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Caline G. Matar Date

MHV68 and *Plasmodium* co-infection

By

Caline Ghassan Matar

Doctor of Philosophy

Graduate Division of Biological and Biomedical Sciences

Microbiology and Molecular Genetics

_____

Samuel H. Speck, Ph.D.
Advisor

_____

Tracey J. Lamb, Ph.D.
Co-mentor

_____

Arash Grakoui, Ph.D.
Committee member

_____

Eric Hunter, Ph.D.
Committee member

_____

Joshy Jacob, Ph.D.
Committee member
Accepted:

_____

Lisa A. Tedesco, Ph.D.

Dean of the James T. Laney School of Graduate Studies

_____

Date

MHV68 and *Plasmodium* co-infection

By

Caline Ghassan Matar

BSc. American University of Beirut, 2009

Advisor: Samuel H. Speck, Ph.D.

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ABSTRACT

MHV68 and *Plasmodium* co-infection

By Caline G. Matar

Plasmodium infection causes nearly 1 million deaths annually, mostly comprising children younger than 5 years of age. Children living in areas endemic for *P. falciparum* transmission are thought to acquire immunity to non-cerebral severe forms of disease within 1-2 infections, however, 10-20% of these children progress to severe disease. *P. falciparum* infection is highly associated with Epstein-Barr Virus (EBV) co-infection in Sub-Saharan Africa. Children repeatedly infected with *P. falciparum* have an increased risk of developing endemic Burkitt's lymphoma (eBL). EBV specific CD8+ T cell responses were shown to be attenuated in eBL patients and are thought to be a consequence of repeated and high exposure rates to *P. falciparum* infection. One aspect of such co-infections that has largely been neglected is the role of a primary asymptomatic EBV infection in modulating immunity to malaria. Asymptomatic EBV infection has been shown to suppress the humoral immune response transiently, and this may render a host susceptible to secondary infections. Co-infection with the Murine gammaherpesvirus 68 (MHV68) transforms the non-lethal *P. yoelii* XNL, but not *P. chabaudi* AS, infection into a lethal one. We demonstrate that MHV68 induces a transient suppression of the humoral immune response, which was marked by suppressed levels of germinal center B cells, plasma cells and T follicular helper cells in the spleen. This effect was dependent on the late acute phase of viral replication, but not long term latency. More importantly, we identify the M2 gene product as a facilitator of the virus induced humoral suppression. Co-infection with the M2 null virus and *P. yoelii* resulted in 94% survival and rescued the parasite specific IgG response by 28 fold. We next evaluated the impact of such co-infection on the development of long term memory B cell responses during a *P. chabaudi* co-infection model by evaluating BCR clonal diversity. Taken together, my thesis work has identified a potential role for EBV, which ubiquitously infects the population, in having substantial effects on the host immune response, thereby predisposing the host to secondary opportunistic infections. This novel finding largely supports efforts directed towards the development of EBV specific anti-viral drugs and vaccines.

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PREFACE

The major focus of my thesis work relates to MHV68 and *Plasmodium* co-infection. However, during my graduate career, I also conducted independent studies relating to host regulatory transcription factors and how they regulate the viral life cycle. Our findings are published in the Journal of Virology and are included as Chapter III in this dissertation.

CHAPTER I

INTRODUCTION

I. Co-infection with EBV and *Plasmodium falciparum* is ubiquitous in sub-Saharan Africa

More than 200 million individuals worldwide are infected with *Plasmodium* on a yearly basis and of these approximately 1 million die, largely children under 5 years of age and pregnant women [1]. In Sub-Saharan Africa *Plasmodium falciparum* is responsible for over 90% of infections and disease severity is spectral, ranging from mild febrile illness to life-threatening conditions such as cerebral malaria (CM) and severe malarial anemia (SMA) [2]. As suggested by the age of fatalities, the most complicated forms of malaria are largely restricted to childhood [3], although not all children living in endemic areas suffer severe disease. Multiple factors contribute to disease severity including host genetics, parasite virulence and transmission intensity [4]. Immune responses to each stage of the parasite life cycle that contribute to the development of protective immunity have been described in more detail elsewhere [5]

Compounding the malaria landscape for children residing in endemic areas is the high prevalence of concomitant infections with other pathogens. Children in sub-Saharan Africa experience their primary Epstein-Barr virus (EBV) infection within the first years of life [6-10]. Contrary to more affluent societies, where primary EBV infection is characterized by the symptomatic manifestation infectious mononucleosis (IM) that occurs mainly during late adolescence and early adulthood [8], infections of young children are typically asymptomatic. EBV and malaria co-infection is postulated to negatively impact the generation of an immune response that effectively controls persistent EBV replication (reviewed in [11]). Deficiencies in immune control of latent EBV infections is thought to be permissive to the development of endemic Burkitt's lymphoma (eBL), a B cell tumor that occurs predominantly in children 5-9

years of age in Equatorial Africa, first described by Dennis Burkitt in 1958 [12]. Studies from children with eBL suggest that this EBV-associated tumor results, in part, from a loss of EBV-specific T cell responses in children after chronic exposure to *Plasmodium falciparum* [13-17] that consequently reactivates latent EBV [18-20]. Here we examine the converse hypothesis that immune modulation by EBV may also have an impact on the severity of malaria infections.

EBV infection has distinct lytic and latent life cycles and maintains persistence throughout the lifetime of the host. Gammaherpesviruses such as EBV are lymphotropic viruses that primarily infect and maintain latency in the memory B cell (**Figure 1**). The humoral immune response has been shown to play a key role in clearance of circulating infected red blood cells (iRBCs) during malaria [21,22]. Given that the B cell compartment is the primary niche for EBV, it is plausible that the humoral immune response to malaria may be altered during EBV co-infection. Indeed, one study noted higher EBV viral loads were associated with increased susceptibility and frequency of malaria attacks in children in Gabon [23]. Although most children under the age of 2 are co-infected with both EBV and malaria, not all children will have acute co-infection with EBV and malaria. This may vary depending on entomological inoculation rates for malaria and the viral shedding status of the mother and other children in the community. The timing of primary EBV infections may lead to diverse outcomes with respect to the generation of anti-malarial immunity and disease severity.

We propose that that risk of developing severe malaria in young non-immune children is dependent on the force of infection (FOI) of EBV as well as malaria, as suggested by epidemiological studies for the risk of developing eBL. Malaria FOI exhibits seasonality and is correlated with age [24]. The peak FOI occurs in children 2-5 years of age coinciding with the age range of highest malaria morbidity [25]. In fact, EBV infection was shown to occur at a younger age in children living in areas holoendemic for *P. falciparum*. This earlier infection rate geographically correlated with an increased risk in developing eBL later in life [26]. The time between primary EBV infection and the development of eBL provides an opportunity for the

virus to replicate during malaria co-infections [27]. We and others speculate that this repeated interplay between pathogens results in progressively diminished EBV-specific T cell control, which has previously been proposed as the mechanism by which malaria induces progression to eBL [28].

II. Can gammaherpesviruses alter the pathogenesis of malaria infection?

To our knowledge, there are currently no published studies that have investigated the impact of a primary EBV and *Plasmodium* co-infection on the development of severe malaria in infants. However it is currently feasible to examine whether a gammaherpesvirus infection could exacerbate the pathogenesis of malaria using well defined rodent models.

a. *Rodent models of malaria infection*

There are several well-described models of malaria infection using parasites isolated from thicket rats in the 1960s [29] that collectively demonstrate different aspects of the pathogenesis of human malaria infections, and that share several key characteristics of immune responses to human *Plasmodium* parasites. *Plasmodium* infection is transmitted through the bite of the female *Anopheles* mosquito. It injects sporozoites that migrate to hepatocytes and replicate asexually, a phase of the infection that lasts close to a week and is clinically asymptomatic. The replicating sporozoites generate large numbers of merozoites that are released from the ruptured hepatocyte and infect red blood cells (RBCs), replicating asexually in a synchronized cycle lasting 24-72 hours, depending on the parasite species. It is this erythrocytic phase of the *Plasmodium* life cycle that is accompanied by febrile illness and the other clinical symptoms of malaria.

Rodent malaria models can be used to examine certain aspects of *Plasmodium* infection reflective of human infections. The *Plasmodium chabaudi* AS and *Plasmodium yoelii* XNL models of malaria are non-lethal in most strains of mice whereas *Plasmodium berghei* ANKA infections of mice on a C57BL/6 or CBA background mimic some features of cerebral malaria

(CM) and is commonly referred to as experimental cerebral malaria (ECM). Immunologically, *Plasmodium* infection induces inflammatory responses within hours of infection in both mice and humans. Reports on human *Plasmodium* infection indicate a positive correlation between IL-12 levels and protection from severe malaria [30-33]. Although there are contradicting results regarding the ability of *P. falciparum* to activate dendritic cells (DCs) [34-37], activation of rodent DCs exposed to iRBCs of rodent malaria parasites [38-41] has been repeatedly shown. In general, DC activation is thought to lead to a Th1 response and this can be measured in peripheral blood mononuclear cells (PBMCs) of individuals infected with malaria and is also a feature of rodent malaria models. Interferon-γ (IFN-γ) can be produced from NK cells, γδ T cells, CD4+ T cells and CD8+ T cells both in humans [42-45] and rodents [46-51] infected with malaria. Antibodies are thought to be a primary mechanism of controlling the erythrocytic stage of malaria infection [21,52] and multiple immunodominant parasite antigens have been identified. In fact, in one study, the breadth of the antibody response with respect to reactivity to multiple antigens has been correlated with protection against developing clinical malaria [52]. The requirement for antibody to control the primary peak of parasitemia in the *P. chabaudi* and *P. yoelii* models of non-lethal malaria differs, with CD4+ T cell-mediated immunity being sufficient to control *P. chabaudi* infection [53]. Although the development of B cell immunity against *Plasmodium* infection appears to require constant challenge infections in humans, the *P. chabaudi* model of malaria has been used to demonstrate that memory B cells can form in rodent malaria infections [54] and contribute to protection against secondary challenges [55,56].

b. *The MHV68 rodent model of gammaherpesvirus infection*

Gammaherpesviruses are capable of maintaining lytic and latent infection in the host. The Murine gammaherpesvirus 68 (MHV68) has been used extensively to understand gammaherpesvirus infection in humans, which exhibits restricted host tropism. The two known human gammaherpesviruses include EBV and Kaposi's sarcoma associated herpesvirus (KSHV). MHV68 encodes for conserved gene products seen in both EBV and KSHV viral genomes. Of

importance, analysis of MHV68 infection in the mouse has been instrumental in understanding the lytic phase of infection, which is difficult to assess in human subjects. Viral transmission is postulated to occur orally as evidenced by the enumeration of EBV titers in the saliva of patients suffering from IM. In the lab setting, intranasal infection with a luciferase expressing MHV68 virus indicated viral replication and presence in the cervical draining lymph nodes, the lungs, the spleen (splenomegaly) and vagina during female C57BL/6 infections [57]. Splenomegaly is observed in EBV infected patients experiencing IM [58] and viral titers have also been detected in female and male genital secretions [59,60]. Intranasal infection of C57BL/6 mice consists of an acute lytic replication in the lungs, peaking around 4-6 days and cleared by day 12 post-infection. Acute lytic replication in the lung seeds B cells travelling to the spleen, which initiates germinal center responses and splenomegaly [61]. Nearly 80% of virus infected B cells show a germinal center phenotype around day 18 post-infection [62]. By day 16-18 post-infection, the virus reaches peak latency levels, with a reduction in productive viral replication, and 1:100 cells being latently infected with virus. Frequency of viral reactivation is approximately 1:1000 at this time point, and is almost undetectable by day 42 post-infection.

The virus maintains latency predominantly in the memory B cell [63] reservoir but is capable of infecting macrophages and DCs [64]. Of importance are the two distinct phases of viral replication. In this review, the initial lytic infection of the virus in a naïve host which consists of fulminant viral replication in the lung and inflammatory response induction will be referred to as: acute primary infection. The strong inflammatory response induced by acute infection generates memory to a primary viral infection. The second phase of viral infection is reactivation from latency, and is considered to be a distinct aspect of the viral life cycle. Low level reactivating virus does not generate potent inflammatory responses in an immune competent host, and is required for seeding the latency reservoir. In the mouse, the second phase of viral reactivation from latency can be assessed by monitoring levels of preformed virus in the lung and spleen 18 days post-infection. If pre-formed infectious viral levels persist, the infection is not considered to

be properly controlled. The equivalent of this type of infection in humans, using our definition, is the asymptomatic primary infection of a naïve child who has lost maternal antibody protection against EBV. This primary infection, although not symptomatic, will generate a memory response, the formation of which is dependent on the innate inflammatory response. This immune response is also distinct from viral reactivation from latency in which stimuli that expand the latently infected pool will induce virus production. In an immune competent host, the anti-viral response is considered to be effective and will ensure proper control of reactivating virus [64]. An ELISA-based technique can discriminate between a primary, recent primary or reactivating EBV infection in children [65]. This assay feasibly could be used to evaluate the stage of EBV infection present in children experiencing severe malaria.

Control of MHV68 infection is not exclusively dependent on CD4+ T cells, CD8+ T cells or B cells during the acute phase. However, all three subsets perform a non-redundant role in host protection during latency and are required to control chronic viral replication [66-70]. MHV68 induces a potent Type I interferon (IFN) response that is required for proper control of acute infection and prevention of host lethality [71]. The virus can be detected by Toll-Like Receptor 9 (TLR9) which senses hypo-methylated CpG motifs in the viral DNA [72]. Infection in TLR9-/- animals results in slightly higher levels of viral titer in the lungs, but does not significantly alter clearance of the infection or establishment of latency [72]. In contrast, MyD88-/- animals clear acute infection normally, but have a defect in the establishment of latency [73] which could be due the requirement of TLR signaling in B cells for the generation and maintenance of germinal center responses during an infection [74]. Recent work also indicates that the unique M2 gene of MHV68 contains an Interferon Sensitive Response Element (ISRE) to the transcriptionally repressive Interferon Response Factor 2 (IRF2) [75]. This ISRE allows the reactivating virus to be responsive to Type I IFN signals and negatively regulate the viral life cycle, minimizing host lethality while maintaining a latency reservoir.

c. *Co-infection with MHV68 and malaria in rodents*

We, and others, have tested the impact of acute MHV68 infection on the pathogenesis of erythrocytic non-lethal *P. yoelii* XNL and *P. chabaudi* AS or lethal *P. berghei* ANKA co-infections in C57BL/6 mice. Primary infection with a gammaherpesvirus can alter the pathogenesis of a secondary malaria infection (Matar *et. al.*, Chapter II)) and this may be because acute gammaherpesvirus infection impairs the ability of mice to mount appropriate immunity to subsequent malaria infections. Of relevance to the pathogenesis of malaria, MHV68 infection rendered a deficiency in the generation of malaria-specific CD8+ T cell responses (Amante *et al.*, unpublished data) and the maintenance of T follicular helper cells that impacted the sustained output of plasma cells in the spleen (Matar *et al.*, Chapter II)). The net effect of this suppression reduced the CD8+T cell-mediated pathology associated with ECM in the *P. berghei* ANKA model (Amante *et al.*, unpublished data) and transformation of the non-lethal *P. yoelii* XNL into a lethal infection [76] as a result of poor anti-malarial humoral immunity, loss of control of the primary peak of parasitemia and the development of lethal SMA (Matar *et al.*, Chapter II). No measurable effect on the primary peak of parasitemia in mice infected with *P. chabaudi* AS was noted (**Table 1**) as might be expected from the ability of B cell deficient animals to control the primary peak of parasitemia with this infection [53] .

Immunologically, it is clear from all three models that MHV68-infection is associated with an immune suppression of specific subsets in the adaptive immune response in mice. C57BL/6 mice acutely infected with MHV68 lose their ability to induce antibody responses to secondary challenge of an unrelated antigen [77]. This transient effect has also been observed in young adults experiencing acute EBV infection [78,79]. Importantly, for the MHV68-malaria co-infection studies described above, the effects of MHV68 infection are dependent on acute and not latent infection, with the detrimental effects of MHV68 infection wearing off after about 30 days. It has been demonstrated for the *P. chabaudi* and *P. yoelii* non-lethal rodent co-infection models that latent MHV68 infection has very little impact on control of the parasite infection [76] (Matar

et al., Chapter II). In this respect, malaria differs from other parasitic infections such as intracellular bacteria *Listeria monocytogenes* [80] and *Yersinia pestis* [81] which show that MHV68 latency, but not acute infection, could protect against infection by maintaining a basal secretion of IFN-γ and activation of macrophages. Given that monocyte and macrophage activation is also considered to play a protective role in phagocytosis of iRBCs and free merozoites [82,83], it is surprising that a bigger effect of latent MHV68 infection on control of malaria infection is not seen. This could arise if iRBCs are better than *L. moncytogenes* or *Y. pestis* at evading macrophage clearance to establish infection, not entirely unfeasible given the sequestration properties of iRBCs which are thought to have evolved to avoid splenic clearance from the circulation [84]. The data in rodent co-infections described above demonstrates that primary gammaherpesvirus infection can alter immunity generated to an incoming infection with *Plasmodium* and in some scenarios this results in an alteration of the pathogenesis of malarial disease in mice.

III. Gammaherpesvirus induced suppression of the innate immune response to malaria

Although the effects of acute MHV68 infection on malaria pathogenesis appear to stem from alterations in the anti-malarial adaptive immune response, adaptive immune responses are generally driven by innate immunity. The ability of gammaherpesviruses to specifically modulate the innate immunity may therefore have interesting implications on the immunobiology of malaria during co-infection (**Figure 2**). Despite the importance of the inflammatory response in curbing the extent of viral infection to minimize damage to the host, the virus has adapted mechanisms to ensure that the innate immune response does not sterilize the infection. These key immune evasion mechanisms consist of an evolutionary adaptation by the virus that supports entrance into and maintenance of latency, which is a unique feature of the herpesvirus family

(reviewed in [85]). Key mechanisms of immune evasion have been documented in both the murine and human gammaherpesviruses. Downstream effectors of the Type I IFN response include the Interferon Regulatory Factors (IRFs). IRFs are integral to TLR signaling and induce NF-κB mediated signaling. The MHV68 encoded ORF36 can directly inhibit cellular IRF3 [86] and the latency associated protein of MHV68, ORF73 (LANA), can directly inhibit NF-κB signaling by targeting the p65 subunit to ubiquitin mediated proteasomal degradation [87,88]. Such features are not unique to this rodent gammaherpesvirus as KSHV can negatively regulate IFN signaling through the expression of homolog viral-IRF (vIRF) and v-IL-6 proteins [89-92]. The immediate early gene product of EBV, BZLF1, is capable of inhibiting IRF7 [93] and BGLF4 is an inhibitor of IRF3 signaling [94]. Collectively, these immune modulating mechanisms are required for proper initiation of the viral life cycle of gammaherpesviruses and thus are primarily employed during acute rather than latent infection (reviewed in [95]). Inhibition of IRF impedes the production of key inflammatory cytokines such as the type 1 IFNs IFN-α and IFN-β which limit viral replication, promote apoptosis of the infected cell and activate antigen presenting cells (APCs) to stimulate the antigen specific T cell helper responses. This virus-induced immune regulation may negatively regulate secondary adaptive immune responses.

