1	latent membrane protein 1
LMP-2	latent membrane protein 2
MHC II	Major Histocompatibility complex, class II

Pf	Plasmodium falciparum
PfEMP1	Plasmodium falciparum Erythrocyte membrane protein 1
pRBC	parasitized red blood cell
TCR	T cell receptor
TLR9	Toll like receptor 9
SHM	somatic hypermutation

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Highlights

- Immunity in young children is functionally and phenotypically different than adults
- Chronic malaria increases EBV load that then impairs anti-viral immune surveillance
- Malaria-derived mediators directly and indirectly increase B cell AID expression

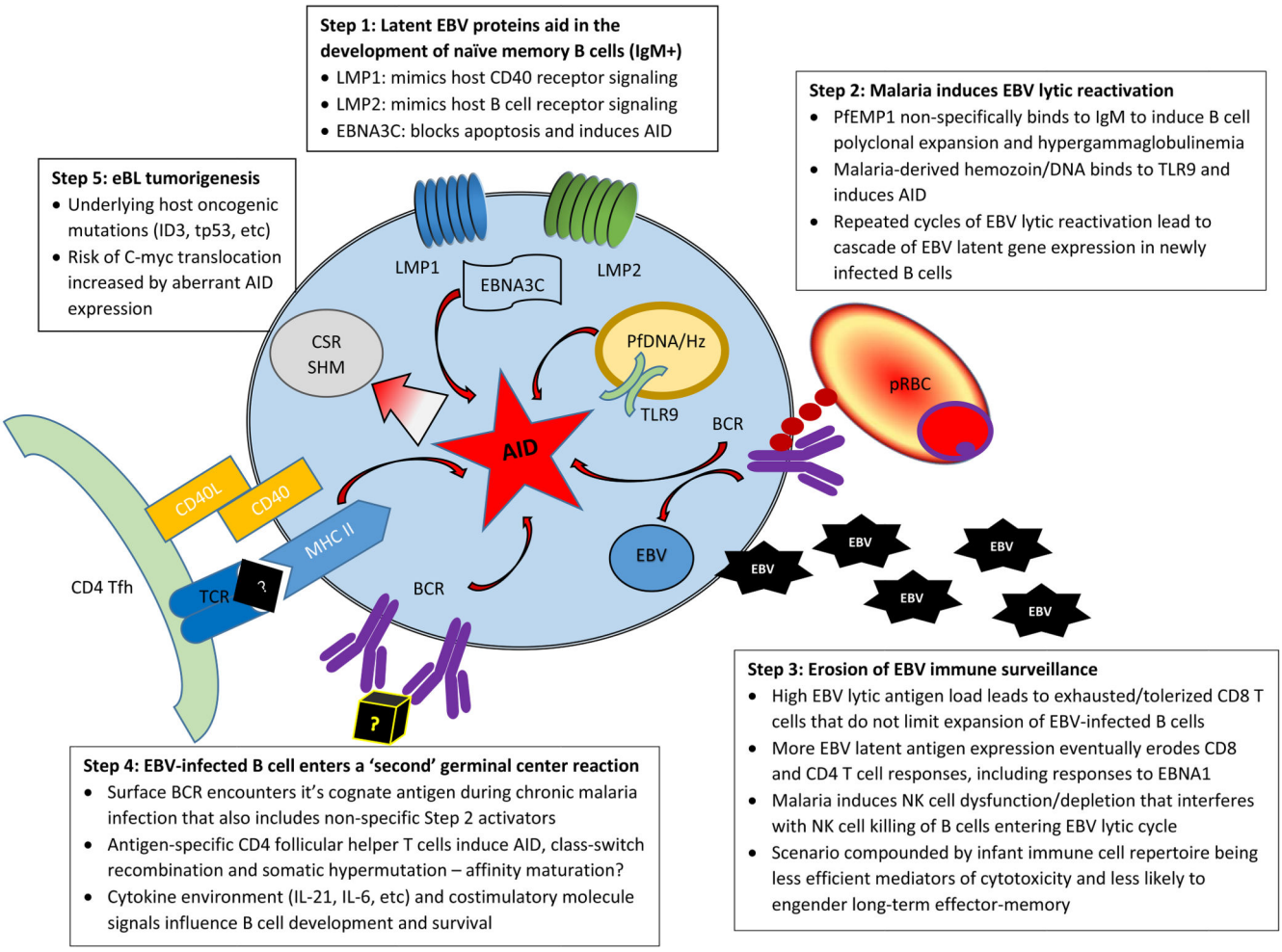

Figure 1. Illustration of the synergistic mechanisms by which *Plasmodium falciparum* malaria could contribute to impaired EBV immune surveillance and aberrant AID expression within an EBV-infected B cell as a prelude to endemic Burkitt lymphoma oncogenesis

Step 1: EBV establishes latency in B cell whereby viral proteins (LMP1, LMP2, EBNA3C and possibly microRNA) mimic host activation signals and development of naïve memory B cells. Question remains if eBL precursor is derived from EBV-infected B cell established independent of a primary germinal center (GC) reaction and thus retains surface IgM that did not undergo cognate antigen-affinity maturation.

Step 2: Recurrent malaria infections induce EBV lytic reactivation, mediated by antigen-independent PfEMP1-IgM interactions, resulting in cyclical episodes of lytic antigen expression and a cascade of EBV latent antigen expression and expansion of the number of EBV-infected B cells. Malaria-derived DNA/hemozoin is a TLR9 ligand that can induce AID expression in the absence of cognate antigen B cell receptor (BCR) crosslinking. Questions remain as to how many malaria infections or the duration of chronic malaria that would provide a threshold of stimulation to induce aberrant AID expression and if there are other malaria-derived mediators of B cell activation.

Step 3: Erosion of EBV immune surveillance presents as a gradual degradation of immune responses to EBV lytic and latent antigens over time. NK cell dysfunction may be induced

