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The company malaria keeps: how co-infection with Epstein-Barr virus leads to endemic Burkitt lymphoma

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Abstract

Purpose of review—Co-infection with *Plasmodium falciparum* (Pf-) malaria and Epstein-Barr virus (EBV) are implicated in the etiology of endemic Burkitt lymphoma (eBL), the most prevalent pediatric cancer in equatorial Africa. Although the causal association between EBV and eBL has been established, Pf-malaria's role is not as clearly defined. This review focuses on how malaria may disrupt EBV persistence and immunity.

Recent findings—Two mutually-compatible theories have been proposed. One suggests that Pf-malaria induces polyclonal B-cell expansion and lytic EBV reactivation, leading to the expansion of latently infected B-cells and the likelihood of *c-myc* translocation; a hallmark of BL tumors. The other advocates that EBV-specific T-cell immunity is impaired during Pf-malaria co-infection, either as a cause or consequence of enhanced EBV replication, leading to loss of viral control. Advancements in our ability to query the complexity of human responses to infectious diseases have stimulated interest in eBL pathogenesis.

Summary—EBV is necessary but not sufficient to cause eBL. A more dynamic model encompasses incremental contributions from both chronic and acute Pf-malaria leading to alterations in EBV persistence and EBV-specific immunity that culminate in eBL. A better understanding of how Pf-malaria modifies EBV infections in children may allow us to anticipate reductions in eBL incidence coinciding with malaria control programs.

Keywords

Malaria; EBV; endemic Burkitt lymphoma; T-cell immunity; B-cell immunity

Introduction

In recent years meaningful decreases in malaria-associated morbidity and mortality have been observed worldwide due to multi-factorial global efforts to control and prevent malaria [1]. These campaigns have significantly decreased malaria in endemic areas however it is far

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from being eradicated. Malaria vaccine efforts are underway with lofty goals of not only preventing disease but ideally preventing infections and transmission. After considerable discussion about clinical endpoints for malaria vaccine efficacy trials, decreases in malaria-associated mortality and morbidity would be acceptable short-term until a vaccine is designed that prevents infection. Thus the possibility exists that a vaccine (or other intervention) which prevents clinical malaria yet does not prevent infections might create a carrier state whereby children harbor untreated infections. One could argue that asymptomatic malaria infections are beneficial and induce naturally acquired protective immunity [2**]. Conversely, chronic malaria infections may have detrimental effects contributing to anemia [3] or malaria-associated diseases such as endemic Burkitt lymphoma (eBL) [4].

Endemic BL has long been associated with early-age Epstein Barr Virus (EBV) infection and geographically linked to holoendemic *Plasmodium falciparum* (Pf)-malaria [4,5]. Several key observations have established a causal role for EBV in eBL tumorigenesis. EBV was first isolated from and subsequently found in nearly 100% of eBL tumors [6,7]. EBV sero-surveys concluded that children in developing countries experience their primary infection before the age of 3 years [8]. And significantly elevated antibody titers to EBV viral capsid antigen (VCA) were found in children who later went on to develop eBL [9]. Since these seminal observations, the role of EBV as a tumor virus and EBV-specific T-cell immune control has been extensively explored [10,11]. In comparison, this review focuses on the role Pf-malaria plays eBL pathogenesis.

There are two prevailing, though not mutually exclusive theories to explain the relationship between EBV and Pf-malaria in eBL etiology [12]. One suggests that Pf-malaria induces polyclonal B-cell expansion and consequently lytic EBV reactivation, thus leading to the expansion of latently infected B-cells and the likelihood of *c-myc* translocation, which is a hallmark of all BL tumors. The other theory argues that EBV-specific T-cell immunity is selectively impaired during Pf-malaria co-infection, either as a cause or consequence of enhanced EBV replication, leading to loss of viral control. Our growing appreciation for the complexity of human immunity allows us to delve further into the question of how Pf-malaria contributes to EBV de-regulation.

Epidemiologic studies to evaluate the role for Pf-malaria in eBL

Whereas the causal association between EBV and eBL has been established, Pf-malaria's role in eBL pathogenesis is not as well understood. Holoendemic malaria is characterized by intense perennial transmission with the highest morbidity and mortality in children under the age of 5 years. Under such conditions, infants and young children are repeatedly infected with Pf-malaria, can harbor chronic asymptomatic often untreated infections. Recurrent infections engender partial immunity to Pf-malaria which allow sustained often high density infections [2]. Since both chronic and acute Pf-malaria infections are common in children residing in holoendemic areas, deconvoluting the multiple opportunities malaria has to influence EBV persistence is challenging.