Similar to gammaherpesviruses, *Plasmodium* induces type 1 IFN production from APCs via ligation of TLR9 by DNA/protein complexes trapped in hemozoin crystals [96,97], an insoluble byproduct of hemoglobin digestion in RBCs. However the role that type 1 IFNs play in anti-malarial immunity appears to vary amongst rodent models. Published studies do not agree on the effect of type 1 IFN on controlling clearance of iRBCs during *P. chabaudi* infection [98,99]. In contrast, type 1 IFN may help to control the frequency of iRBCs during *P. yoelii* infection due to its suppressive effects on erythropoiesis [100] which reduces the release of reticulocytes into the circulation [101], the target cell in a *P. yoelii* XNL infection. Nevertheless type 1 IFN has been shown to promote hematopoietic stem cell exhaustion [102,103] and in the context of viral infections, type 1 IFN has been shown to have suppressive properties on hematopoiesis during

lymphocytic choriomeningitis virus (LCMV) infections [104]. This suggests a potential role for chronic Type I IFN signaling in the exacerbation of severe malarial anemia (SMA), a condition where erythropoietic and hematopoietic suppression is a key feature [105]. Indeed the human immunodeficiency virus (HIV), although not a herpes virus, is associated with induction of type 1 interferon [106] and published studies suggest that *P. falciparum* and HIV co-infection can be associated with exacerbation of SMA [107,108].

Type 1 interferon can suppress the CD4+T cell response in the *P. berghei* ANKA model of malaria [76]. Although the mechanism by which this occurs is not clearly understood, it is likely to be via an effect on the antigen presenting cells (APCs) which activate T helper cells. In fact, recent work has demonstrated that MHV68-infected DCs could not up-regulate activation markers and present antigen as efficiently as those not exposed to MHV68 [109]. In a similar vein, EBV infection of monocytes prevented their maturation into DCs and promoted apoptosis [110]. Of interest, the expression of DC activation markers during an MHV68 and *P. berghei* co-infection was reduced as compared to a single *P. berghei* infection (Amante *et al.* unpublished) supporting a model that implicates virus-modulated immune responses as negative regulators of APC activation during a co-infection with malaria.

Gammaherpesviruses have been shown to increase levels of IL-10 production during an infection. MHV68 does this via the unique gene product M2 [111,112]. Similarly EBV encodes for a viral IL-10 homolog [113]. From the perspective of EBV, virus-induced IL-10 production is postulated to increase B cell proliferation, thus expanding the latency reservoir. With regards to the effect of virus infection-derived IL-10 on a malaria infection, there are multiple effects on anti-malarial immunity that may be observed depending on which cell types may be affected. For example, IL-10 is generally regarded as having an immunosuppressive effect on Th1 responses [114] and rodent models of malaria suggest that it can impair control of circulating *Plasmodium* parasitemia. However, IL-10 seems to play a critical role in protection against over-exuberant inflammatory immune responses which lead to pathogenesis during a malaria infection [46]. The

effect of virus-induced IL-10 on T cell activation has not been formally demonstrated in either mouse models of infection or in human EBV infection. Interestingly, levels of IL-10 production from CD4 T cells were not altered during MHV68 co-infection with either *P. yoelii* or *P. chabaudi* (Matar *et al.* Chapter II).

Chronic influenza infection was recently shown to induce Indoleamine 2,3 dioxygenase (IDO) production [115]. IDO is an enzyme that catalyzes the breakdown of tryptophan (TRP) via the kynurenine pathways [116] and negatively regulates Th1 CD4/CD8 T cells, while activating the CD4+ FOXP3+ T regulatory subset. One of the signals that promote IDO induction is IL-10, a by-product of gammaherpesvirus infection, and in the absence of IDO expression, greater proliferation of effector CD8 T cells eliminated the viral infection [112]. Multiple pathogens, including *Plasmodium*, are capable of inducing IDO production to create localized immunosuppressive niches that promote pathogen survival [117-120]. There is no current evidence suggesting a role for gammaherpeviruses in induction of IDO expression. However, there were no alterations to the CD4+ FoxP3+ T reg subset during MHV68 and *P. yoelii* or *P. chabaudi* co-infection (Matar *et al.* Chapter II). Additionally, Th1 CD4+ T cells in both these co-infection models did not exhibit any signs of exhaustion and produced normal levels of IFN-γ and IL-10 in response to CD3/CD28 stimulation as compared to single *Plasmodium* infection.

IV. Modulation of CD8+T cell responses during EBV/*P. falciparum* co-infection: does it matter to malaria?

It has been demonstrated that T cell memory to specific pathogens may be impaired during secondary successive and chronic secondary viral infections [121,122]. Thus, it is postulated that repeated exposure to *P. falciparum* infection in endemic areas results in the exhaustion of EBV-specific CD8+ T cell responses. Indeed, the primary molecular link between EBV and *P. falciparum* co-infection is related to the defective anti-viral CD8+ T cell response that results in

the outgrowth of virally transformed B cells and results in eBL [13-16,18,19,123]. Amante *et al*. have shown that MHV68 and *P. berghei* co-infection resulted in reduced *P. berghei* specific CD8+ T cell responses (unpublished data) suggesting that EBV infection could also impair the generation of pathogenic malaria-specific CD8+T cells.

CD8+T cells are expanded in malaria infection, but much of the focus on the target and function of these cells has centered on liver stage malaria whereby CD8+ T cell-mediated lysis of infected hepatocytes containing developing parasites is considered to be a mechanism of protection against pre-erythrocytic malaria (reviewed in [5]). In this context, defective development or maintenance of CD8+T cell responses during primary EBV infection may lead to increased numbers of liver stage parasites in turn increasing blood stage parasitemia or multiplicity of infection. However, there is emerging evidence that CD8+ T cells also directly contribute to the control of blood-stage parasitemia (Lamb et al. unpublished data). Studies on the role of CD8+ T cell responses to erythrocytic malaria with respect to control of iRBCs are scarce. Only one report has described any role for CD8+ T cell activation in a non-lethal *P. chabaudi* malaria infection [124] suggesting that CD8+ T cell activation by CD11c+CD8α+ DCs is essential for control of peripheral parasitemia and prevention of hyperparasitemia-induced death that is associated SMA. Transfer of activated CD8+T cells from *P. yoelii* infected mice was able to provide some protection against a lethal *P. yoelii* strain [125]. The mechanism by which CD8+ T cell responses play a role in controlling blood stage parasitemia during non-lethal infections is unclear, however, IFN-γ production and its contribution in generating cytophilic antibody responses and activating phagocytic macrophages for iRBC clearance is likely to be instrumental.

The suppression of CD8+ T cell responses against malaria may have beneficial effects in reducing the propensity for the development of organ-specific manifestations of malaria. T cell trafficking to target organs where there is sequestration of iRBCs in ECM is thought to be a key event in the development of damage in the brain [126,127] and lung [128,129] but not liver [130].

CM is a serious condition resulting from infection with *P. falciparum* and is a diffuse of encephalopathy that is characterized by deep coma with seizures that is often fatal and affects around 3 million children world-wide in areas where malaria is endemic [1]. iRBCs are thought to sequester and adhere to the brain endothelium via interactions between variant molecules exported to the surface of the infected RBC such as *Plasmodium falciparum* erythrocyte membrane protein-1 (*Pf*EMP-1) and associated families of molecules that can interact with endothelial expressed ICAM-1 [131] and CD36 [132]. Localized inflammatory responses to sequestered iRBCs occur and, at least in the *P. berghei* ANKA model of ECM, IFN-γ driven inflammation is a critical component of adverse outcome for infected mice [126,127]. Both CD4+ and CD8+ T cells are thought to be important cellular mediators of ECM [133] driving an inflammatory immune response resulting in death between 8 and 10 days post-infection. CD8+ T cells are activated by blood stage malaria infections by cross-presentation of antigens in the spleen [50,134,135]. Thus it is possible that if the suppression of T cells extends to anti-malarial CD8+T cells the chances of developing CM during acute EBV infection may be reduced, as demonstrated in *P. berghei* / MHV68 co-infections (Amante *et al.*, unpublished data).

V. Impairment of the anti-malarial humoral immune response by gammaherpesvirus co-infection

In the context of a co-infection with malaria, some studies suggest that polyclonal B cell stimulation by *P. falciparum* results in the expansion of virally-infected B cells, thus increasing viral load in the host [18,136-139]. B cells require specific IRF signaling pathways for proper germinal center responses during infections. T-dependent antigen specific responses requires TLR stimulation of the B cell in addition to T helper cell function [74]. Since EBV is capable of inhibiting IRF and TLR signaling pathways in B cells, it is a logical assumption that virus mediated regulation of the infected B cells may impact humoral responses to secondary unrelated

antigenic challenges. The immune suppressive effects exerted by acute MHV68 and EBV infection on humoral responses have previously been reported *in vivo* [77-79] and it appears that the transient immune suppression observed was contingent on acute viral replication and specifically impacted the humoral response. This suppression was still operational in mice deficient in the majority of TLR signaling (MyD88-/-) which presumably abrogated a large portion of the TLR9-dependent type 1 interferon response to MHV68, as well as in the absence of a stimulator of interferon genes (STING) [140]. This suggests that, although type 1 interferons may be important in suppressing CD4+ T cell responses against malaria [98], the induction of type 1 interferon by MHV68 may not explain the deficiency in the generation of anti-malarial humoral immunity in the *P. yoelii* XNL and *P. chabaudi* AS models (Matar *et al*., Chapter II). Interestingly, the suppression of the humoral immune response in this study was not dependent on the anti-inflammatory cytokine IL-10 [77] but this does not rule out a dependency on other immunosuppressive molecules such as TGF-β or IDO in MHV68-induced suppression of the humoral response to secondary antigens.

In the *P. yoelii* and MHV68 co-infection model SMA causes lethality and is due to impaired humoral responses that result in uncontrolled parasitemia in the periphery (Matar *et al*. Chapter II). The observation that acute MHV68 infection can profoundly suppress the humoral response to malaria in this mouse model demonstrates that interactions can occur between these pathogens that may be important in the development of clinical malaria. This is supported by evidence for the requirement of the humoral response in clearing iRBCs [21,141] and the established link between poor humoral responses and severe malarial disease in young children [142]. MHV68 co-infection with *P. chabaudi* induced the same humoral suppression (Matar *et al*., Chapter II). However, since the humoral response is not required for control of primary peak of infection in this model, the co-infection did not impair acute malarial disease but we speculate impairment in the development of malaria specific B cell memory responses [53,56].

VI. CONCLUSION

It has been previously demonstrated that immunity to the severe form of malaria is acquired within 1-2 infections in areas of endemic transmission for *P. falciparum* [143]. Yet, despite this minimal number of infections, almost 1 million children die annually as a result of complications associated with severe malaria. This subset of individuals is also restricted to a narrow age bracket consisting of children younger than 5 years of age. Could primary EBV infection impact immune responses to a secondary malarial infection? The biology of EBV and malaria infections suggests that there are multiple points of immune syndemism that could impact the pathogenesis of malaria infection. Rodent models of EBV and malaria co-infection demonstrate that it is possible for a primary gammaherpesvirus infection to negatively modulate the generation of anti-malarial immunity, thus transforming non-lethal infections into lethal ones (Matar *et al.*, Chapter II). However, the suppressive effects of gammaherpesviruses diminish after 30 days of infection. There is no direct human evidence that primary EBV infection negatively impacts the development of *Plasmodium*-specific adaptive immune responses, nor whether the timing of primary EBV infection in relation to malaria infections lead to more severe malarial disease. The latter could be examined using longitudinal birth cohort studies to determine whether timing of primary EBV infection during the development of an infant's anti-malarial immunity impacts on the effectiveness of a response that protects from severe clinical malaria. Addressing the details of this co-infection combination has important implications if early-age primary EBV infection is a risk factor for the poor development of anti-malarial immunity and development of clinical malaria in infants.

FIGURES

Figure 1.

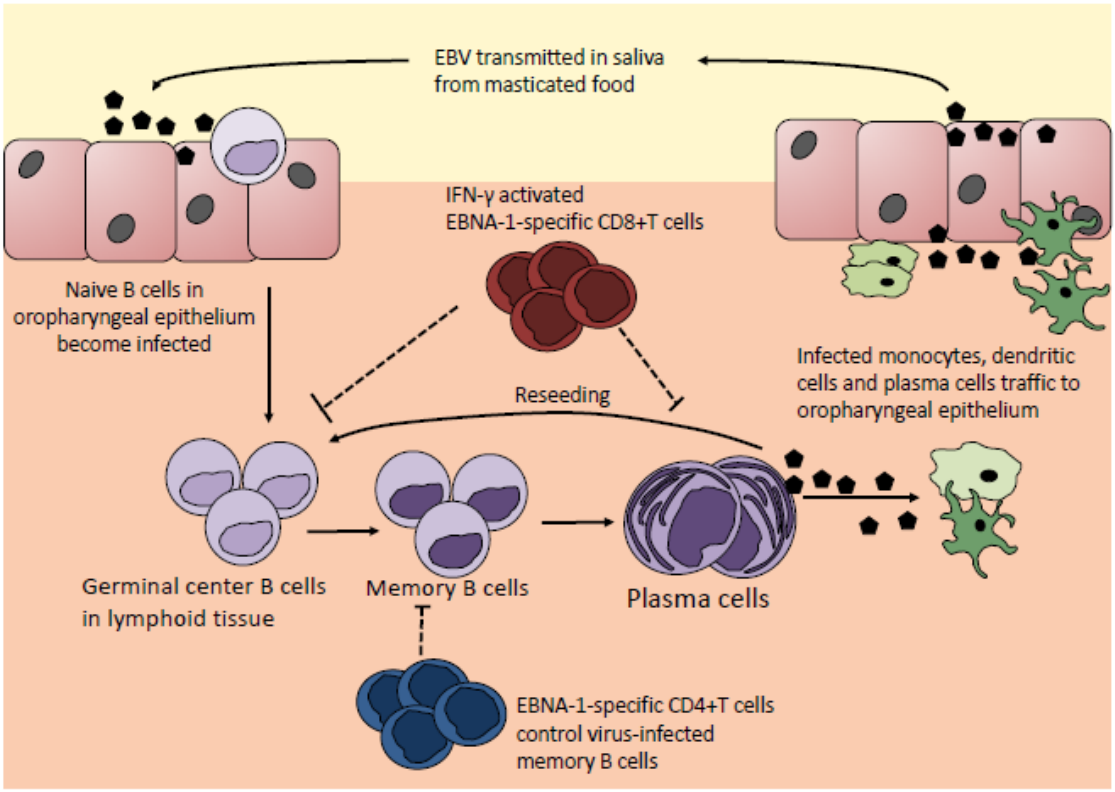

Figure 2.

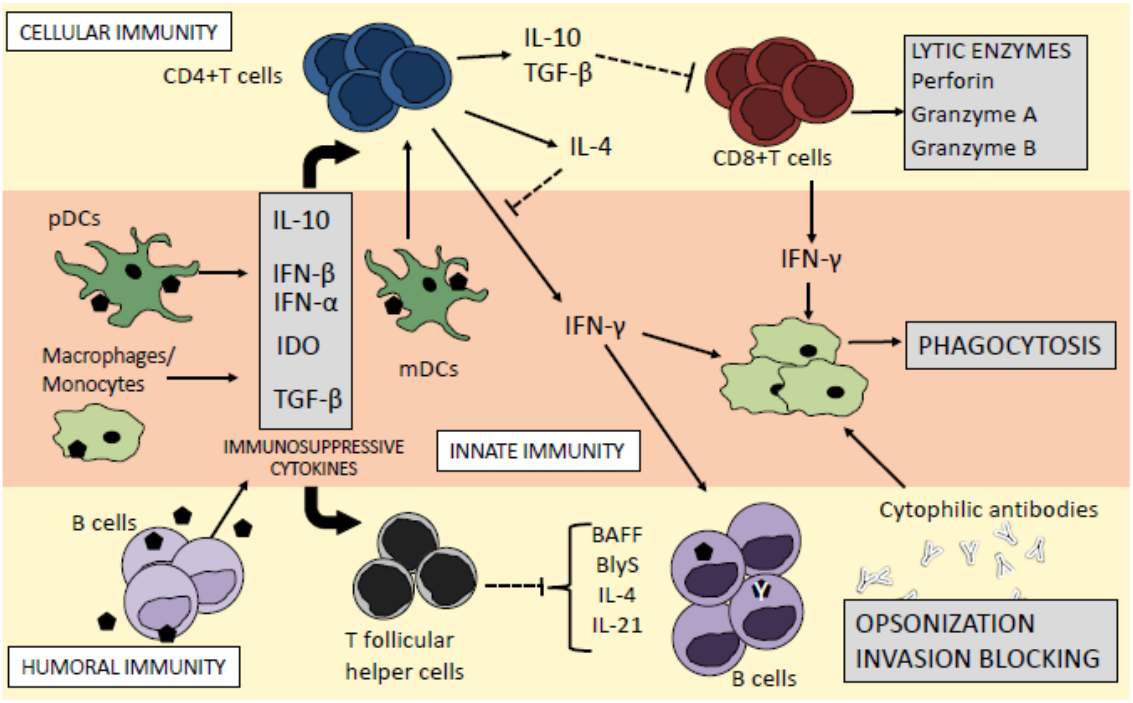
CELLULAR IMMUNITY
CD4+T cells
IL-10
TGF-β
IL-4
CD8+T cells
LYTIC ENZYMES
Perforin
Granzyme A
Granzyme B
pDCs
IL-10
IFN-β
IFN-α
IDO
TGF-β
mDCs
IFN-γ
IFN-γ
PHAGOCYTOSIS
Macrophages/
Monocytes
IMMUNOSUPPRESSIVE
CYTOKINES
INNATE IMMUNITY
B cells
Cytophilic antibodies
BAFF
BlyS
IL-4
IL-21
T follicular
helper cells
B cells
OPSONIZATION
INVASION BLOCKING
HUMORAL IMMUNITY

Table 1.

	Effect of type 1 interferon	**Requirement for cell mediated immunity**	**Requirement for humoral responses**	**Effect of overlapping acute MHV68 infection** **(less than 16 days post MHV68 - infection)**	**Effect of a latent MHV68 infection** **(more than 16 days post-MHV68 infection)**
***Plasmodium yoelii* XNL** (Matar *et al*., unpublished)	Inhibits infection by suppressing reticulocytosis [101]	Requires CD4+ T cells to control the primary peak of infection [144] CD8+T cells may provide some protection against *P. yoelii* XL [48]	Requires antibody for control of primary peak of infection [53]	Non-lethal infection becomes lethal [76] due to uncontrolled primary peak of infection	Immunosuppressive effects on humoral responses wanes after 60 days
***Plasmodium chabaudi* AS** (Matar *et al*., unpublished)	Suppresses protective IFN-γ production ([98]	Requires CD4+T cells to control the primary peak of infection [145] CD8+T cells may provide some protection against erythrocytic malaria [124,146]	Does not require antibody for control of acute peak of infection [53] Less able to control secondary peaks of infection [56]	Non-lethal infection remains non-lethal across primary peak of infection	No data
***Plasmodium berghei* ANKA** (Amante *et al*., unpublished data)	Suppresses CD4+ T cell responses and control of erythrocytic parasitaemia [98]	CD4+T cells required for development of ECM [147] CD8+T cells T cells trafficking to the brain are pathogenic [126,127,148]	Required for control of iRBCs for related species *Plasmodium berghei* NK65 [149]	Protection from death during the inflammatory phase of experimental cerebral malaria (during or just after lytic infection)	No measureable affect on death from ECM (6 weeks post-infection)

FIGURE LEGENDS

Figure 1: The life cycle of Epstein-Barr Virus

Figure 2: Hypothesized points of interaction in the immunobiology of malaria and EBV infection. EBV primarily infects B cells but also innate immune cells such as dendritic cells and monocytes/ macrophages. This leads to the induction of immunosuppressive cytokines that in turn affect CD4+T cells that are no longer effective at providing help for CD8+T cells, phagocytes, or B cells in turn suppressing the humoral response. Additional suppression of T follicular helper cells further impairs the maintenance of B cells transformation to becoming antibody-producing plasma cells. EBV is represented by the black pentagons. Points of interaction are represented by the dashed lines. Key: BAFF, B cell activating factor; BlyS, B lymphocyte stimulator; IDO, indoleamine 2,3 dioxygenase; IFN, interferon; IL, interleukin; mDC, myeloid dendritic cells; pDC, plasmacytoid dendritic cells; TGF, transforming growth factor

Table 1: Rodent models of erythrocytic malaria infection and the outcome of overlapping co-infections with MHV68 in C57BL/6 mice

CHAPTER II

Gammaherpesvirus co-infection promotes malaria lethality by impairing the generation of anti-parasitic humoral immunity

Matar CG[1,3], Anthony NR[2], O'Flaherty BM[1,3], Jacobs N[2,4], Priyamvada L[2,3], Engwerda CR[6], Speck SH[1,5,*] and Lamb TJ[2,*]

[1] Department of Microbiology and Immunology, Emory University School of Medicine, , Atlanta, Georgia 30322, USA
[2]Division of Pediatric Infectious Disease, Department of Pediatrics, Emory University School of Medicine, Emory Children's Centre, Atlanta, Georgia 30322, USA
[3]Microbiology and Molecular Genetics Graduate Program, Laney Graduate School, Emory University, Atlanta, GA 30322
[4]Population Biology, Ecology and Evolution Graduate Program, Laney Graduate School, Emory University, Atlanta, GA, 30322
[5]Emory Vaccine Center, Emory University, Atlanta, GA, 30322
[6]Immunology and Infection Laboratory, QIMR Berghofer Medical Research Institute, Herston, Brisbane, Queensland, Australia.