by malaria with a bystander effect of an inability to control secondary EBV infections. EBV lytic and then latent T cell responses become exhausted or tolerized to this persistent viral infection when antigen load is high. This scenario is further hindered by the human infant immune system being inherently less efficient at controlling infectious diseases and less likely to induce long-term effector-memory T cell subsets. Questions remain as to the threshold of EBV antigen-load that results in loss of T cell function and if this can be rendered moot by decreasing malaria exposure alone.

Step 4: EBV-infected memory B cells enter 'second' germinal center reaction. The EBV-infected B cell encounters its cognate antigen but now in the presence of antigen-specific CD4 follicular helper T cells (the development of which do not occur until children are older, years after their primary exposures to EBV and repeated malaria infections). Combined, these normal B cell signaling pathways induce class-switch recombination (CSR) and somatic hypermutations (SHM) mediated by AID. Questions remain as to the antigen-specificity of the BCR on eBL tumors and if they have undergone proper affinity maturation if they bypassed the GC reaction in Step 1, or if the chronic malaria infection and repeated peaks of parasitemia are more relevant to antigen-independent AID expression as describe in Step 2. In addition, the inflammatory environment present during the GC reaction could influence B cell development and survival. If these events occur in concert it could have a synergistic effect by over amplifying the expression of AID and may also explain the rarity of one EBV-infected B cell becoming an eBL precursor.

Step 5: Endemic Burkitt lymphoma (eBL) tumorigenesis. Underlying host mutations have been described for BL tumors; combined with aberrantly high or prolonged AID expression would likely facilitate the risk of a C-myc translocation resulting in oncogenesis. The success of one B cell becoming a BL tumor is all the more likely when immunosurveillance has been impaired as described in Step 3.

In summary, malaria appears to play numerous roles in eBL tumorigenesis, with EBV as the promotor and human mutations the underlying landscape.