Anti-malarial treatment in holoendemic area has been associated with a decrease in eBL incidence [13,14] although few studies have directly addressed the causality. A historical 'malaria-suppression' trial was conducted in Tanzania from 1977 to 1982 in an area monitoring eBL and malaria prevalence [15]. This intervention study distributed chloroquine to ~100,000 children below 10 years of age every two weeks for five years in an explicit attempt to reduce eBL incidence. They observed a decrease in eBL but could not conclude that treating malaria was the sole mediator because eBL incidence had been declining prior

to the study. However, the prevalence of malaria cases also declined prior to the intervention mirroring the decline in eBL incidence.

Other attempts to determine if relative peaks in malaria act as a 'trigger' of eBL by analyzing space-time clustering or seasonality of cases were also inconclusive [16]. However using spatial analytical models, significant high-risk clusters for eBL incidence were found within a malaria holoendemic region thereby highlighting the heterogeneity of individual-malaria exposure within holoendemic areas [17]. In addition, though the incidence of eBL is significantly lower in areas with hypoendemic malaria, eBL may still occur [18,19]. These observations support the premise that timing of co-infection and duration of Pf-malaria infections on an individual-level is critical for understanding the dysregulation of EBV leading to eBL.

The coincidence of Pf-malaria infection preceding eBL further supports a temporal interaction with EBV. The average age of eBL patients in malarious areas was noted to be 8.1 years while in low risk malarious areas it was 16.2 years [20]. In addition, immigrants from malaria hypoendemic areas who moved to holoendemic areas presented with eBL in late adolescents and adulthood [20]. A similar pattern was also found as most adult cases of eBL were born in hypoendemic areas [21]. Figure 1 depicts the chronology of EBV and Pf-malaria co-infections in relation to eBL incidence in malaria holoendemic areas. The peak age-related incidence of eBL is 4-9 years of age [19,20], after the age at which Pf-malaria-associated morbidity, mortality and parasite densities are the highest. Even though these children experience their primary EBV infection before three years of life [8] it is extremely rare for a child to develop eBL before two years of age suggesting a prolonged interaction is necessary for these co-infections to promote tumorigenesis. In support of this, the Tanzanian study [15] reported a two-year lag between Pf-malaria burden and eBL incidence with a concomitant increase in eBL incidence after the intervention was stopped. BL is one of the most rapidly dividing human tumors [10], thus the eBL 'triggering event' must occur proximal to tumorigenesis. Malaria and EBV co-infections are common in children residing in high-risk eBL areas, although the incidence of eBL is only 1-5 per 100,000 children annually. Pf-malaria may play multiple roles in eBL pathogenesis whereby co-infection during primary EBV infection sets the stage for deregulation of EBV latency and repeat malaria infections exacerbates EBV homeostasis and T-cell immunity.

EBV-specific T-cell immune deregulation by Pf-malaria

Based on other studies of acute infectious mononucleosis and healthy EBV seropositive adults, we know that EBV-specific T-cells restrict viral replication and limit the number of latently infected B-cells [22]. Thus, EBV load in peripheral blood has been used as surrogate measure for loss of T-cell mediated viral control [23]. The first study to measure EBV load in children living in a malaria holoendemic area found the youngest children had significantly higher EBV loads compared to older children, adults and children from a hypoendemic area [24]. Ensuing studies measured EBV before and after episodes of acute clinical malaria and even though viremia was consistently higher in acute cases than controls, changes in viral load resulting from an episode of acute malaria could not be consistently measured after the episode resolved [24-28]. These contradictory findings could be the results of differences in cumulative Pf-malaria exposure and the frequency of latently infected B-cells present. A recent study using multi-level mixed modeling found that children residing in a malaria holoendemic area experienced their primary EBV infection significantly earlier compared to children from a hypoendemic area and that individual malaria burden over time were significant predictors of higher EBV viral load (Rochford, unpublished data). These findings highlight the need for prospective studies designed to

measure individual-level exposure to both Pf-malaria and EBV in order to understand how the heterogeneity in timing and intensity of these co-infections interact.

In immune competent individuals, EBV persistence is an elegant balance between viral immune evasion strategies and regulation mediated by $CD8^+$ and $CD4^+$ T-cells specific to a hierarchy of latent and lytic EBV antigens [23*,29]. The notion that Pf-malaria interferes with immunological control over EBV occurred early on [30] even before studies directly assessing the impact of malaria on EBV-specific T-cell function were conducted. These seminal studies measured *in vitro* regression of EBV-induced B-cell transformation and concluded that acute malaria infection and chronic malaria exposure resulted in loss of EBV-specific T-cell immunity [31-33]. This assay measured T-cell immunity against latent but not lytic cycle antigens [34] and were not compared to T-cell responses to Pf-malaria antigens or other measurements of global immune competence.