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Immune fluorescence images of the spleen in Figure 3 were collected in assistance with Dr. Neil Anthony. Lung histology images and pathology analysis in Figure S1 were performed by Drs. Cynthia Courtney and Anna Patricia Garcia.

INTRODUCTION

Nearly 1 million individuals die annually as a result of severe malaria, largely children under the age of 5 [1]. In regions that are endemic for *Plasmodium falciparum* transmission, mathematical modeling data suggests that immunity to severe non-cerebral malaria requiring hospitalization in children may be attained after 1-2 infections [143]. However, it is not fully understood why some children are unable to acquire immunity to severe lethal disease. Multiple factors may account for this (reviewed in [2,4]) and the presence of co-infecting pathogens in the host could be one such factor. In Sub-Saharan Africa, infants are often co-infected with Epstein-Barr virus (EBV), a gammaherpesvirus that infects B cells and maintains latency throughout the lifetime of the host [150]. Children are often seropositive to EBV by the age of 6 months in this region of the world [9] and it is well established that children infected with EBV living in areas endemic for transmission of *P. falciparum* have increased chances of developing endemic Burkitt's Lymphoma (eBL). eBL is the most lethal of childhood cancers in equatorial Africa, with the highest prevalence in children aged 5-9 years old. eBL is characterized by a c-myc translocation that results in over-expression of the oncogene (reviewed in [123]). It is postulated that repeated infections with *P. falciparum* results in a weakened anti-viral CD8 T-cell response that allows for the outgrowth of transformed B cells [13-16].

Despite the compelling evidence indicating a role for *P. falciparum* in modulating the immune responses that control EBV infection, little is known regarding the impact of acute EBV infection on the development and functionality of the immune responses that control *P. falciparum* infection. It is well appreciated that the humoral response is protective during *Plasmodium* infection. Passive immunization of children in The Gambia [21] and adults in Thailand [151] with *P. falciparum* hyper-immune serum from adult donors living in Sub-Saharan Africa allowed for control of peripheral parasitemia. Additionally, numerous studies in humans

have identified a role for increased breadth and diversity in the anti-*Plasmodium* humoral response that provides a protective advantage during clinical malaria [52,142,152,153]. Although acute EBV infection is generally asymptomatic in young children [8], virus-induced humoral immune deficiencies have been observed in one case of co-infection with a secondary pathogen [78] and in young adults experiencing a primary EBV infection and manifesting symptoms of Infectious Mononucleosis (IM) [79,154]. Although there are few reports of this phenomenon, these documented cases provide key evidence of the ability of EBV to suppress humoral responses during the acute phase of infection. This data, combined with the known role of antibody in resolution of *P. falciparum* parasitemia (refs), suggests that overlapping acute EBV infection could suppress anti-malarial humoral responses in some children and thus be a contributing factor in the development of severe malarial disease.

Acute murine gammaherpesvirus 68 (MHV68) infections of mice, like acute EBV infections in humans, can induce a transient immune suppression of the humoral response during secondary antigenic challenge [77]. Using MHV68 as a model for acute EBV infection, we have investigated whether gammaherpesvirus infection can suppress the humoral immune response to a secondary malarial infection. We have used the well-established non-lethal murine models of malaria infection *P. yoelii* XNL and *P. chabaudi* AS, and determined that acute gammaherpesvirus infection can suppress the anti-malarial humoral response during co-infection with either of these *Plasmodium* infections. This suppression results in loss of control of peripheral parasitemia in *P. yoelii* XNL, but not *P. chabaudi* AS, infection and transforms the non-lethal infection into a lethal one. This is in agreement with the course of infection in B cell deficient μMT mice where *P. chabaudi*, but not *P. yoelii*, parasitemia is controlled [53]. The reduced anti-malarial antibody response during co-infection was accompanied with a virus induced failure to maintain the T follicular helper cells subset in the spleen. As such, loss of this

critical T helper cell in the germinal center follicle resulted in loss of germinal center B cells and a failure to develop sufficient plasma cells to produce anti-malarial antibody.

We have identified that the MHV68-derived latency associated protein M2 is essential for the failure of co-infected animals to mount anti-malarial humoral responses and that this effect in the mouse model lasted up to 30 days. This identifies the acute phase of infection as necessary for virus-mediated immune suppression of the humoral response. In terms of EBV infection in humans, one case study identified the asymptomatic acute phase of EBV infection to induce immune suppression that can last up to 4 months [78]. This potentially gives a 4 month time window of humoral suppression that could substantially influence the outcome of a *P. falciparum* infection. As such, our data provides novel and compelling evidence for a need to evaluate primary acute EBV infections as a potential risk factor in the development of non-cerebral severe malaria.

MATERIALS AND METHODS

Ethics Statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Emory University Institutional Animal Care and Use Committee, and in accordance with established guidelines and policies at Emory University School of Medicine (Protocol Number: YER-2002245-031416GN).

Mice and infections

Female C57BL/6 mice (6-8 weeks) were purchased from Jackson Laboratories. μMT and RAG2-/- mice were purchased from Jackson laboratories and bred in house. IL21R-/- mice were a gift of Dr. Warren J. Leonard and were bred in house. Mice were infected with 1000 Plaque Forming Units (PFU) of MHV68 in DMEM without fetal bovine serum (FBS) either Intra-Nasally (IN) or Intra-Peritoneally (IP). Animals are infected IN with MHV68 throughout the manuscript, unless where otherwise noted. Frozen stabilates of *P. yoelii* XNL or *P. chabaudi* AS were administered IP in Kreb's saline with glucose (KSG) into donor mice. After day 7-9 after infection of the donor mouse, experimental mice were infected at a dose of 1 x 10^5 parasitized Red Blood Cells (pRBCs) via the IP route in KSG. Anemia was measured by counting RBCs from tail blood diluted in KSG using a haemocytometer [155]. Parasitemia was enumerated from Giemsa stained thin blood smears.

Limiting dilution analysis of viral lung titer and lung tissue histology

The left lung was collected for analysis of viral replication using a limiting dilution analysis as previously described [156]. Briefly, lungs were homogenized in 1ml of complete DMEM media and 1.0 mm Zirconia/Silicon beads (BioSpec products) using a BioSpec mini bead-beater 16.

Samples were homogenized 4 times (1 minute homogenization followed by 1 minute rest on ice). Samples were then transferred to a new tube with 0.5 mm Zirconia/Silicon beads and homogenization was repeated as above. Homogenate was then plated on Mouse Embryonic Fibroblasts (MEFs) in 96 well plates in serial 2 fold dilutions, up to 12 dilutions. Plates were incubated for 2 weeks in a low evaporation incubator (5% CO_2, 37^oC) and analyzed for Cytopathic effect (CPE). Results are plotted as percent of wells displaying CPE at each plated dilution. For lung tissue histology, the left lung tissue was collected for lung histology analysis. Whole tissue was fixed in 10% (v/v) Normal Buffered formaldehyde for 24 hours at room temperature. Tissue was then put into 70% ethanol solution until samples were sectioned. Samples were paraffin embedded and prepared for Hematoxylin and Eosin Staining as previously described [157].

Flow cytometry

Splenocytes were blocked with anti-CD16/32 (BD bioscience). Surface stains were performed in PBS-2% FBS- 1mM EDTA for 20 minutes on ice. Markers used: CD138-PE (BD bioscience), B220-Pacific Blue (Biolegend) or Pacific Orange (Invtirogen), CD95-PE-Cy7 (BD biosciences), GL7-Alexa Fluor 660 or FITC (eBioscience), CD3-PerCP/ Pacific Blue (BD bioscience), CD4-PerCP (BD Bioscience), CD8-Pacific Orange (Invitrogen), IL-10-PE (Biolegend), IFN-γ-APC (ebioscience), IL-2 PE-Cy7 (ebioscience) or FITC (Biolegend), purified anti-CXCR5 (BD biosciences), Biotin-SP-AffiniPure F(ab')2 Fragment Goat Anti-Rat IgG (H+L) (Jackson immunoresearch), Streptavidin-APC (Molecular probe), CD279-PE (Biolegend), CD44-Pacific Blue (Biolegend), CD25-Pacific Blue (Biolegend), FoxP3-APC (ebioscience) and CD19-FITC/PE/PE-Cy7/PerCP/ APC/Pacific Blue (BD bioscience). Intracellular cytokine stains for IL-2, IL-10 and IFN-γ were performed using the BD bioscience Cytofix/Cytoperm staining kit. Cells were stimulated (5 hours, 5% CO_2, 37^oC) in anti-CD3 coated 96 wells tissue culture plates and supplemented with soluble anti-CD28 and Brefeldin A. Intracellular staining for FoxP3 was

performed using the eBioscience FoxP3 staining kit. Staining for Tfh cells was performed as previously described [158]. Fixable live dead stains were purchased in FITC from Life Technologies and Zombie Yellow (Pacific Orange) from Biolegend and used according to manufacturer's guidelines. Stained splenocytes were fixed in 2% formaldehyde prior to analysis. Samples were read on a BD LSRII. Data was analyzed using FACS Diva and FloJo software.

Blood collection and ELISA

For serum samples, blood was collected by cardiac puncture during terminal bleeds. Blood was allowed to clot at 4^{o}C for 1 hour. Tubes were spun at 4^{o}C at 14,000 rpm for 2 minutes. Serum was transferred to a fresh tube and stored at -80^{o}C. For plasma collection, 100µl of blood was collected from the tail vein into Lithium-heparin coated tubes (BD microtainer). Tubes were spun at 4^{o}C at 10,000 rpm for 10 minutes. Plasma was transferred to a fresh tube and stored at -80^{o}C. ELISA assays were performed as previously described [70]. Briefly, 96 well Nunc ImmunoMaxisorp ELISA plates were coated with 0.5 ug/well of goat anti-mouse IgG or IgM antibody (Southern Biotech) or sucrose gradient purified MHV68 in PBS. Serum was serially diluted (3 fold, beginning at 1:100) and 6 dilutions were plated for each sample. Alkaline Phosphatase conjugated goat anti-mouse IgG or IgM (Southern Biotech) was used as a secondary antibody. For parasite specific ELISAs, mice were infected with 10^5 pRBC of *P. yoelii* XNL or *P. chabaudi* AS. Blood was harvested and pooled from infected mice. pRBCs were purified using a Percoll gradient. Purified pRBCs were then cultured for schizont maturation for 4 hours in a shaking 37^{o}C water bath in RPMI media supplemented with 10% FBS, 100ug/ml Streptomycin, 100U/ml Penicillin, L-Glutamine (2 mM), HEPES (6 mM), β-mercaptoethanol (50 µM) and sodium pyruvate (0.5 mM). pRBC schizonts were then spun out of culture and lysed with lysis buffer (50 mM Tris/HCl + 1 mM EDTA + 0.5% SDS). Optical Density (OD) of homogenate was read at 206 nm. Plates were coated with homogenate at an OD of 0.1-0.2. ELISA protocol for the parasite specific response was performed as previously reported [159] Color was developed using

p-nitrophenyl phosphate (Sigma) in a diethanolamine substrate buffer. Absorbance at 405 nm was read on a Biotek Synergy HT reader. Data is represented as absorbance at 405 nm.

Immunofluorescence staining and Microscopy

Whole spleen was collected at day 8 (post parasite infection) from animals that were MHV68, *P. yoelii* XNL, MHV68 + *P. yoelii* XNL or mock infected. The whole spleen was embedded in Tissue-Tek OCT media (Sakura-Finetek) and frozen in chilled Isopentane. 7µm tissue sections were mounted on slides and allowed to dry at room temperature for 12 hours after which they were frozen at -80^{o}C for long term storage. For staining, slides were allowed to equilibrate to room temperature then rehydrated in PBS for 10 minutes. Sections were stained using B220-FITC (BD bioscience), GL7-AF660 (eBioscience) and a primary purified biotinylated CD3 (eBioscience) with secondary anti-Streptavidin in AF-568 (Life Technologies). Sections were blocked in 5% normal mouse serum in PBS for 20 minutes at room temperature. Primary stains were incubated in block solution for 1 hour and secondary stains for 30 minutes. Sections were washed 3 times with PBS and then mounted with Prolong anti-fade without DAPI (Cell signaling) and #1.5 Fisherbrand microscope slides. Mounted sections were allowed to cure in the dark at room temperature for 24 hours. Fluorescence images were taken on an Olympus Fluoview FV1000 with a 10X 0.3 NA objective and utilizing the multi-area time lapse (MATL) xy-stitching functions. The confocal pinhole was opened to 300 µm to increase the thickness of the optical section facilitating the single plane image. Entire spleen sections required ~100 to 200 images, 1600 x 1600 pixels (~850 x 850 µm) at a zoom of 1.5 and zero overlap. This exceeds the 15000 x 15000 stitching pixel limit of the Fluoview software, and as such a Fiji plugin was written to convert MATL log files for use with the stitching plugin within Fiji [link to http://ici.emory.edu/Resources/plugins].

RESULTS

Acute MHV68 infection impairs the development of malaria specific antibody responses

The humoral response is generally considered to be a critical effector mechanism for controlling peripheral parasitemia in both human and mouse malaria infection [53]. To understand the impact of acute MHV68 infection on the humoral response to a *Plasmodium* infection, we infected C57BL/6 mice with 1000 PFU of MHV68 intra-nasally (IN) on day -7 and 10^5 parasitized red blood cells (pRBCs) of *P. yoelii* XNL or *P. chabaudi* AS intra-peritoneally (IP) on day 0 (Fig. 1A). Single infection with either of the *Plasmodium* species was non-lethal but, in the context of an MHV68 infected mouse, *P. yoelii* XNL, but not *P. chabaudi* AS, caused 100% lethality (Fig. 1B). This corroborates a previous observation by Haque et al. who also observed lethality during MHV68 and *P. yoelii* XNL co-infection [76]. Knowing the importance of a robust humoral response in protection during *Plasmodium* infection [21,151], we hypothesized that MHV68 may impair the generation of an effective antibody response to control *P. yoelii* XNL parasitemia. Total IgM levels were reduced in co-infected animals relative to singly infected animals in *P. yoelii* XNL co-infection at day 23 post malaria infection (Mann Whitney-U test $p<0.05$) and at days 11 and 15 post malaria infection in *P. chabaudi* AS co-infection (both Mann Whitney-U test $p<0.05$) (Figs. 1B and 1C). Total IgG levels were similarly affected and reduced at day 23 post malaria infection in *P. yoelii* XNL co-infected animals and at day 11 post malaria infection in *P. chabaudi* AS co-infected animals (both Mann Whitney U-test $p<0.05$; Fig 1B and 1D). This reduction in total IgG was mirrored in parasite-reactive IgG in both co-infection models compared with the relevant singly-infected animals (both Mann Whitney U-test $p<0.05$; Fig. 1E and 1F). This observation shows that MHV68 acute infection can suppress the humoral response to malaria infection in mice. In one of the mouse malaria models tested (*P. yoelii* XNL), this suppression is correlated with the transformation of a non-lethal malaria infection into a lethal one (Fig 1B). This observation prompted us to evaluate how suppression of

the anti-malaria humoral response impacts the control of peripheral parasitemia and to investigate why the acute phase of MHV68 co-infection impacted the virulence of *P. yoelii* XNL, but not *P. chabaudi* AS infection.

Acute MHV68 co-infection leads to loss of control of *P. yoelii* XNL, but not *P. chabaudi* AS parasitemia

To extend the above observations, we evaluated the impact of an acute MHV68 infection on clearance of the primary peak of parasitemia during secondary challenge with *Plasmodium.* During the initial stages of malaria infection, MHV68 and *P. yoelii* XNL co-infected animals had comparable peripheral parasitemia when compared with *P. yoelii* XNL singly infected animals (Fig. 2A). However, by day 17 post-infection, singly infected animals began to control peripheral parasitemia while co-infected animals were unable to do so (Fig. 2A; Mann Whitney-U test $p<0.05$). There was a trend for co-infected animals to have more severe malarial anemia during *P. yoelii* XNL and MHV68 co-infection compared to *P. yoelii* XNL singly infected animals, but this did not reach statistical significance (Fig. 2B; Mann Whitney-U test on the area above the curve $p=0.056$). There was no difference in *P. chabaudi* AS parasitemia or anemia in singly infected or MHV68 co-infected groups (Mann Whitney-U test on the area under or above the curve respectively $p>0.05$) (Fig. 2A, 2B).

It is possible that elevated persistent replication of MHV68 in the lungs of co-infected mice could contribe to the lethality of *P. yoelii* XNL infection. However, although we observed that levels of preformed virus were significantly higher in the co-infected animals compared to singly infected animals (Mann Whitney-U test on area under the curve $p <0.05$ at day 23 post *P. yoelii* XNL co-infection and day 15 post *P. chabaudi* AS co-infection MHV68 infection; Fig. S1A), we also observed a similar increase in viral titer in the lungs of *P. chabaudi* AS and MHV68 co-infected animals which was not lethal (Fig. S1B). The elevated persistent viral

replication in the lungs of co-infected animals correlated to a decrease in virus-specific IgG in co-infected mice compared to mice infected with MHV68 alone (Fig. S1 panels C & D). Notably, the virus specific antibody response is critical in long term control of viral replication [64]. Assessment of lung tissue at day 23 post co-infection with *P. yoelii* XNL and MHV68 indicated increased Type II hyperplasia, which is indicative of interstitial pneumonia (Fig. S1E) and hemosiderin deposition, as compared to animals singly infected with MHV68. In contrast, single MHV68 infection caused greater levels of inflammation in the lung as defined by larger numbers of histiocytes (macrophages and dendritic cells) in the lung tissue (Fig. S1E). Animals co-infected with *P. chabaudi* AS and MHV68 showed little to no obvious lung tissue damage as compared to single MHV68 infection (Fig. S1E). Thus, at this point we cannot rule out the possibility that increased persistent replication of MHV68 contributes to the lethality of P. yoelii in co-infected mice.

Based on previously published work [53,160], we hypothesized that the reason why the abolishment of the anti-malarial humoral immunity is lethal for *P. yoelii* XNL, but not *P. chabaudi* AS infected mice, is because each infection has a differential requirement for a parasite specific antibody response to control the primary peak of parasitemia. To test this hypothesis, we compared the course of infection for both species of rodent malaria in μMT (B cell-deficient) and C57BL/6 mice. We observed that, in the absence of B cells, *P. chabaudi* AS infected animals could control the primary peak of parasitemia, whereas, *P. yoelii* XNL infected animals developed fulminant parasitemia (Fig. 2C). This was also mirrored in the development of a more severe SMA in *P. yoelii* XNL co-infected animals (Fig. 2D). This data supports the hypothesis that suppression of the anti-*Plasmodium* humoral response in MHV68 co-infected animals is a key factor in why MHV68 co-infection alters the lethality of *P. yoelii* XNL, but not *P. chabaudi* AS, malaria infection.

MHV68 impairs the formation of plasma cells in response to secondary malaria infection

We hypothesized that the impairment of the anti-malarial humoral response in MHV68 infected animals was due to a defect in the generation or function of plasma cells upon infection with malaria. We assessed the populations of plasma cells and germinal center (GC) B cells (a precursor of memory and plasma cells) in the spleen at different times post-infection with malaria. Mice that were co-infected with MHV68 and malaria had a comparable number of GC B cells compared to singly infected animals at day 7-8 post infection with malaria (Figs. 3A,3B and S2; co-infected compared with singly infected animals Mann Whitney-U test $p>0.05$ in both models). However, by day 12 post-infection with *P. yoelii* XNL or day 15 post-infection with *P. chabaudi* AS, GC B cell numbers were significantly reduced as compared to a single *Plasmodium* infection (both Mann Whitney-U test $p<0.05$). At day 8 post co-infection, the GC B cells present were located in T cell-containing germinal centers in representative *P. yoelii* XNL singly infected and MHV68 co-infected animals (Fig. 3C), suggesting that the defect was in the maintenance of the germinal center rather than a follicular structural defect. The impaired GC response correlated with greatly reduced numbers of plasma cells by day 11/12 post-infection with malaria in MHV68 co-infected animals compared with malaria singly infected animals (Mann Whitney-U test $p<0.05$ in both cases). This observation suggests that the defect in anti-malarial antibody responses to the *Plasmodium* infection in MHV68 co-infected animals is likely due to a defect in the generation and/or maintenance of GC B cells.

Impaired germinal center maintenance is correlated with reduced Tfh survival

Since germinal center formation and maintenance is dependent on CD4+ T follicular helper cells (Tfh) (reviewed in [161]), we hypothesized that GC B cell numbers may not be sustained if there are detrimental changes in the splenic Tfh population. One notable observation from the representative spleen sections shown in Figure 3C is that the day 8 co-infected mouse

appears to have a reduced number of CD3+ T cells within the germinal center follicles compared to the day 8 *P. yoelii* XNL singly infected mouse (Fig 3C). Thus, although levels of GC B cells are comparable to a single *P. yoelii* XNL infection at this early time point (Fig. 3A), a reduction in CD3+ T cells, which would include the Tfh subset, may explain the subsequent decay of the splenic GC population. As such, we next evaluated the T cell repertoire in the spleen that is required for germinal center formation and survival.

We analyzed how total Tfh cells (CD4+ CXCR5+ PD-1+), activated/antigen specific Tfh (CD4+ PD-1+ CD44hi CXCR5+) and germinal center Tfh (GL7+ CXCR5+) cells (Fig. 4A) changed over time in the spleen. It was evident that by 23 days post-malaria infection there were defects in the maintenance of all three Tfh subsets in co-infected animals when compared to *P. yoelii* XNL singly infected animals (Fig. 4B; Mann Whitney-U test $p<0.05$ in all cases). This was mirrored in the MHV68 and *P. chabaudi* co-infected animals (Fig. 4C). The MHV68 and *P. yoelii* XNL co-infected animals displayed defects in the total and activated Tfh subsets as early as 12 days post co-infection (Fig. 4B; Mann Whitney-U test $p<0.05$ in all cases) indicating that MHV68 co-infected animals are capable of generating Tfh responses within the first week after co-infection, but they failed to maintain this cellular subset. The MHV68 induced defect in the Tfh population by day 12 post *P. yoelii* XNL co-infection (Fig. 4B) also corresponds to the time point at which the GC B cell population begins to decline (Fig. 3A). This also applies to the decrease in the Tfh population by day 15 post *P. chabaudi* AS challenge (Fig. 4C) in MHV68 co-infected animals compared with *P. chabaudi* AS singly-infected animals and the corresponding reduction in GC B cell numbers (Fig. 3A). This correlation supports the hypothesis that the failure to maintain the population of GC B cells in MHV68 co-infected mice is correlated with a failure to maintain a Tfh cell population.

The defective anti-malarial humoral response induced by an acute pre-existing MHV68 infection may have been a result of alteration of other T cell subsets known to be involved in generating an antibody response, or in the control of parasitemia during malaria infection. To address this, we enumerated numbers of regulatory T cells (Tregs) that can negatively regulate the Tfh response [162] and CD4+T cells that co-express IFN-γ and IL-10 which play an important role in the control of *P. yoelii* parasitemia [163]. Acute MHV68 co-infection did not lead to an increase in these subsets in response to malaria infection (Figs. S3 and S4). In fact, Treg numbers were significantly decreased by MHV68 co-infection at day 15/16 post-infection with malaria in both models (Fig. S3B; Mann Whitney-U test $p<0.05$ in both cases), which we reasoned should enhance, rather than suppress the Tfh response [162], making it an unlikely explanation for suppression of anti-malarial humoral immunity in MHV68 co-infected mice. To further assess the consequences of Tfh deficiency on *P. yoelii* XNL compared with *P. chabaudi* AS malaria infection, we infected IL-21R-/- mice which can generate comparable levels of Tfh cells and germinal center responses during the early stages of an LCMV infection (around 15 days), but fail to maintain both Tfh and germinal center responses after 2 weeks of infection [164], recapitulating the immunological phenotype of MHV68 and malaria co-infected animals. Similar to μMT mice, *P. yoelii* XNL infection of IL21R-/- mice was lethal and this was associated with impaired control of parasitemia and a concomitant increase in the severity of SMA (Fig. S5 A and B). In contrast, IL-21R deficiency did not affect the kinetics of a *P. chabaudi* AS single infection (Fig. S5). Collectively, this data supports the hypothesis that the failure of MHV68 co-infected animals to maintain the Tfh cellular subset in the spleen is associated with a defective humoral response against a secondary malaria infection, which in the case of a *P. yoelii* XNL infection, results in lethality.

Acute, but not latent MHV68 infection, is required for exacerbated malarial disease during co-infection

Given that all children in an endemic area would likely be co-infected with EBV and malaria by the time they are 2 years of age, we hypothesized that gammaherpesvirus induced suppression of the establishment of an anti-malarial humoral responses would depend on the timing of co-infection. Gammaherpesvirus infections, such as EBV and MHV68, can be divided into 2 distinct phases: a lytic phase in which there is acute virus replication and dissemination, followed by the establishment of viral latency in B cells and some other cell types. Latent infection consists of a quiescent phase of viral gene expression, but still results in an underlying inflammatory response [150]. Therefore we investigated whether latent infection as well as acute infection resulted in a defect in the development of the anti-malarial humoral response during co-infection. C57BL/6 mice were infected with MHV68 at 60 (latency), 30 (latency), 15 (late lytic-acute) or 7 (early lytic- acute) days prior to being co-infected with *P. yoelii* XNL (Fig 5A). We measured the number of GC B cells and plasma cells (Fig. 5B), the levels of circulating *P. yoelii* XNL specific IgG responses (Fig. 5C) and the numbers of Tfh cellular subsets in the spleen (Fig. 5D).

As expected, animals infected at day -7 prior to *P. yoelii* XNL infection showed a marked reduction in all of these parameters compared to mice singly infected with *P. yoelii* XNL (Kruskal-Wallis $p<0.05$; Dunn's pairwise comparison $p<0.05$ in all cases). This pattern was repeated in animals infected with MHV68 at 15 days prior to *P. yoelii* XNL infection (Kruskal-Wallis $p<0.05$; Dunn's pairwise comparison $p<0.05$ in all cases) and there was a trend towards this pattern in mice infected with MHV68 for 30 days prior to *P. yoelii* XNL infection that did not reach significance (Kruskal-Wallis $p<0.05$; Dunn's pairwise comparison $p>0.05$ in all cases). However, it is clear that the suppressive effects of an acute MHV68 infection were not present in mice that were latently infected with MHV68 60 days prior to infection with *P. yoelii* XNL. This

data suggests that an established latent MHV68 infection does not suppress the generation of the humoral immune response to an incoming malaria infection in mice.

The mechanism by which acute MHV68 infection can suppress the generation of a humoral response to malaria during co-infection is unclear, but GC B cells from animals with MHV68 co-infection 15 or 7 days prior to *P. yoelii* XNL infection had increased expression of PD-L1 (Fig. S6B), a ligand for PD-1 that negatively regulates Tfh expansion [165], relative to *P. yoelii* XNL singly-infected animals (Kruskal-Wallis $p<0.05$; Dunn's pairwise comparison $p>0.05$ in both cases). Thus, one possibility is that virus induced PD-L1 expression on GC B cells may contribute to the loss of Tfh functionality during co-infection.

The MHV68 M2 gene product plays a role in suppression of anti-parasitic humoral responses.

The data presented above clearly points to a suppressed humoral response as being a critical mediator of lethality during *P. yoelii* XNL co-infection with MHV68. As such, we hypothesized that if we could restore the parasite specific humoral response, we could rescue mice from lethality caused by an MHV68 and *P. yoelii* XNL co-infection. It has previously been shown that the M2 gene product of MHV68 can induce significant levels of IL-10 production from B cells and modulate the surface phenotype of infected B cells [112,166]. IL-10 is known to have multiple immunomodulatory roles, one of which is to negatively regulate T cell responses [167,168]. We hypothesized that one reason Tfh cells did not function correctly could be due to M2-induced suppression, a hypothesis supported by published work showing that in the absence of M2, mice are able to mount enhanced virus specific CD8+ T cell responses [112]. Given that the downstream effect of MHV68 induced immunosuppression was a result of impaired anti-malarial antibody responses, we initially asked whether levels of virus specific IgG responses were enhanced in the absence of M2 expression. To avoid a known defect in the establishment of

splenic infection following intranasal inoculation of M2-deficient MHV68 mutants [169]we opted to infect mice with the same dose of virus (1000 PFU) as used in the previous experiments, but via the intraperitoneal route – a route and dose of virus which allow the M2-deficient mutant to efficiently infect the spleen [169]. As a proper control, the marker rescue virus (i.e, a recombinant MHV68 in which the genetic mutation introduced into the M2 null mutant was restored to the wild type virus sequence) [111], was also administered via the IP route. Notably, we have extensively compared IN versus IP MHV68 co-infection with *P. yoelii* and found there to be no difference in outcome. M2.Stop (M2 null virus; M2.St) infection in a C57BL/6 mouse induced a nearly 2-fold higher MHV68 specific IgG response as compared to infection with the marker rescue control virus (M2.MR; MR) (Fig. 6A; day 21 post-infection Mann Whitney-U test $p<0.05$). It is important to note that day 21 post-MHV68 infection in this experiment corresponds to day 14 post-co-infection with *P. yoelii* XNL. The time point at which the virus specific humoral response is suppressed overlaps with the timing at which parasite specific IgG responses become severely compromised during co-infection (Figs. 1C and 1E). This observation suggests that M2 may be mediating the virus-induced suppression of anti-malarial humoral immune responses.

To evaluate whether loss of M2 expression could alleviate the MHV68 induced suppression of the anti-malarial humoral response, we infected mice with either the M2 null mutant (M2.Stop) or the marker rescue virus control (MR) 7 days prior to challenge with *P. yoelii* XNL. While co-infection with the MR virus suppressed the generation of anti-*P. yoelii* XNL specific IgG (Kruskal Wallis $p<0.05$; Dunn's pairwise comparison test $p<0.05$), mice that were co-infected with M2.Stop showed a 28-fold increase in *P. yoelii* XNL specific IgG at day 23 post co-infection relative to the lethal MHV68 and *P. yoelii* XNL co-infection model (Kruskal Wallis $p<0.05$; Dunn's pairwise comparison test $p>0.05$) (Fig. 6B). Importantly, 94% of mice co-infected with M2.Stop and *P. yoelii* XNL survived (1 out of 15 mice died) (Fig. 6C) compared to

100% lethality in mice co-infected with MR and *P. yoelii* XNL. Consistent with this observation, circulating parasitemia was not detectable by microscopy at day 28 post co-infection in the M2.Stop and *P. yoelii* XNL co-infected mice (Fig. 6D). This data clearly establishes a strong link between MHV68 mediated suppression of the humoral response to *P. yoelii* XNL and survival. In addition, it argues for a role of the MHV68 M2 protein in mediating the suppression of the anti-malarial humoral response in MHV68 co-infected mice.

DISCUSSION

The risk factors postulated to contribute to disease severity in young children infected with malaria are numerous and include co-infection with other pathogens [4]. Many children in Equatorial African countries are seropositive for EBV by the age of 6 months as protective maternal antibodies wane [6,9], indicating that primary infection with EBV coincides with the time at which the risk of developing severe malaria is greatest [8,9]. Acute infection with EBV is asymptomatic in young children [8] and results in a latent infection that persists for the life time of the host. Children who experience recurrent infection with *P. falciparum* while latent for EBV show an impairment in virus specific CD8 T cell responses [15,19,170-172] which contributes to an increased risk of developing eBL [reviewed in [123]]. While the impact of repeated *P. falciparum* infections can abate an EBV-specific adaptive immune response, little has been known regarding the impact of acute EBV infection on severe malarial disease during childhood.

The risk of co-infection with *P. falciparum* before the age of 1 is extremely high for children living in Sub-Saharan Africa [8,9] and for some children it is likely that their primary infections of EBV and *P. falciparum* will overlap. There are several reports that asymptomatic EBV infection can have suppressive effects on the host's humoral adaptive response. One prominent example involves a case study on a child aged 2 ½ years presenting with a recurrent case of otitis media and pneumonia. It was established that this child exhibited suppressed humoral responses when immunized with bacteriophage ϕX174 or Keyhole limpet hemocyanin (KLH) during an asymptomatic infection with EBV. Increased EBV specific antibody titers correlated with a suppression in secondary humoral responses to unrelated antigens [78]. The same observation has been documented in young adults experiencing a primary EBV infection and manifesting symptoms of Infectious Mononucleosis (IM) [79,154]. Similarly, Holder et al. recently described a role for acute EBV infection in attenuating vaccine specific antibody

responses in Gambian children as compared to children who had concurrent CMV infection [173]. These observations were also extended to the marmoset model where Wedderburn et al. demonstrate that acute co-infection of EBV and *P. brasilianum* resulted in severe morbidity and death [174]. Collectively, these documented cases provide key evidence of the immune suppressive nature of an acute EBV infection on the development of humoral immunity. More importantly, several studies have shown a correlation between non-cerebral severe disease (particularly severe malarial anemia [SMA]) and attenuated parasite specific antibody responses [52,175].

Here, using well characterized mouse models, we provide evidence that acute gammaherpesvirus infection can suppress the development of humoral immunity to a secondary *Plasmodium* infection in two different non-lethal models of rodent malaria (Fig. 1). Interestingly, this defect transformed non-lethal malaria infection into a lethal one in the case of *P. yoelii* XNL co-infection, but had no obvious impact on the pathogenesis of *P. chabaudi* AS infection (Fig. 2). This result is likely to be due to the differential role of antibody mediated parasite clearance mechanisms in controlling the primary peak of parasitemia in each of these models (Figs. 2 and S5) [53,160], although the effects of a primary acute MHV68 infection on other cells types such as macrophages cannot be ruled out. The reason why *P. yoelii* XNL infection is more dependent on antibodies for the control of parasitemia than *P. chabaudi* AS is unknown, but could be related to the kinetics of infection. In our hands, *P. yoelii* XNL parasitemia in intact C57BL/6 mice peaked significantly later than *P. chabaudi* AS infection (15.5 ± 1.5 days compared with 8.2 ±0.5 days; Mann Whitney-U test $p<0.05$). T cells, in particular IFN-γ-producing T cells [176-179], have been implicated in orchestrating the control of peripheral parasitemia in both models, whereas IL-10 producing T cells have been shown to exacerbate *P. yoelii* XNL parasitemia [163]. Therefore, it is possible that MHV68 infection resulted in an alteration of T cell phenotypes generated against a secondary malaria infection that impacted the pathogenesis of the infection.

However, these splenic CD4+ T cell populations measured at different time points post-infection were not significantly altered (Fig. S4), and IL-10-producing CD4+T cells were less in number during MHV68 and *P. yoelii* XNL co-infected animals as compared to *P. yoelii* XNL singly infected animals (Fig. S4B), which theoretically should lead to better control of peripheral parasitemia [163]. Furthermore, T cells have been shown to play a critical role in the control of the primary peak of *P. chabaudi* AS infection [178,179], yet MHV68 co-infection did not alter the peripheral parasitemia in this model suggesting that the relevant defect lies within the failure to mount an appropriate humoral response.

Despite the differential outcome of the two co-infection models, the impact of acute viral infection on the suppression of the humoral response is a common feature of MHV68 and malaria co-infection in C57BL/6 mice. Our data suggests that co-infected mice have a profound defect in the ability to form antibody producing plasma cells (Fig. 3). GC B cells are precursors of plasma cells and are dependent on the T follicular helper subset for development and maintenance (reviewed in [180,181]). Analysis of germinal center formation at day 8 post-infection with *P. yoelii* XNL demonstrates that germinal centers can form in the spleens of co-infected mice, but are not maintained (Fig. 3). This may be due to a defective ability of GC B cells to communicate with Tfh cells due to elevated expression of the suppressive ligand PD-L1 (Fig. S6B). PD-L1 mediates its inhibitory role by ligating the PD-1 (Programmed Death-1) receptor on T cells. Recent studies suggest that the reduction in HIV specific antibody responses during chronic infection is correlated with an up-regulation of PD-L1 expression on germinal center B cells [182]. It is interesting that the possible cause of the Tfh impairment may be associated with a change in the surface phenotype of the GC B cell. This is particularly significant in the case of MHV68 infection, since B cells are the primary cell infected by this virus. However, it is unclear whether MHV68 directly affects the GC B cell, or whether soluble mediators of infection suppress the function of Tfh cells in their ability to support the transformation of GC B cells to

plasma cells. Our data does not support a role for suppressive effects of FoxP3+ Tregs (Fig. S3) in mediating this effect since the expansion of Tregs is comparable during co-infection compared to a single *Plasmodium* infection. (Fig. S3 Mann Whitney-U test $p<0.05$ in both cases).