A more recent study measured EBV-specificity and function by IFN-γ ELISPOT, concluding that children 5-9 years of age, after recurrent Pf-malaria infections, had impaired response to both lytic and latent EBV CD8+ T-cell epitope-peptides compared to children from a hypoendemic area [35]. A transient impairment of IFN-γ ELISPOT recall responses around an episode of acute Pf-malaria was consistent with earlier regression studies. However the decrease in T-cell immunity did not accompany a concomitant increase in EBV load, although viremia remained significantly higher in Pf-malaria cases compared to healthy controls even though T-cell responses were restored to normal levels [26]. It is possible that this loss of EBV-specific T-cells in the peripheral circulation was due to T-cell trafficking to the site of EBV replication during acute infection. A third study examined the duration of IFN-γ ELISPOT recall responses over a 2-year period using both an ecologic and individual-level definition for Pf-malaria exposure and found that T-cell responses gradually diminished to EBV lytic but not latent antigens in 5-9 year old malaria-exposed children compared to other age groups and non-malaria exposed children (Snider and Moormann, unpublished data). In contrast, IFN-γ recall responses to EBV lytic and latent antigens in children diagnosed with eBL were robust; however, the effector cell secreting IFN-γ cannot be identified by ELISPOT [36**]. Table 1 summarizes relevant studies directly investigating the impact of Pf-malaria on immunity to EBV.

Advancements in polychromatic flow cytometry have enabled us to learn more about human T-cell differentiation and function [37] and EBV-specific T-cell phenotypes, especially when blood sample volumes are limited. Using a 16- parameter flow cytometry panel developed to quantify differentiation, exhaustion, senescence and homeostatic potential for EBV-specific $CD8^+$ T-cell restricted by HLA A*0201to epitopes derived from latently-expressed and lytic phase proteins, we found that individuals residing in a malaria holoendemic area have significantly more differentiated EBV-specific $CD8^+$ T-cells compared to those residing in a hypoendemic area and this profile shift was strongest for responses to EBV latent antigens (Moormann, Price and Chattopadhyay unpublished data). Taken together, these studies suggest that Pf-malaria co-infections during the establishment of EBV-latency may negatively impact EBV-latent antigen specific T-cell lineage commitment and that recurrent Pf-malaria infections over time diminish the functional capacity of EBV-lytic antigen-specific T-cells.

Malaria's influence on B-cells (where EBV latently resides)

The development of humoral immunity is important for mediating protection against Pf-malaria, yet identifying which antibodies confer protection given the plethora of immunogenic parasite antigens remains enigmatic [2]. Sero-prevalence to malaria antigens by the age at which a child is at risk for eBL reflects cumulative lifetime exposure and could

be used to infer differences in transmission intensity when comparing populations. The caveat to applying single Pf-malarial antibody titers as a surrogate for the number or severity of past Pf-malaria infections is they are not quantitative and depend on the immunogenicity of the malaria antigen and the longevity of the antibody response being measured [38-40].

Antibodies also play a role in limiting EBV infection and reflect the clinical status of the individual [41]. During primary EBV infection, immunoglobulin (Ig) M to VCA appears first, followed by VCA IgG that persists through life. Epstein Barr nuclear antigen (EBNA1) IgG is detectable 2-4 months after infection and remains throughout life. The Z transactivation antigen (ZEBRA or Zta) is an immediate early protein indicative of EBV replication and signifies the switch from latent to lytic replication [42*]. Elevated VCA IgG antibody levels have been associated with eBL incidence [9]. A recent study found elevated anti-ZEBRA and anti-VCA levels in eBL patients compared to healthy children, and elevated ZEBRA antibodies were associated with higher EBV load in eBL children [43**]. However, there is no consensus on the cumulative effect of Pf-malaria on EBV antibody titers in otherwise healthy children. A number of early studies on acute Pf-malaria found no difference in anti-VCA titers during and after Pf-malaria infection or among residents of different malaria transmission areas [44,45]. Another study on acute Pf-malaria reported that ZEBRA IgG levels were significantly higher among children infected with Pf-malaria compared to controls [25]. The only study to examine the effect of chronic Pf-malaria on EBV antibody titers found higher IgG titers to EBNA1, VCA, and ZEBRA in children from a malaria holoendemic area compared to children from a hypoendemic area [46**]. Meanwhile, a significant decrease in IgG titers for all EBV antigens with increasing age was observed in children from the hypoendemic area [46**]. These observations suggest that antibody titers may be useful to monitor cumulative EBV latency and lytic reactivation but are not supple enough to directly measure the influence of Pf-malaria on EBV-infected B cells.