The suppression of the anti-malarial humoral response is evident during acute, but not latent, MHV68 infection (Fig. 5). MHV68 has evolved elaborate immune evasion strategies to survive the potent innate inflammatory responses that it induces during acute infection [85]. One interesting observation previously made in the Speck laboratory noted that loss of M2 expression *in vivo* resulted in a more robust anti-viral CD8 T cell response [112]. M2 is a unique viral protein expressed by MHV68 which shares some functional homology with the LMP1 and LMP2a EBV gene products - which mimic CD40 and BCR signaling, respectively [183,184]. M2 is able to promote signals downstream of the BCR receptor [185,186], induces IL-10 production from B cells [111,112] and promotes differentiation of infected B cells into plasma cells (note that $\leq$ 1% of B cells are MHV68 infected at the peak of latency) [166]. One notable effect of M2 expression *in vivo* is the dramatic increase of IL-10 levels in the serum of infected mice [112]. IL-10 is an immune-modulatory cytokine which is capable of suppressing T cell activation [168]. M2 mediated reduction of the anti-viral CD8 T cell response likely reflects an evolutionary viral adaptation that allows for the evasion of the immune response, and more importantly, allows for establishment of viral latency. Since the humoral response is dependent on a robust T cell response, we predicted that M2 may also influence the generation of a virus specific IgG response, a critical branch of the adaptive response involved in long term clearance of the virus [64]. Our data shows that in the absence of M2 expression, the development of an anti-viral humoral response was enhanced 2-fold (Fig. 6). Additionally, co-infection with M2.Stop mutant virus and *P. yoelii* XNL showed a 28-fold increase in the anti-malarial IgG response, which also correlated with survival during co-infection. We compared virus specific IgG levels in mice infected with another unrelated viral mutant that is null for M1 protein

expression. Both the M1.Stop and M1.MR viruses showed equivalent levels of virus specific IgG responses over 2 months of infection (Supplementary figure 7)), suggesting an M2-specific role in suppressing the virus specific humoral response. This further corroborates data shown by Getahun et al. [77], which demonstrated that infection with M1.Stop or M3.Stop (a viral chemokine) could not alleviate the virus-induced immune suppression. We currently do not understand the mechanism by which M2 is mediating this effect, although we hypothesize that increased IL-10 production from B cells in the splenic follicle may negatively regulate Tfh survival and consequently affect germinal center maintenance and development. Although B cell expansion is beneficial for seeding viral latency and persistence, it is evident that the virus also negatively regulates the production of virus specific IgG responses, a key immune evasion mechanism which would support viral persistence. Although M2 expression is associated with IL-10 production, we cannot rule out the possibility that M2 is mediating its effect in a non-IL-10 dependent manner. However, our novel discovery implicating M2 in mediating the virus induced humoral suppression against secondary parasitic infection is a key observation for future efforts in dissecting the mechanism behind this observed phenotype.

As with every model system, certain limitations exist. Our studies rely on infection of 6-8 week old mice since this age group has been extensively studied. The limited studies in neonatal mice suggest that BALB/c mice, and not C57BL/6 mice, are more susceptible to infection and may develop myocarditis and neurologic disorders [187,188]. It appears that neonate C57BL/6 mice, which is the background used in our studies, do not experience altered immune responses compared to adults. As such, there is little premise to suggest that younger mice would react differently to acute MHV68 infection. However, the impact of co-infection on malarial disease severity in neonate mice has not been explored and is worth pursuing in other studies. Another important aspect worth noting is that the isolated system used to model human co-infection cannot encompass the myriad of factors influencing malarial disease severity in humans. It has

been extensively demonstrated that various other viral, bacterial, and helminth co-infections impact malarial disease [189-194]. Additionally, factors such as parasite virulence, nutrition, host health and genetics can also contribute to the variation in malarial disease severity [2,4]. As such, acute EBV infection is unlikely to be the sole contributor in modulating malarial disease. However, the results reported in this manuscript aim to elucidate previously neglected co-infections, such as ubiquitous asymptomatic EBV infection, in altering non-cerebral malarial disease severity. More importantly, multiple human reports indicate that asymptomatic acute EBV infection has the ability to alter the generation of a humoral response during secondary pathogen challenge [78,79,154,173,174] and demonstrated by us and others [77] during MHV68 acute infection. Collectively, our observations in the mouse model and supporting literature of human studies provide a strong premise for investigating the role of acute EBV in malarial disease. Undoubtedly, detailed longitudinal studies are required in humans to conclusively establish this correlation.

In conclusion, our work provides compelling evidence that acute gammaherpesvirus infection can negatively modulate the humoral immune response to malaria infection. This data provides justification to investigate how EBV infection might impact the development of *P. falciparum* humoral immunity in young children living in malaria endemic areas. If found to be a risk factor for developing severe malaria, tackling EBV infection via the development and use of an EBV vaccine or anti-viral therapies in malaria endemic areas, may provide some relief in the development of non-cerebral severe disease during childhood malaria infection.

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FIGURES

Figure 1

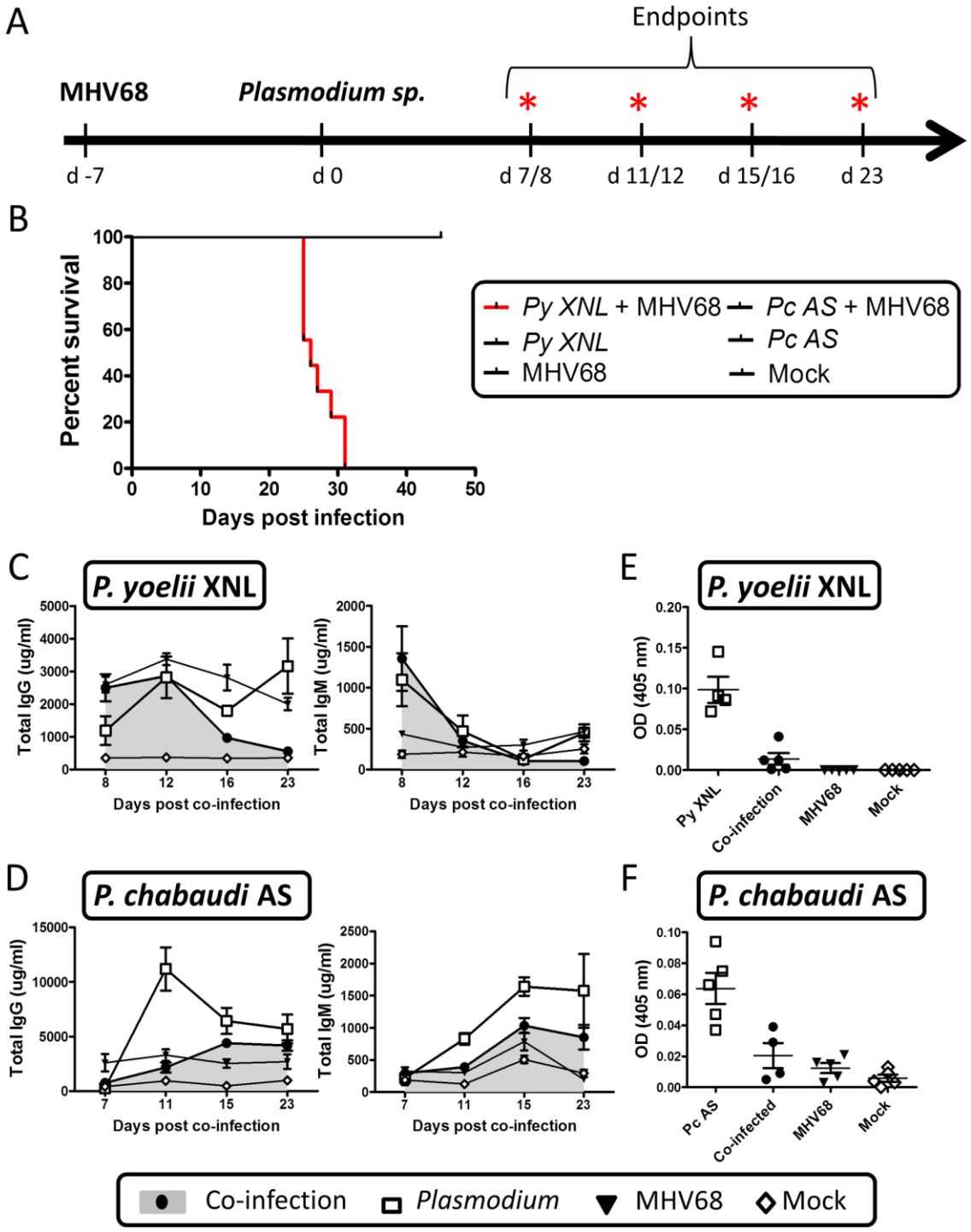

Figure 2

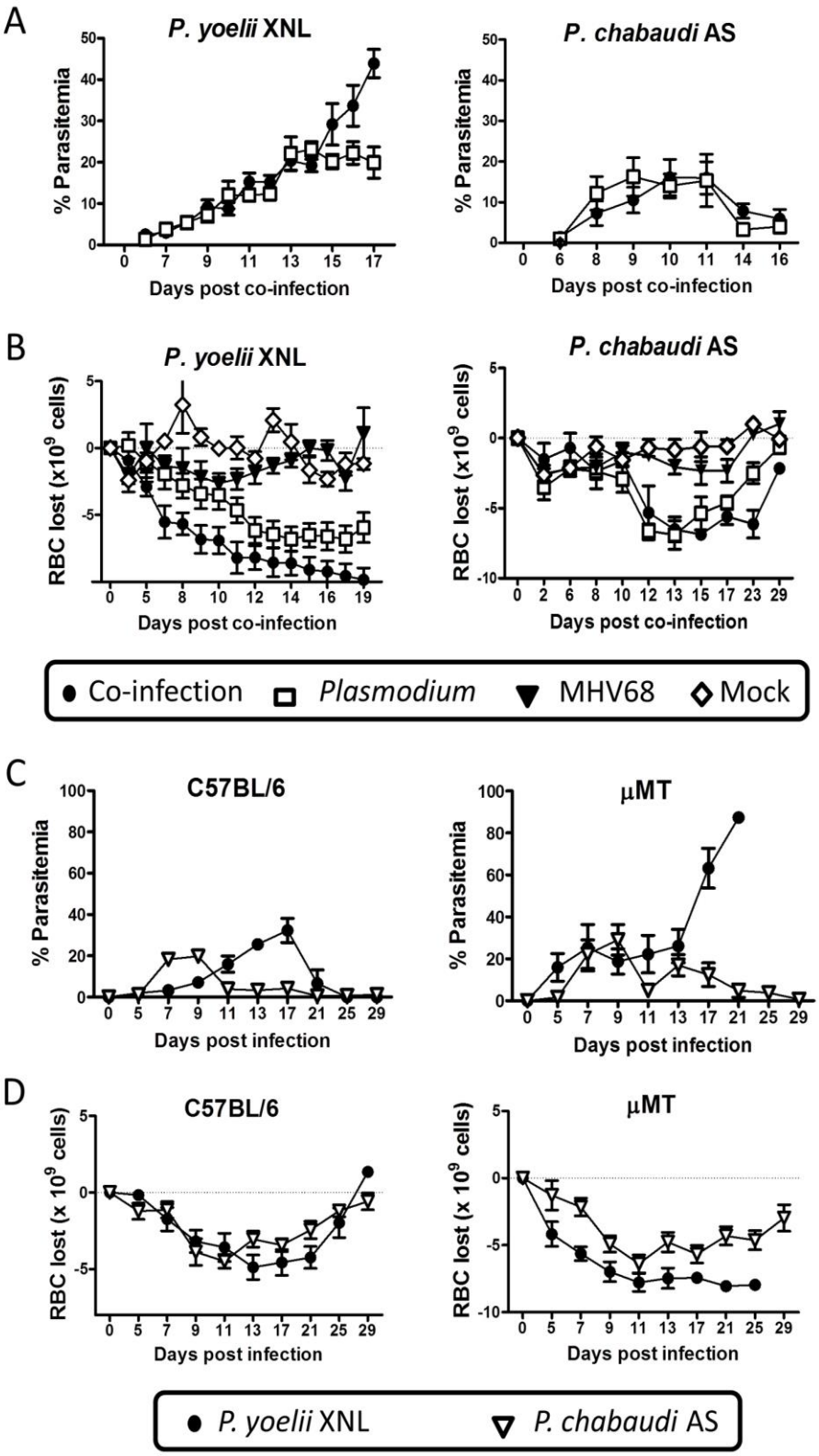
A
P. yoelii XNL
% Parasitemia
Days post co-infection
P. chabaudi AS
% Parasitemia
Days post co-infection
B
P. yoelii XNL
RBC lost (x10^9 cells)
Days post co-infection
P. chabaudi AS
RBC lost (x10^9 cells)
Days post co-infection
Co-infection
Plasmodium
MHV68
Mock
C
C57BL/6
% Parasitemia
Days post infection
µMT
% Parasitemia
Days post infection
D
C57BL/6
RBC lost (x 10^9 cells)
Days post infection
µMT
RBC lost (x 10^9 cells)
Days post infection
P. yoelii XNL
P. chabaudi AS

Figure 3

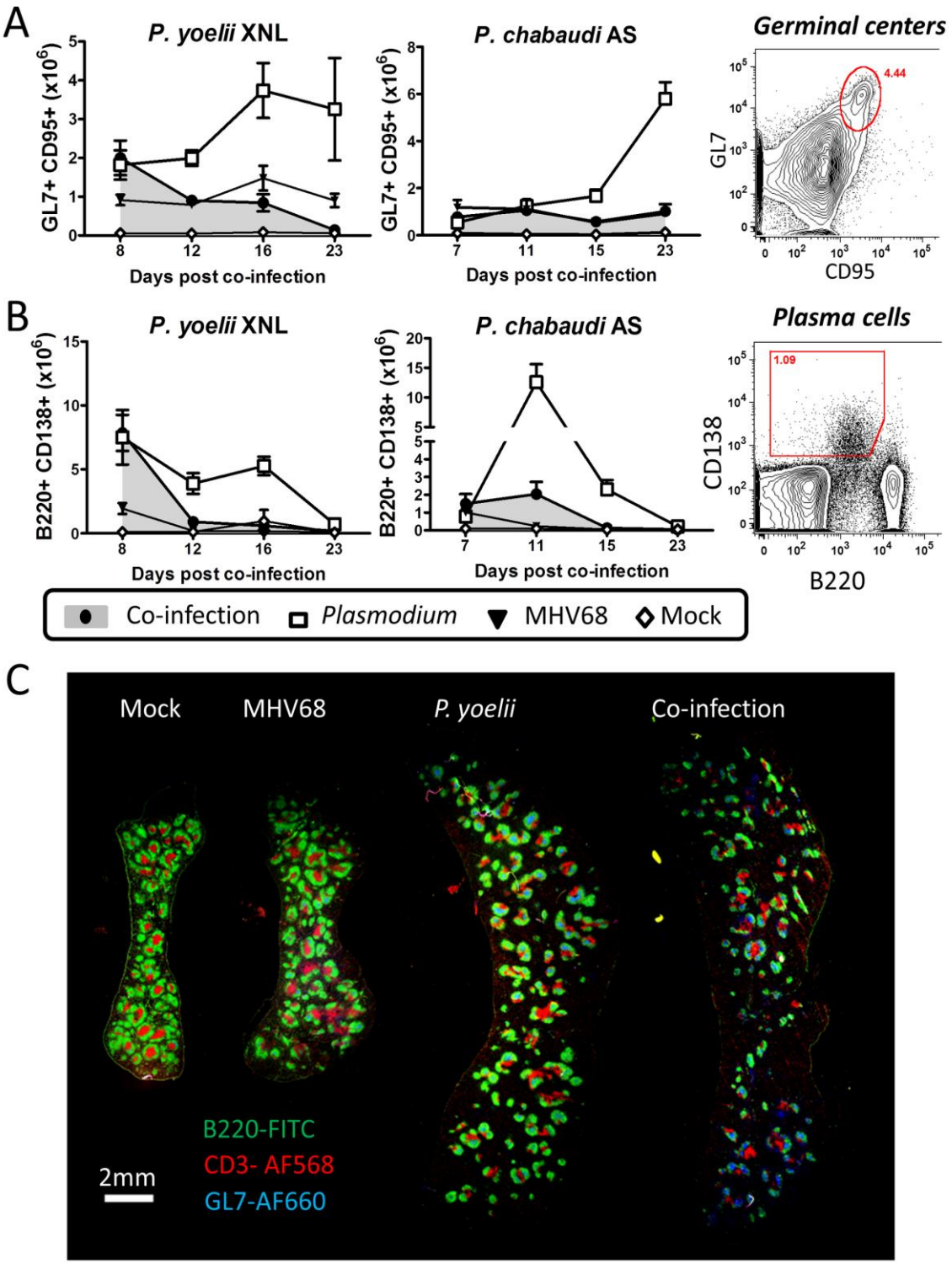
A
P. yoelii XNL
P. chabaudi AS
Germinal centers
GL7+ CD95+ (x10^6)
Days post co-infection
4.44
GL7
CD95
B
P. yoelii XNL
P. chabaudi AS
Plasma cells
B220+ CD138+ (x10^6)
Days post co-infection
1.09
CD138
B220
Co-infection
Plasmodium
MHV68
Mock
C
Mock
MHV68
P. yoelii
Co-infection
B220-FITC
CD3- AF568
GL7-AF660
2mm

Figure 4

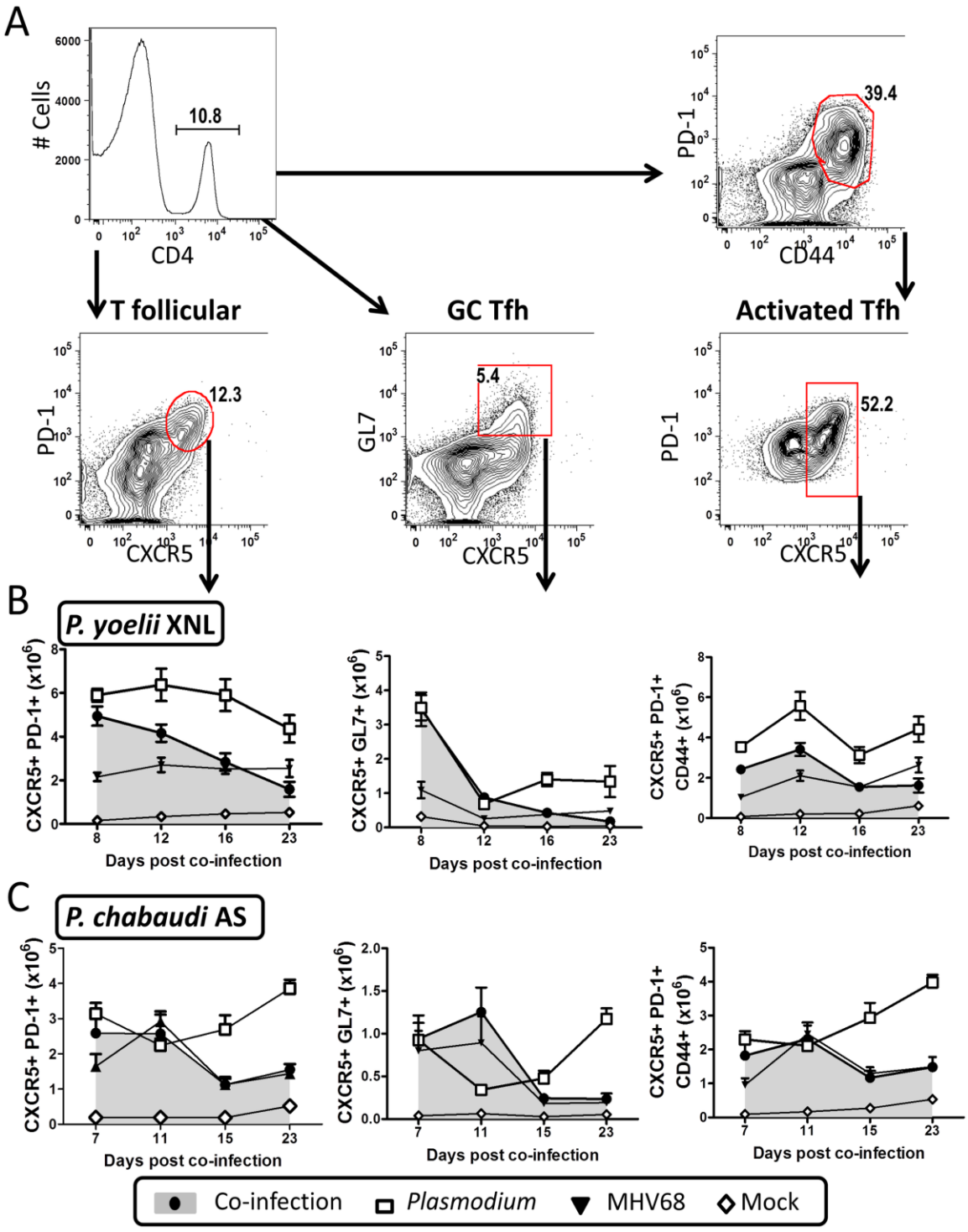
A
Cells
10.8
CD4
PD-1
39.4
CD44
T follicular
GC Tfh
Activated Tfh
PD-1
12.3
CXCR5
GL7
5.4
CXCR5
PD-1
52.2
CXCR5
B
P. yoelii XNL
CXCR5+ PD-1+ (x10^6)
Days post co-infection
CXCR5+ GL7+ (x10^6)
Days post co-infection
CXCR5+ PD-1+ CD44+ (x10^6)
Days post co-infection
C
P. chabaudi AS
CXCR5+ PD-1+ (x10^6)
Days post co-infection
CXCR5+ GL7+ (x10^6)
Days post co-infection
CXCR5+ PD-1+ CD44+ (x10^6)
Days post co-infection
Co-infection
Plasmodium
MHV68
Mock

Figure 5

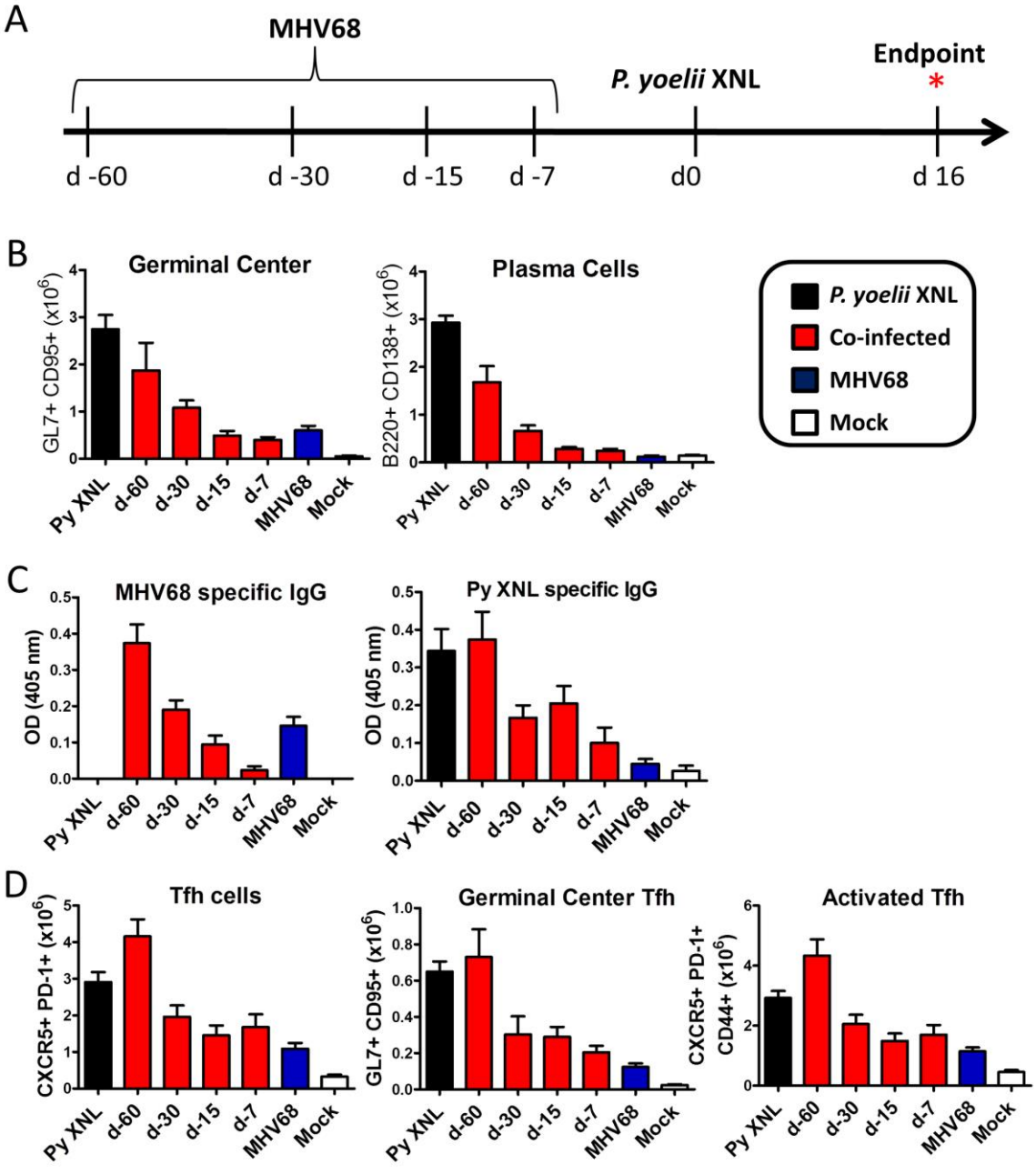
A
MHV68
P. yoelii XNL
Endpoint
*
d -60
d -30
d -15
d -7
d0
d 16
B
Germinal Center
GL7+ CD95+ (x10^6)
Plasma Cells
B220+ CD138+ (x10^6)
P. yoelii XNL
Co-infected
MHV68
Mock
Py XNL
d-60
d-30
d-15
d-7
MHV68
Mock
C
MHV68 specific IgG
OD (405 nm)
Py XNL specific IgG
D
Tfh cells
CXCR5+ PD-1+ (x10^6)
Germinal Center Tfh
GL7+ CD95+ (x10^6)
Activated Tfh
CXCR5+ PD-1+ CD44+ (x10^6)

Figure 6

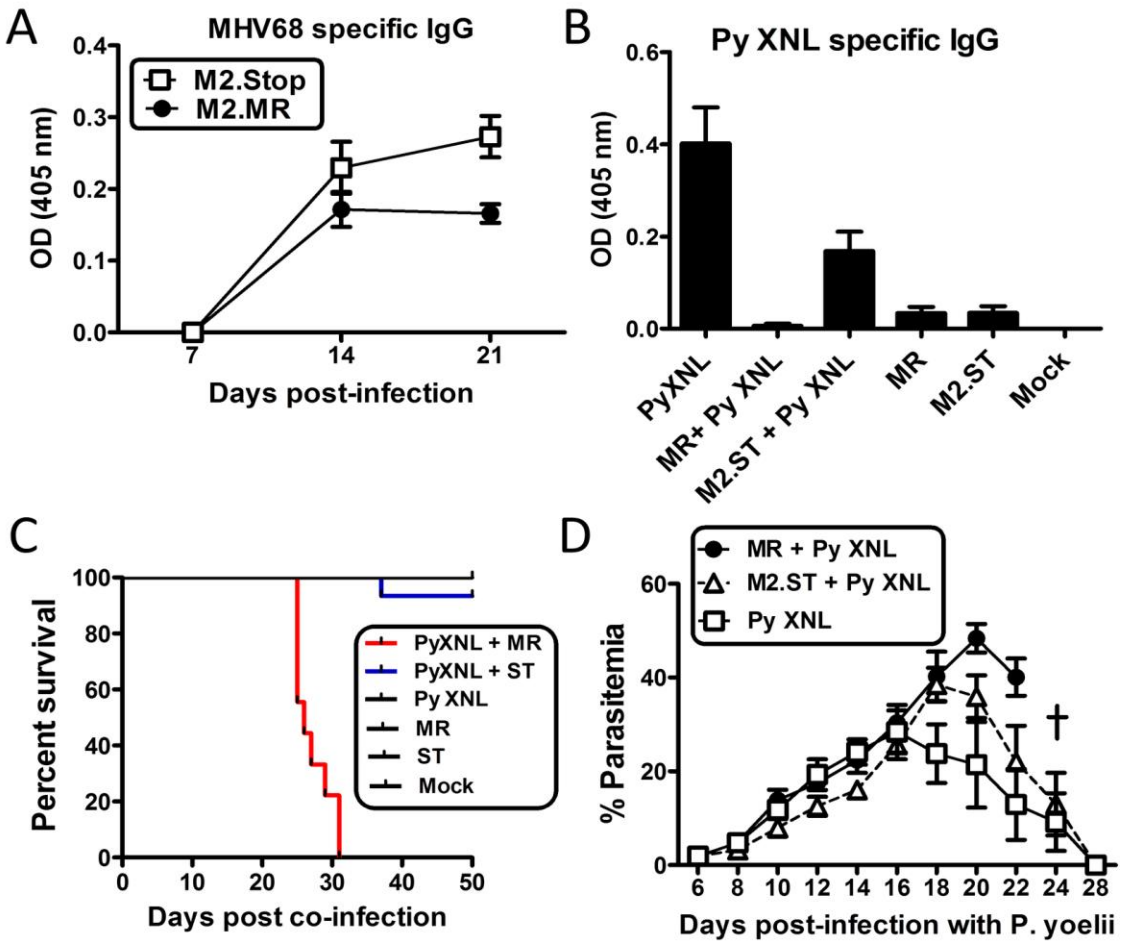
A
MHV68 specific IgG
M2.Stop
M2.MR
OD (405 nm)
0.4
0.3
0.2
0.1
0.0
7
14
21
Days post-infection
B
Py XNL specific IgG
OD (405 nm)
0.6
0.4
0.2
0.0
PyXNL
MR+ Py XNL
M2.ST + Py XNL
MR
M2.ST
Mock
C
Percent survival
100
80
60
40
20
0
0
10
20
30
40
50
Days post co-infection
PyXNL + MR
PyXNL + ST
Py XNL
MR
ST
Mock
D
% Parasitemia
60
40
20
0
6
8
10
12
14
16
18
20
22
24
28
Days post-infection with P. yoelii
MR + Py XNL
M2.ST + Py XNL
Py XNL
†

Figure S1

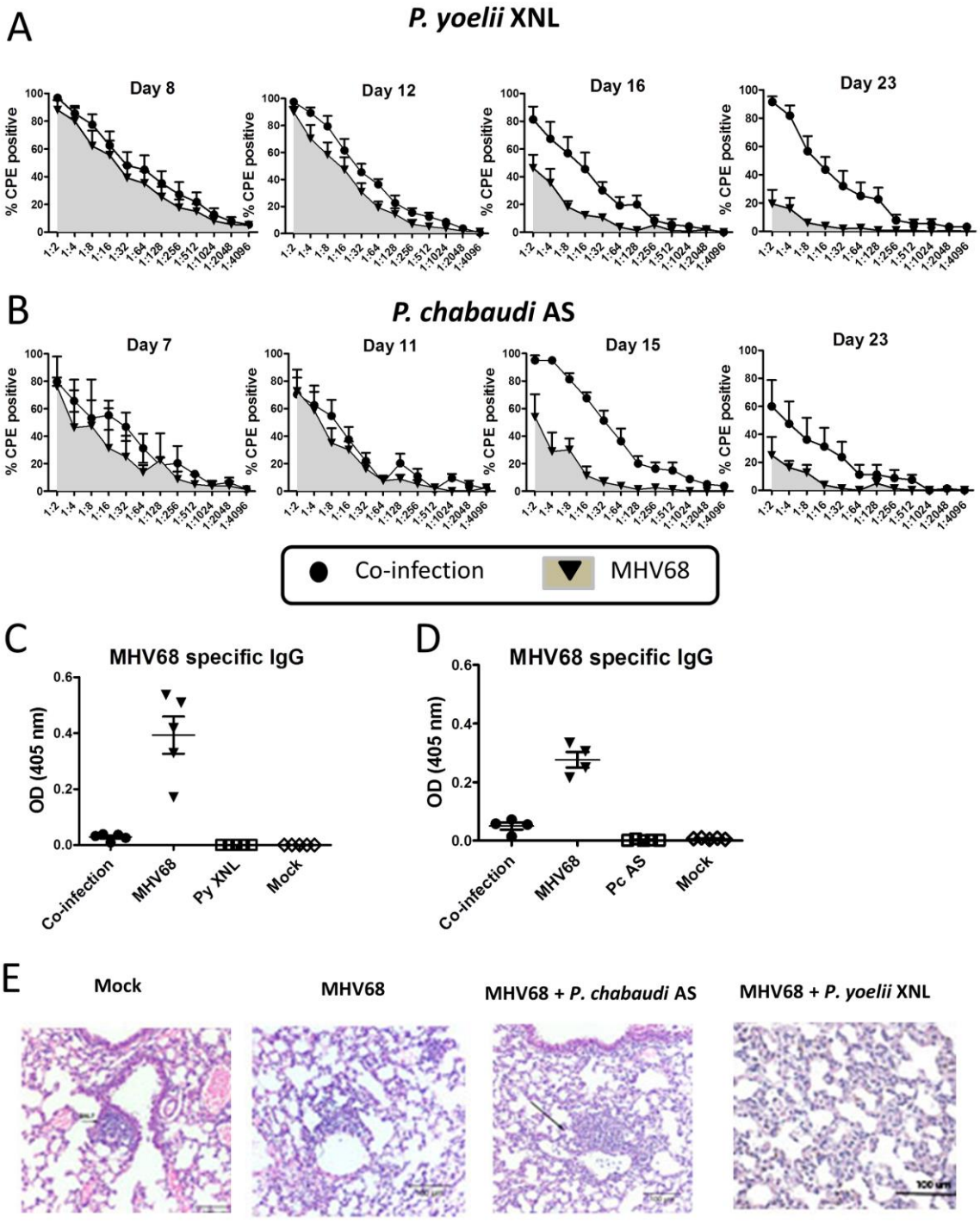
A
P. yoelii XNL
Day 8
Day 12
Day 16
Day 23
% CPE positive
B
P. chabaudi AS
Day 7
Day 11
Day 15
Day 23
Co-infection
MHV68
C
MHV68 specific IgG
OD (405 nm)
Co-infection
MHV68
Py XNL
Mock
D
MHV68 specific IgG
OD (405 nm)
Co-infection
MHV68
Pc AS
Mock
E
Mock
MHV68
MHV68 + P. chabaudi AS
MHV68 + P. yoelii XNL

Figure S2

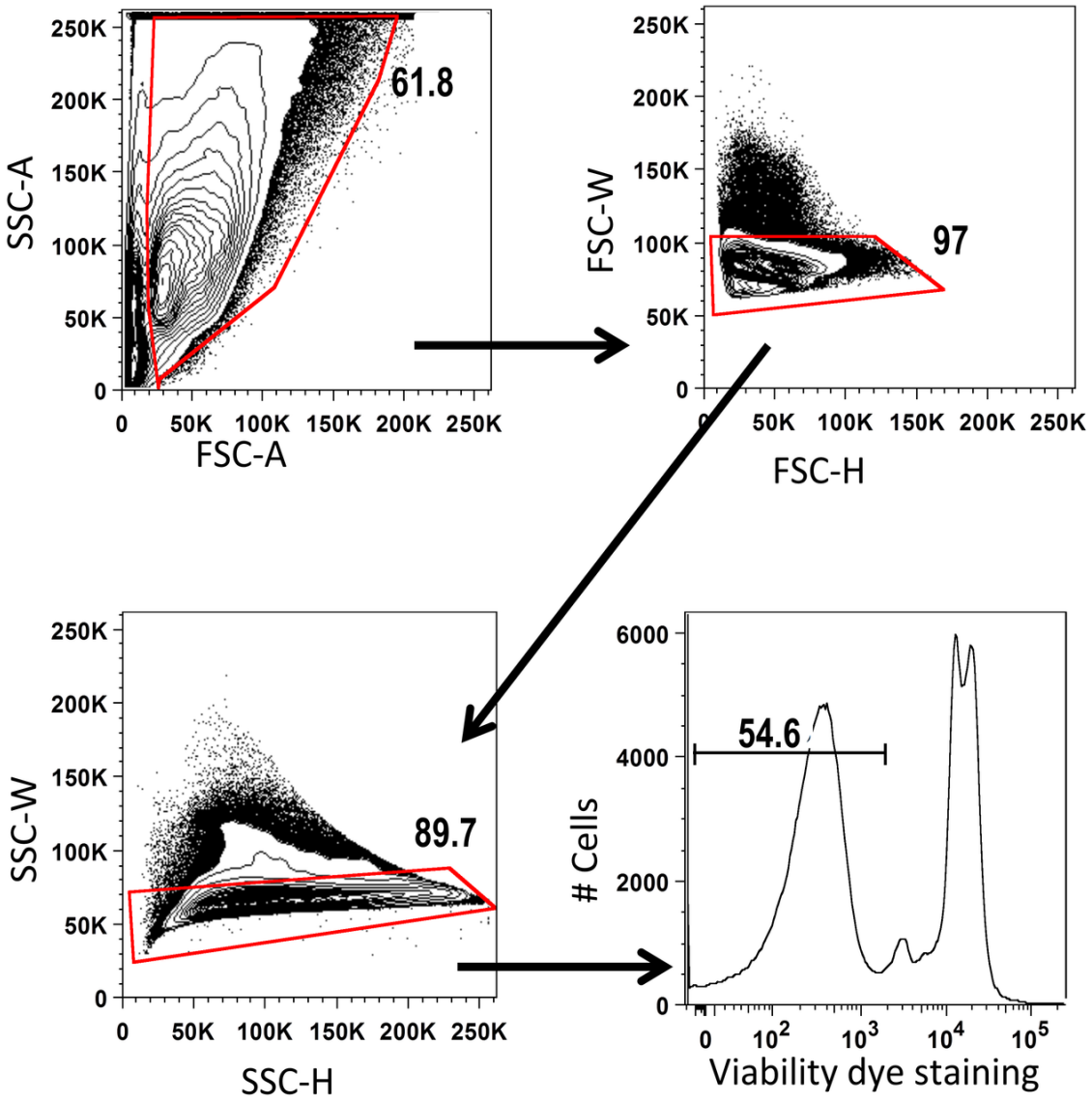
SSC-A
FSC-A
61.8
FSC-W
FSC-H
97
SSC-W
SSC-H
89.7
Cells
Viability dye staining
54.6
0
50K
100K
150K
200K
250K
10^2
10^3
10^4
10^5
2000
4000
6000