Pf-malaria has been shown to be a powerful B-cell mitogen inducing hypergamma-globulinemia [47-49]. The cysteine-rich interdomain region 1α (CIDR1α) domain of the Pf erythrocyte membrane protein 1 (PfEMP1) has been shown *in vitro* to activate proliferation of B-cells from individuals with no previous malaria exposure, preferentially targeting activation of memory B-cells, and providing protection against apoptosis [50]. A direct link between CIDR1α and EBV-infected B-cells has also been demonstrated suggesting CIDR1α could trigger reactivation of EBV during Pf-malaria infection [51]. Directly testing this interaction in vivo presents the obvious limitation for human studies, nonetheless the possibility remains that chronic Pf-malaria stimulation could continue to reactivate latently infected B-cells even in the absence of acute malaria infections.

More recent attention has focused on the generation of human B-cell memory subsets and deciphering signals that determine if antibodies are secreted from long- or short-lived plasma cells [52-54]. As an immune evasion strategy malaria appears to directly influence B-cell differentiation by impeding the development of long-lived memory B-cells and allowing the generation of atypical short-lived memory B-cells [55*,56]. Considering that B-cells are where EBV persists, integrating the influence of EBV on the B-cell compartment with signals from Pf-malaria poses choreographic challenges [57*]. Flow cytometry allows the delineation of naïve B-cells into memory subsets. CD10 is a classical marker for germinal center B-cells and eBL tumor cells [10]. However, CD10 is also expressed on immature transitional B-cells present during chronic infection [58*]. In healthy EBV sero-positive individuals, EBV is strictly cell-associated and found in the memory B-cell compartment [10]. Children with acute clinical malaria are found to have a significantly higher number of transitional B-cells in their peripheral circulation [59]; and this subset is more susceptible to EBV infection compared to other memory B-cell subset and also

expresses elevated levels of microRNA which is important in blocking B-cell differentiation [60*]. Malaria-induced alterations in B-cell differentiation in conjunction with identifying possible alternative EBV reservoirs has yet to be fully investigated.

Conclusion

There is still much to learn about the host orchestration of recurrent Pf-malaria infections and EBV persistence that crescendo into eBL. Unexplored moderators of immunity include innate immune signals, other lymphocyte subsets such as natural killer cells, gamma-delta T cells and regulatory T cells. Exploring human genetic susceptibility for eBL necessitates large DNA repositories linked with medical informatics than may shed new light on etiologic pathways. Designing studies using a systems biology approach will allow us to encompass the complexity and diversity of human immune responses to infectious diseases whereby our understanding of eBL pathogenesis will achieve a new zenith.

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Key points

- Acute and chronic Pf-malaria infections may differentially impact T-cell immunity to EBV latent and lytic antigens.
- A systems biology approach simultaneously measuring T-cell immune control over EBV latency and lytic reactivation during acute and chronic Pf-malaria infections will improve our understanding of eBL etiology.
- An improved understanding of the sequence of events shaped during Pf-malaria and EBV co-infections will help target eBL prevention strategies.

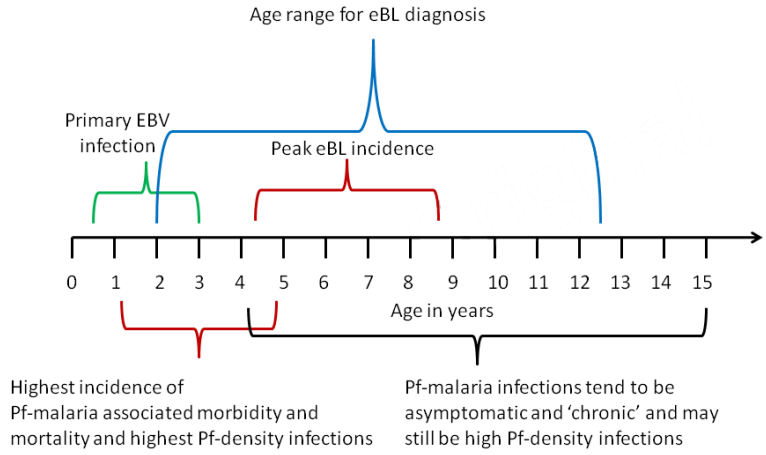

Figure 1. Chronology of Pf-malaria and EBV infections in relation to eBL incidence

Timing of co-infections prior to peak age-related incidence of eBL suggest a cumulative interaction that results in the deterioration of immune control over EBV.