Figure S3

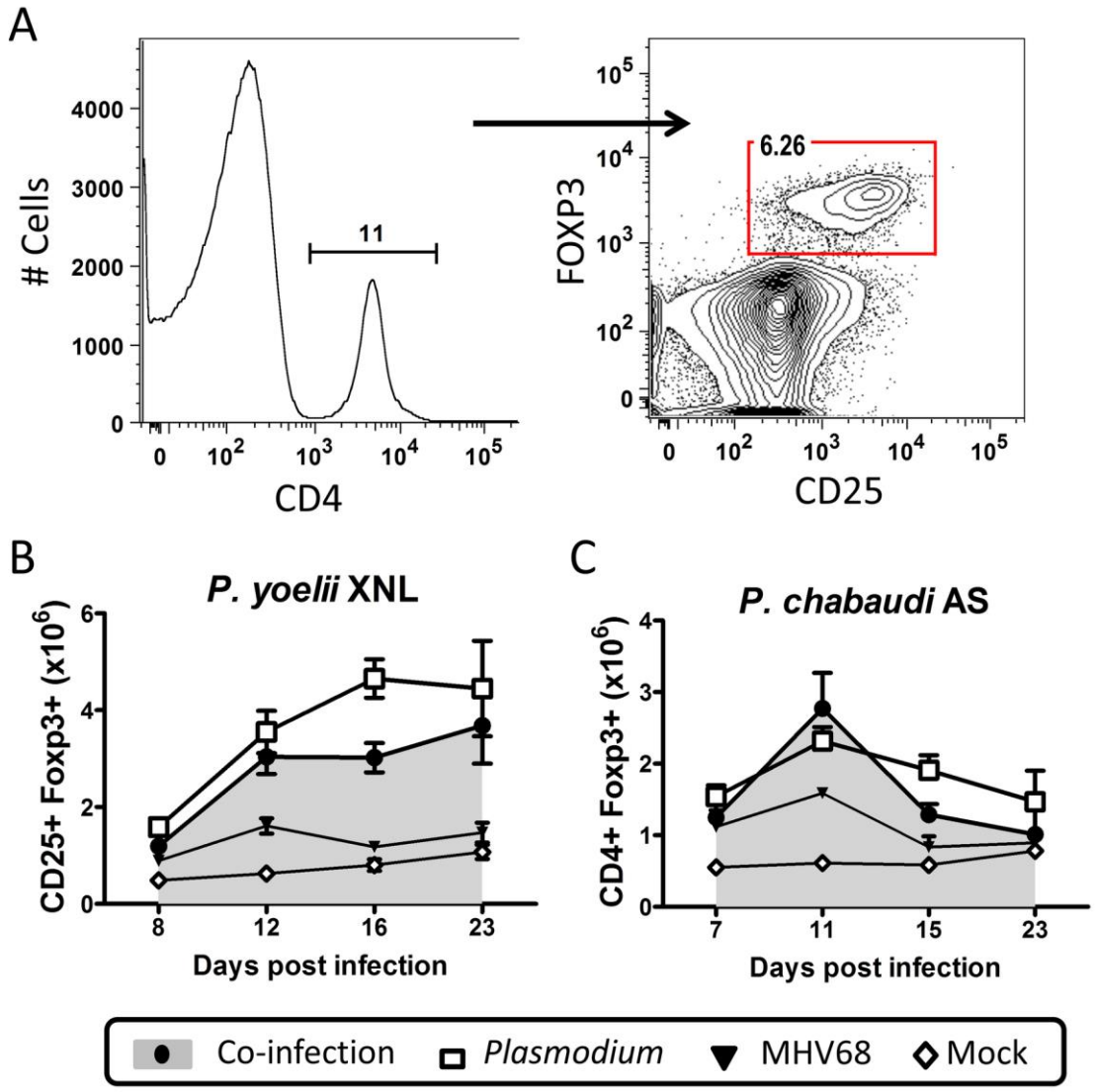
A
Cells
CD4
11
FOXP3
CD25
6.26
B
P. yoelii XNL
CD25+ Foxp3+ (x10^6)
Days post infection
C
P. chabaudi AS
CD4+ Foxp3+ (x10^6)
Days post infection
Co-infection
Plasmodium
MHV68
Mock

Figure S4

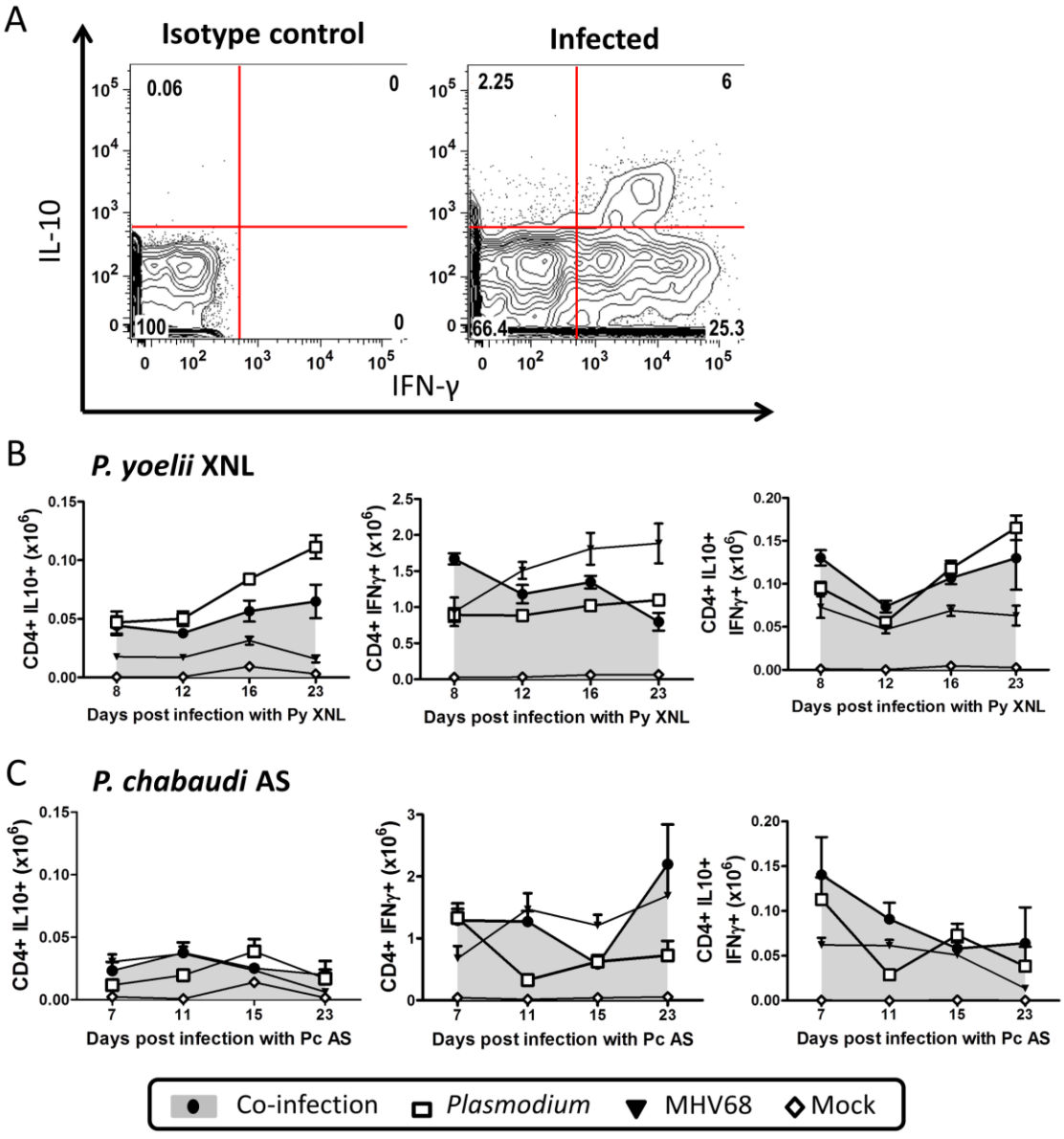
A
Isotype control
Infected
0.06
0
100
0
2.25
6
66.4
25.3
IL-10
IFN-γ
B
P. yoelii XNL
CD4+ IL10+ (x10^6)
CD4+ IFNγ+ (x10^6)
CD4+ IL10+ IFNγ+ (x10^6)
Days post infection with Py XNL
C
P. chabaudi AS
Days post infection with Pc AS
Co-infection
Plasmodium
MHV68
Mock

Figure S5

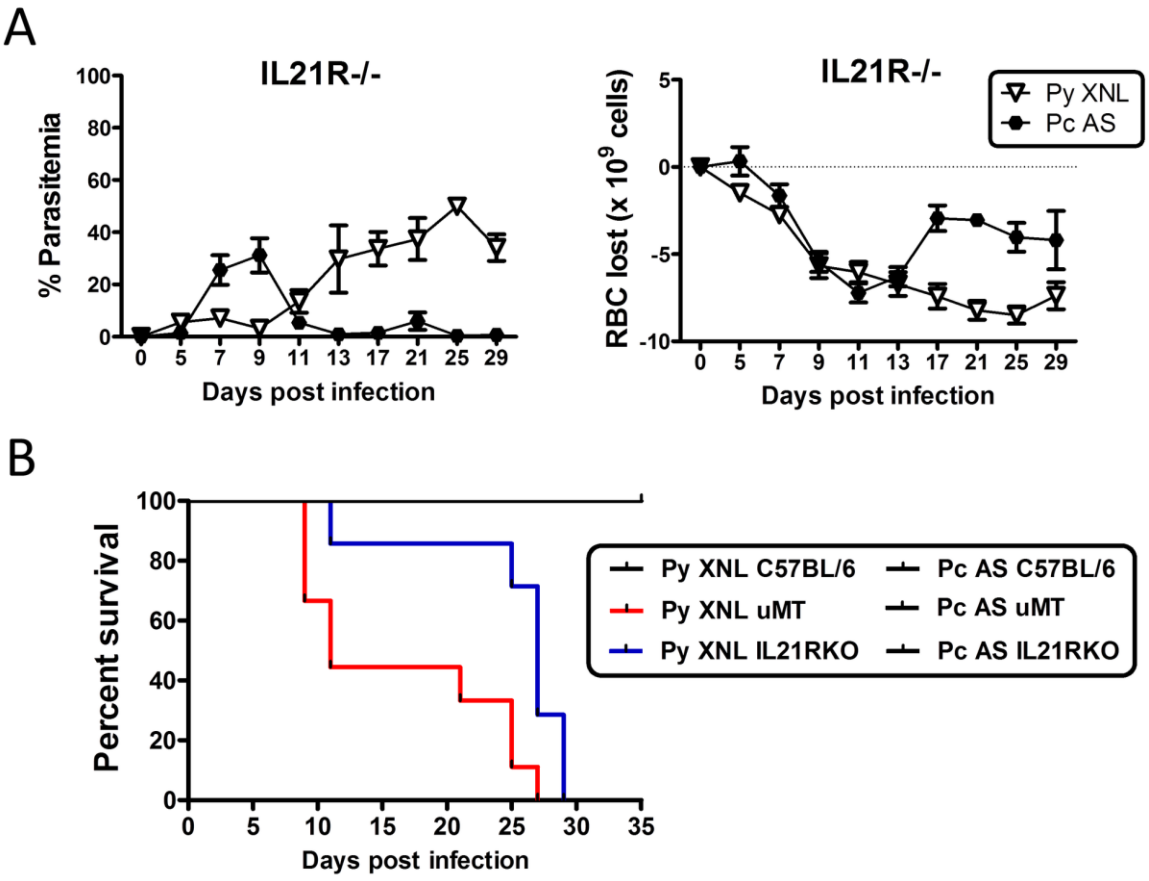
A
IL21R-/-
% Parasitemia
Days post infection
IL21R-/-
RBC lost (x 10^9 cells)
Py XNL
Pc AS
Days post infection
B
Percent survival
Days post infection
Py XNL C57BL/6
Py XNL uMT
Py XNL IL21RKO
Pc AS C57BL/6
Pc AS uMT
Pc AS IL21RKO

Figure S6

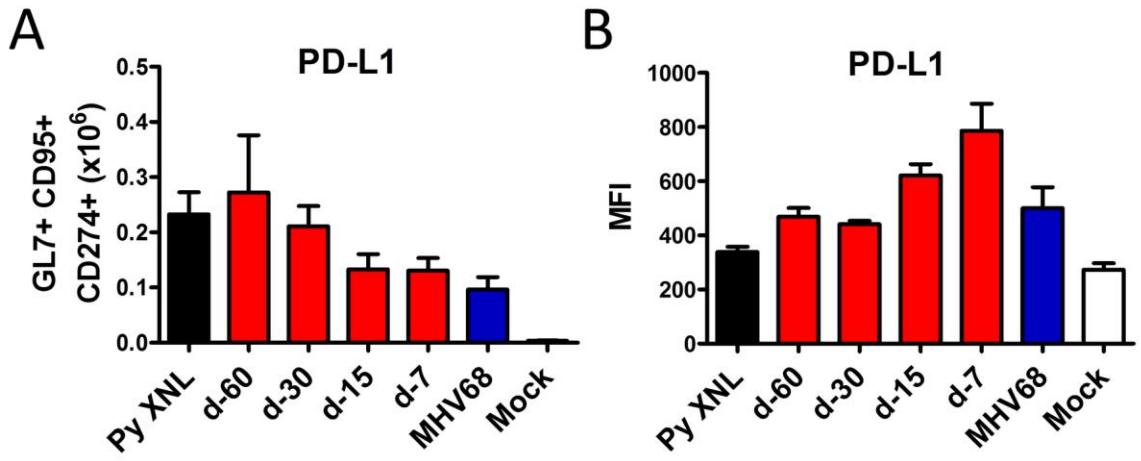
A
PD-L1
GL7+ CD95+ CD274+ (x10^6)
0.5
0.4
0.3
0.2
0.1
0.0
Py XNL
d-60
d-30
d-15
d-7
MHV68
Mock
B
PD-L1
MFI
1000
800
600
400
200
0
Py XNL
d-60
d-30
d-15
d-7
MHV68
Mock

Figure S7

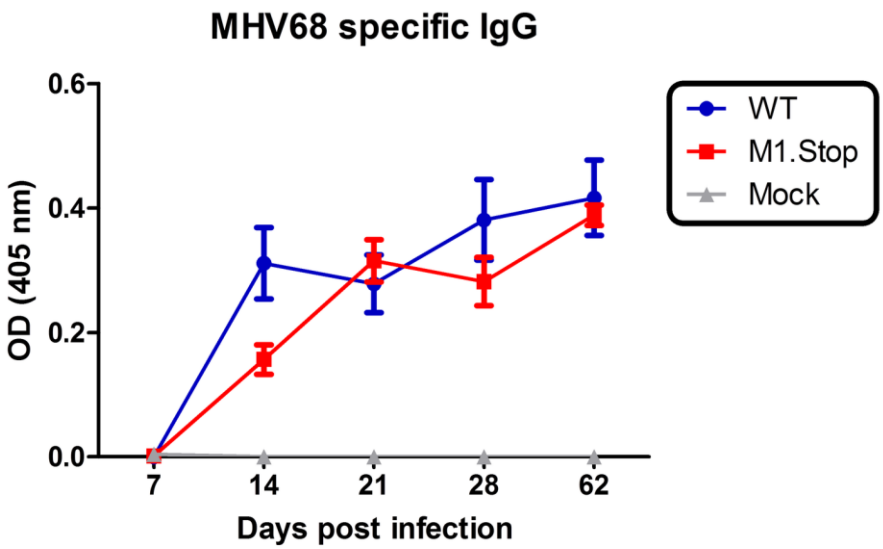
MHV68 specific IgG
OD (405 nm)
0.6
0.4
0.2
0.0
7
14
21
28
62
Days post infection
WT
M1.Stop
Mock

FIGURE LEGENDS

Figure 1: MHV68 co-infection with the non-lethal *P. yoelii* XNL in C57BL/6 results in lethal malarial disease and suppressed *Plasmodium* specific IgG response. (A) Timeline of infection. 6-8 week old C57BL/6 mice were infected with 1000 PFU of MHV68 on day -7 followed by infection with 10^5pRBCs of non-lethal *P. yoelii* XNL or *P. chabaudi* AS. Infections consisted of 5 experimental groups: MHV68 + *Plasmodium*, *Plasmodium*, MHV68 or mock infected. Each experimental group consisted of n=5 and was repeated twice. Animals were sacrificed at days 8, 12, 16 and 23 post *P. yoelii* XNL infection or day 7, 11, 15 and 23 post *P. chabaudi* AS infection for collection of spleen, lung and blood. (B) Survival analysis of animals co-infected with MHV68 and *P. yoelii* XNL or *P. chabaudi* AS. Total IgG and IgM levels in serum in (C) *P. yoelii* XNL (Day 23 IgG – *P. yoelii* vs co-infected: $p<0.05$ Mann Whitney U-test) or (D) *P. chabaudi* AS co-infection model (Day 11 IgG – *P. chabaudi* vs co-infected: $p<0.05$ Mann Whitney U-test). Parasite specific IgG levels in serum during (E) *P. yoelii* XNL (day 23 post infection, *P. yoelii* vs co-infected: $p<0.05$ Mann Whitney U-test) or (F) *P. chabaudi* AS (day 11 post infection, *P. chabaudi* vs co-infected: $p<0.05$ Mann Whitney U-test) co-infection.

Figure 2: *P. yoelii* XNL requires *Plasmodium* specific IgG response to clear primary peak of parasitemia. (A) Percent parasitemia in the periphery during *P. yoelii* XNL ($p<0.05$; area under curve, Mann Whitney U-test) or *P. chabaudi* AS co-infection models ($p>0.05$; area under the curve, Mann Whitney U-test). (B) Anemia during *P. yoelii* XNL ($p>0.05$; area over curve, Mann Whitney U-test, *P. yoelii* vs. co-infected) or *P. chabaudi* AS co-infection ($p>0.05$; area over curve, Mann Whitney U-test, *P. chabaudi* vs. co-infected). (C) Percent parasitemia in periphery during infection of single *P. yoelii* XNL or *P. chabaudi* AS in C57BL/6 or μMT (B cell-deficient) mice. (D) Anemia during infection of single *P. yoelii* XNL or *P. chabaudi* AS in C57BL/6 or μMT mice.

Figure 3: MHV68 suppresses splenic B cell responses during co-infection with *Plasmodium*. The timeline and experimental set up was identical to that shown in Figure 1A. (A) Absolute numbers of splenic GC B cell populations (B220+ GL7+ CD95+) during *P. yoelii* XNL and *P. chabaudi* AS co-infection models with representative gating strategy (Day 12 post *P. yoelii* or Day 15 post *P. chabaudi*; *Plasmodium* vs. co-infected, $p<0.05$, Mann Whitney U-test). (B) Absolute numbers of splenic plasma cell populations (CD3- B220int CD138+) during *P. yoelii* XNL AND *P. chabaudi* AS co-infection models with representative gating strategy (Day 12 post *P. yoelii* or Day 11 post *P. chabaudi*; *Plasmodium* vs. co-infected, $p<0.05$, Mann Whitney U-test). (C) Spleen section for mock infected, MHV68 infected, *P. yoelii* XNL infected and MHV68 and *P. yoelii* XNL co-infected animals at day 8 post infection with *P. yoelii* XNL (or day 15 post-infection with MHV68). Green: B220-FITC (B cells), Blue: GL7-AF660 (Germinal center B cells) and Red: CD3-AF568 (T cells).

Figure 4: MHV68 and *Plasmodium* co-infection results in defective splenic T follicular helper (Tfh) response. The timeline and experimental set up was identical to that shown in Figure 1A. (A) Representative flow plots for gating strategies used to define the global Tfh population (CD4+ PD-1+ CXCR5+), germinal center Tfh (CD4+ GL7+ CXCR5+) and activated/antigen specific Tfh (CD4+ CD44+ PD-1+ CXCR5+). (B) Absolute values for all three Tfh subsets are plotted for the *P. yoelii* XNL (Day 23, all Tfh subsets, *P. yoelii* vs. co-infected, $p<0.05$ Mann Whitney U-test) or (C) *P. chabaudi* co-infection models at multiple time points (Day 23, all Tfh subsets, *P. chabaudi* vs. co-infected, $p<0.05$ Mann Whitney U-test).