Table 1

Studies of Pf-mediated loss over EBV-specific T-cell control

Author and country of study	Study Design and population	Malaria endemicity	Key points
Klein [30], 1976 USA	***Symposium*** "Immunological Control of Virus-associated Tumors in Man: Prospects and Problems"	eBL incidence geographically associated with holoendemic Pf-malaria	**1** Endemic (ie African) BL was identified as the only tumor where EBV was consistently detected. **2** Pf-malaria proposed as a co-factor but mechanisms unknown. **3** "Immunologic orchestra" to control EBV mediated by cytotoxic T cells and T-cell memory.
Moss [32], 1983 Papua New Guinea	Cross-sectional study of asymptomatic adults	Compared holoendemic (n=55), hypoendemic (n=35) and non-malaria exposed (n=27) adults	**1** Higher in vitro regression endpoints for adults from holoendemic area compared to hypoendemic control groups. No difference between two control groups. **2** Spontaneous transformation of B-cells in individuals from holoendemic area but not control groups.
Whittle [33], 1984 Gambia	Acute Pf-malaria cohort of children	Hypo- to meso-endemic malaria with repeat measures around a single episode of febrile Pf-malaria in children 5-18 years old. (n=9)	**1** Higher in vitro regression indices during acute Pf-malaria infection compared to convalescence. **2** During acute Pf-malaria infection, number and proportion of T-helper cells was reduced. **3** Proportion of B-cells higher during acute Pf-malaria infection.
Gunapala [31], 1990 UK	Case-control study of adults	Acute Pf-malaria cases in UK residents with no previous history of malaria (n=20) compared to healthy controls (n=18).	**1** Higher in vitro regression indices for acute Pf-malaria cases than malaria-naïve controls. **2** Malaria cases had higher numbers of B-cells and CD8+ T-cells; ratio of CD4:CD8 reduced compared to controls. **3** Greater proportion of spontaneously transformed B-cells from Pf-malaria cases compared to control groups.
Moormann [35], 2007 Kenya	Cross-sectional study of asymptomatic, EBV-seropositive children	Compared holoendemic (n=104) and hypoendemic (n=127) malaria exposed children 1-14 years old.	**1** No difference in EBV-specific cytotoxic T-cell responses measured by IFN-γ ELISPOT between two groups of children. **2** When stratified by age group, children 5-9 years old from malaria holoendemic area had significantly fewer IFN-γ responses to EBV lytic and latent antigens compared to other age groups and children from the hypoendemic area. **3** Children from holoendemic area had significantly less Interleukin-10 responses to EBV lytic peptides compared to children from hypoendemic area.
Njie [26], 2009 Gambia	Case-control study of children	Hypo- to meso-endemic malaria with repeat measures around a single episode of febrile Pf-malaria in children 3-11 years old (n=57), age-matched with asymptomatic, aparasitemic children (n=40), Gambian (n=9) and UK (n=6) aparasitemic adult	**1** HLA-B*3501 restricted IFN-γ ELISPOT responses were not significantly different between Gambian and UK adults, though there was a trend for median EBV lytic but not latent responses to be lower in Gambians. **2** Gambian children had fewer IFN-γ ELISPOTs to pooled EBV lytic and latent antigens during acute Pf-malaria infection compared to recovery levels and to

Author and country of study	Study Design and population	Malaria endemicity	Key points
	and adults	controls	adult controls (no significance testing due to small sample size).
Moormann [36], 2009 Kenya	Cross-sectional study of eBL and healthy children.	Compared holoendemic (n=60) and hypoendemic (n=60) malaria exposed children to children with eBL (n=44)	**1** First study to directly measure EBV-specific T cell immunity in children diagnosed with eBL and discovered that eBL children were deficient in IFN-γ ELISPOT responses to EBNA1 (the sole EBV antigen expressed by eBL tumor cells) but not EBV lytic and latent CD8-epitope peptides. **2** eBL patients had IgG1 subclass antibodies to EBNA1, similar to control groups suggesting loss of EBNA1-specific Th1-polarized T cells. **3** Lack of IFN-γ ELISPOT to EBNA1 was associated with higher EBV load.
Snider and Moormann (unpublished data)	Repeat cross-sectional study from same children over 2 year period.	Compared holoendemic (n=66) and hypoendemic (n=83) malaria exposed children over time	**1** Using both a geographic and individual-level definition of malaria exposure found a gradual decrease in EBV lytic antigen but not latent antigen responses measured by IFN-γ ELISPOT. **2** Children 5-9 years of age had significantly diminished IFN-γ responses to EBV lytic but not latent antigens associated with malaria exposure compared to other age and malaria exposure groups.