Figure 5: Acute, but not latent, MHV68 infection results in suppressed humoral response. (A) Timeline of infection. C57BL/6 mice were infected with 1000 PFU of MHV68 IN at day -60, -30, -15 or -7 and challenged with 10^5 pRBCs on day 0. Absolute number of (B) splenic GC B

cell (B220+ GL7+ CD95+) and plasma cell (CD3- B220int CD138+) populations at day 16 post *P. yoelii* XNL infection (For GC and PC: Day -7 and Day -15 co-infected vs. *P. yoelii,* Kruskal Wallis $p<0.05$; Dunn's pairwise comparison test $p<0.05$/ Day -30 co-infected vs. *P. yoelii,* Kruskal Wallis $p<0.05$; Dunn's pairwise comparison test $p>0.05$). (C) MHV68 and *P. yoelii* XNL specific IgG responses at day 16 post *P. yoelii* XNL infection (Day -7 and Day -15 co-infected vs. *P. yoelii,* Kruskal Wallis $p<0.05$; Dunn's pairwise comparison test $p<0.05$/ Day -30 co-infected vs. *P. yoelii*, Kruskal Wallis $p<0.05$; Dunn's pairwise comparison test $p>0.05$). (D) Global Tfh population (CD4+ PD-1+ CXCR5+), germinal center Tfh (CD4+ GL7+ CXCR5+) and activated/antigen specific Tfh (CD4+ CD44+ PD-1+ CXCR5+) in the spleen at day 16 post *P. yoelii* XNL infection.

Figure 6: The MHV68 M2 gene product is necessary for virus mediated humoral suppression and lethality during *Plasmodium* co-infection. (A) MHV68 specific IgG titers from serum of animals infected with the MR (M2.Marker Rescue) or M2.Stop (ST, M2-null) viruses. Serum was collected and analyzed on days 7, 14 and 21 post infection with either virus (n=10/ virus) (Day 21, MR vs. M2.Stop, Kruskal Wallis $p<0.05$; Dunn's pairwise comparison test $p<0.05$). (B) *P. yoelii* XNL specific IgG response during *P. yoelii* XNL co-infection with either the M2.MR or M2.Stop virus. Serum was collected at day 20 post infection with *P. yoelii* XNL (WT + *P. yoelii* co-infected vs. *P. yoelii,* Kruskal Wallis $p<0.05$; Dunn's pairwise comparison test $p<0.05$/ WT + *P. yoelii* co-infected vs. M2.Stop + *P. yoelii,* Kruskal Wallis $p<0.05$; Dunn's pairwise comparison test $p>0.05$). (C) Survival curve during *P. yoelii* XNL co-infection with either the M2.MR or M2.Stop virus. Note: data representing *P. yoelii* XNL + MHV68 co-infection is the identical data set to that in Fig 1B. It was added in panel C for comparative purposes. (D) % parasitemia in the periphery during *P. yoelii* XNL, *P. yoelii* XNL +MR and *P. yoelii* XNL + M2.Stop infection.

Supplementary Figure 1 (S1): Increased viral persistence and reduced virus specific IgG response during MHV68 and *Plasmodium* co-infection The timeline and experimental set up was identical to that shown in Figure 1A. Limiting dilution analysis of viral lung titers in the (A) *P. yoelii* XNL or (B) *P. chabaudi* AS co-infection models at multiple times post co-infection. MHV68 specific IgG titers in the serum at day 23 post *Plasmodium* infection in (C) *P. yoelii* XNL or (D) *P. chabaudi* AS co-infected mice. (E) Hematoxylin and eosin stain of lung tissue sections from animals sacrificed at day 23 post co-infected with MHV68 and either *P. yoelii* XNL or *P. chabaudi* AS (mock and MHV68 infected lung sections are also shown). Scale bar 100 μm.

Supplementary Figure 2 (S2): Gating strategies for removing doublets and discriminating between and live vs. dead lymphocyte populations in the spleen. Identification of live cells was done using a fixable viability dye (Life Technologies). SSC-A, side scatter area; FSC-A, forward scatter area; FSC-W, forward scatter width; FSC-H, forward scatter height; SSC-W, side scatter width; SSC-H, side scatter height.

Supplementary Figure 3 (S3): MHV68 and *Plasmodium* co-infection does not alter CD4+ Th1 responses in the spleen. The timeline and experimental set up was identical to that shown in Figure 1A. (A) Representative flow panels show gating strategies. Cells were gated on the live population, and CD4+ cells were analyzed for cytokine production. Absolute number of CD4+ T cells producing IL-10, IFN-γ, or both in the (B) *P. yoelii* XNL and (E) *P.chabaudi* AS co-infection models.

Supplementary Figure 4 (S4): MHV68 and *Plasmodium* co-infection does not alter the CD4+ T regulatory (Tregs) subset in the spleen. The timeline and experimental set up was identical to that shown in Figure 1A. (A) Representative flow plots showing gating strategy for

Tregs (CD4+ CD25+ FoxP3+). Absolute numbers of Tregs in the spleen at indicated time points for (B) *P. yoelii* and (C) *P. chabaudi* co-infection models.

Supplementary Figure 5 (S5): *P. yoelii* XNL infection IL-21R-/- mice is lethal. (A) % parasitemia and anemia during *P. yoelii* XNL or *P. chabaudi* AS infection of IL-21R-/- mice. (B) Survival curve during *P. yoelii* XNL or *P. chabaudi* AS infection of C57BL/6, µMT and IL-21R-/- mice.

Supplementary Figure 6 (S6): Increase in level of PD-L1 expression on GC B cells during lethal MHV68 and *P. yoelii* XNL co-infection. The timeline and experimental set up was identical to that shown in Figure 5A. (A) Absolute number of PD-L1 (B220+ GL7+ CD95+ CD274+) expressing splenic GC B cells at day 16 post-infection with *P. yoelii* XNL. (B) Mean Fluorescence Intensity (MFI) of the PD-L1 (CD274) marker on GC B cells at day 16 post-infection with *P. yoelii* XNL.

Supplementary Figure 7 (S7): M1 does not alter kinetics of MHV68 specific IgG response during infection. C57BL/6 mice infected with 1 x 10^5 PFU via the IN route with either the M1 null mutant (M1.Stop virus) or the marker rescue (MR) virus. Blood was collected at multiple times post viral infection. Plotted are MHV68 specific IgG responses as a function of days post viral infection. Serum from naïve mice was used as a negative control.

CHAPTER III

Murine Gammaherpesvirus 68 Reactivation from B Cells Requires IRF4 but Not XBP-1

Caline G. Matar,[a,c] Udaya Shankari Rangaswamy,[a,c] Brian S. Wakeman,[b,c] Neal Iwakoshi,[d] Samuel H. Speck[c,e]

Microbiology and Molecular Genetics Graduate Program,[a] Immunology and Molecular Pathogenesis Graduate Program,[b] Department of Microbiology and Immunology, Emory School of Medicine,c Department of Surgery, Division of Transplantation, Emory School of Medicine,[d] and Emory Vaccine Center,[e] Emory University, Atlanta, Georgia, USA

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Data presented in Figure 8 of the manuscript was generated by Dr. Udaya S. Rangaswamy.

INTRODUCTION

Gammaherpesviruses are lymphotropic viruses that maintain distinct lytic and latent life cycles [95]. Primary infections are generally asymptomatic in immune competent hosts, although Epstein-Barr virus (EBV) acute infection can result in the self-limiting lymphoproliferative syndrome infectious mononucleosis in young adults. Furthermore, EBV is associated with the development of several cancers in humans – including endemic Burkitt's lymphoma, nasopharyngeal carcinoma and some Hodgkin's lymphomas [8,195-200] . The other known human gammaherpesvirus, Kaposi's sarcoma-associated herpesvirus (KSHV), has also been linked to various cancers – most notably Primary Effusion Lymphoma (PEL) and multicentric Castleman's disease [201-204]. Murine gammaherpesvirus 68 (MHV68) infection of laboratory strains of inbred mice has been extensively studied to characterize basic aspects of gammaherpesvirus pathogenesis *in vivo*. Similar to the human viruses, MHV68 has been associated with the induction of B cell lymphomas in immunosuppressed mice [205,206] and to immortalize fetal liver-derived B cells *in vitro* [207].

Members of the gammaherpesvirus family predominantly infect and maintain latency in B cells [63,195,208,209]. In fact, persistence of the latency reservoir is postulated to be dependent on viral reactivation from latency. Several reports have indicated a close link between plasma cell differentiation and viral reactivation from latency [166,210,211]. Previous work from our lab has demonstrated a distinct requirement for Blimp-1 mediated plasma cell differentiation in viral reactivation, long term maintenance of latency, and persistence of long-term MHV68 specific antibody responses [212]. Recently, a strong link between plasma cell transcription factor X-box binding protein-1 (XBP-1) and viral reactivation has been reported for both EBV [213,214] and KSHV [215-217]. These studies demonstrated that overexpression of XBP-1s in latently infected EBV or KSHV cell lines could induce viral reactivation. Additionally, these data

collectively demonstrate that XBP-1s binds to specific residues in the BZLF-1 and RTA promoters, as well as synergizes with RTA expression.

XBP-1, a basic region leucine zipper (bZIP) transcription factor [218], has been shown to be essential for plasma cell function [219,220]. XBP-1 is a member of the CREB/ATF family of transcription factors that was initially discovered due to its ability to bind cyclic AMP (cAMP) response sequences in the MHC class II human gene locus [218]. Functionally, it plays an integral role in mediating the unfolded protein response (UPR) in the endoplasmic reticulum. Initially described in yeast, Inositol Requiring Enzyme 1 (IRE-1) is a transmembrane protein with kinase and endonuclease activity. It is activated in response to the accumulation of unfolded proteins and chaperones during cellular stress. Oligomerization and trans-autophosphorylation activates the endoribonuclease function of IRE-1. The only identified substrate for IRE-1 processing are the homologs Hac1 (yeast) and XBP-1 (metazoans). IRE-1 activation results in the removal of a 26 nucleotide intron in the XBP-1 transcript, changing it from the un-spliced form XBP-1u (inhibitor of the UPR) to the spliced form XBP-1s (activator of the UPR). XBP-1 splicing results in the acquisition of a C-terminal transactivation domain [221-223]. As a result, XBP-1s targets expression of UPR genes, such as chaperone proteins, that mediate a survival response in the stressed cell. B cell differentiation into a plasma cell results in large quantities of immunoglobulin production which induces the UPR. Additionally, XBP-1 expression is an integral component of mediating the UPR during plasma cell differentiation and permits for immunoglobulin (Ig) secretion [219,220,224].

This direct interaction between a plasma cell host transcription factor and the immediate early gene promoter of gene 50 indicates a viral evolutionary adaptation that senses changes in the viability of the host cell and signals an escape response, such as reactivation. However, due to the species specific nature of the human gammaherpesviruses, there are no direct *in vivo* studies addressing the role of XBP-1 in plasma cell differentiation-mediated reactivation. As such, using

MHV68 as a model system, we have evaluated the *in vivo* implications of loss of XBP-1 expression specifically in B cells on MHV68 latency and reactivation. A valuable transgenic model which selectively deletes XBP-1 expression from B cells has been previously described [225] and used in this study. Loss of XBP-1 expression in B cells was demonstrated to have no effect on germinal center or plasma cell frequencies, but showed a defect in immunoglobulin secretion from plasma cells [225]. Here we report that XBP-1s transactivation of the MHV68 gene50 proximal promoter can be observed *in vitro*, consistent with studies on the impact of XBP-1 expression on EBV and KSHV immediate-early gene expression [213-217]. However, we did not observe a requirement for B cell specific XBP-1 expression to promote viral reactivation or maintenance of MHV68 latency *in vivo*. This unexpected observation points to the value of *in vivo* pathogenesis models, which help reveal the often complex and perhaps redundant network of host transcription factors that can modulate the viral life cycle. Additionally, in contrast to the apparent lack of a role for XBP-1 in MHV68 reactivation, we show that another host transcription factor IRF4 – which is essential for plasma cell differentiation - plays a critical role in MHV68 reactivation from B cell latency. Notably, IRF4 has been implicated in EBV transformation of human B lymphocytes [226]. However, the role of IRF4 in reactivation from B cells for the human gammaherpesviruses still remains unknown.

MATERIALS AND METHODS

Promoters and expression vectors: Proximal ORF50 promoter (position 66142-66552 in viral genome) of MHV68 was cloned into a pGL4.10[luc 2] vector from Promega (pGL410-PpORF50). Primers used to amplify and clone the proximal promoter include Forward primer: 5'tcagggattcagccaacaa3' and Reverse primer: 5'AAGGTGGTGGTTGCCAGC3'. NheI and EcoRV restriction enzymes were used to clone the promoter upstream of the luciferase expression construct. The XBP-1s expression vector was a kind gift of Dr. Xiaozhan Liang, and was initially cloned into the MSCV-IRES-GFP vector (Addgene). For experiments outlined in this manuscript, the XBP-1s insert was cloned into the pCMV.Tag2b (Addgene) vector using the following primers: F-XBP-1s-BglII: 5'ccatcgaagatctATGGTGGTGGTGGCAGCGG3' and R-XBP-1s-PstI: 5'ccatcgactgcagTTAGAGGCTTGGTGTATACATGGTCA3'.

Tissue culture and promoter assays: The M12 B cell murine lymphoma cell line was used for promoter assays. Cells were plated in 6 well Corning tissue culture dishes at a density of 2.5 x 10^5 cells/ 2 ml of media 12 hours prior to transfection. The lipid based transfection was performed using the Transfectin Lipid Reagent (BioRad). Protocol was designed based on manufacturer's instructions. Briefly, a total of 2µg of DNA (pGL410-PpORF50 + XBP-1s.pCMV.Tag2b) was added to 7 ul of the lipid reagent and diluted in neat DMEM. The mixture was allowed to incubate at room temperature for 20 minutes. 500µl of the master mix was dripped onto the plated B cells and gently swirled. 12 hours post transfection cells were treated with 20ng/ml of 12-O-tetradecanoylphorbol-13-acetate (TPA) and/or a final concentration of 0.5 µM of Ionomycin. Cells were allowed to incubate for an additional 12 hours before harvesting and analyzing luciferase activity (Promega luciferase assay system).

Animals and Infections: XBP-$1^{Flox/Flox}$ $CD19^{CRE/+}$ C57BL/6 mice were a kind gift of Dr. Neal Iwakoshi. The mice were generated as previously described [225] and were bred in house. In

order to confirm LoxP site at the XBP-1 exon 2 locus, we used the following primers; forward 3'loxS: 5'ACTTGCACCAACACTTGCCATTTC3' and Reverse 3'loxA: 5'CAAGGTGGTTCACTGCCTGTAATG3'. In order to confirm CD19-CRE recombinase insertion, we used the following primers: Forward primer CRE-F: 5' GCGGTCTGGCAGTAAAAACTATC3' and reverse primer CRE-R: 5' GTGAAACAGCATTGCTGTCACTT 3'. As littermate controls in our experiments, we used XBP-$1^{fl/fl}$ *CD19*$^{+/+}$ that contain the floxed allele without Cre expression.

$IRF4^{fl/fl}$ mice and $CD19^{Cre/Cre}$ mice were purchased from Jackson labs (Catalog numbers 009380 and 006785, respectively). $Irf4^{fl/fl}$ mice were crossed with $CD19^{+/CRE}$ mice to generate Cre expressing mice that were homozygous for the conditional IRF4 allele. The resulting $IRF4^{fl/fl}CD19^{Cre/+}$ mice express Cre recombinase under the CD19 promoter, thus lacking IRF4 expression in CD19 expressing B cells. As littermate controls in our experiments, we used $IRF4^{fl/fl}$ $CD19^{+/+}$ that contain the floxed allele without Cre expression. Genotyping was performed as described in Jackson labs protocol. Additionally, the $IRF4^{fl/fl}$ mice contain an EGFP cassette flanking the loxP sites, such that Cre mediated excision of the floxed alleles results in EGFP expression. Therefore, mice were confirmed by genotyping of ear tissue followed by flow cytometry analysis on peripheral blood to confirm expression of EGFP expression in the knockouts.

Animals were housed at Emory University Division of Animal Resources in accordance with guidelines specified by Institutional Animal Care and Use Committee at Emory University (IACUC protocol number: YER-2002245-031416GN). Mice were infected at 8-12 weeks of age. Animals were anesthetized with Isoflurane prior to infection. The MHV68-H2bYFP virus [62] was administered intranasally in a 20µl volume at a dose of 1000 Plaque Forming Units (PFU). For infections of $IRF4^{fl/fl}CD19^{+/Cre}$ and $IRF4^{fl/fl}CD19^{+/+}$, the M2stop.HY and M2MR.HY viruses

described in [111] were used as indicated. Animals were sacrificed between days 16-18 post infection for analysis of splenic populations.

Limiting Dilution Analysis: Limiting dilution PCR (LD-PCR) was used to quantify latent levels of viral genome as previously described [64]. Briefly, frozen splenocytes were thawed (after storage at -80^{0}C in cDMEM + 10% Dimethyl Sulfoxide), washed in an isotonic buffer and plated in a 96 well PCR plate at a starting density of 10^4 splenocytes and serially diluted 3-fold on a background of uninfected 3T12s. Each dilution underwent 12 PCR reactions, and each sample was serially diluted 6 times. Samples were digested with Proteinase K overnight (8-12 hours), followed by two rounds of nested PCR. Standards included 0, 0.1, 1 and 10 copies of a control DNA plasmid to determine viral DNA copy number. Samples were then run on 2% agarose gel and stained with ethidium bromide. Wells positive for viral genome at each dilution were plotted using the Poisson distribution. Limiting dilution reactivation assay was performed to evaluate the frequency of viral genome reactivating from latency [64]. Briefly, 5 x 10^6 splenocytes were explanted on a monolayer of Mouse Embryonic Fibroblasts (MEFs) in a 96 well format. Splenocytes were serially diluted two-fold in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS). Cells were plated in 24 wells per dilution, and diluted up to 12 dilutions. After 14-21 days of incubation, wells were monitored for Cytopathic Effect (CPE) and frequencies were plotted using the Poisson distribution. As a control, splenocytes were physically disrupted to release preformed virus using 0.5 mm silicon beads in a bead beater (Beat for 1 minutes 4x, rest on ice after every disruption cycle for 1 minute). Homogenates were plated on MEFs as described earlier.

Antibodies: Splenocytes were blocked with anti-CD16/32 (BD bioscience). Surface stains were performed in PBS-2% FBS- 1mM EDTA for 20 minutes on ice. Markers used: CD138-PE (BD bioscience), B220-Pacific Blue (Biolegend), CD95-PE-Cy7 (BD biosciences), GL7-Alexa Fluor

660 (eBioscience), CD3/4/8-PerCP (BD bioscience). Fixable live dead stains in Zombie Yellow (Pacific Orange) was purchased from Biolegend and used according to manufacturer's guidelines.

Enzyme Linked Immunosorbent Assay (ELISA): Blood was collected during terminal bleeds. Serum was collected by allowing the blood to clot at 4^0C for 1 hour. Blood was centrifuged at 14000 RPM at 4^0C for 2 minutes. Serum was collected and frozen at -80^0C for long term storage. ELISA protocol was used as previously described [70] to measure global IgG and IgM levels. Briefly, 96 well Nunc ImmunoMaxisorp ELISA plates were coated with 0.5 ug/well of goat anti-mouse IgG or IgM antibody (Southern Biotech). Serum was serially diluted (3 fold, beginning at 1:100) and 6 dilutions were plated for each sample. Alkaline Phosphatase conjugated goat anti-mouse IgG or IgM (Southern Biotech) was used as a secondary antibody. Color was developed using p-nitrophenyl phosphate (Sigma) in a diethanolamine substrate buffer. Absorbance at 405 nm was read on a Biotek Synergy HT reader.

RESULTS

Murine XBP-1s can transactivate the MHV68 ORF50 proximal promoter *in vitro.*

Previous reports indicated the potential for the active spliced form of XBP-1 to transactivate the BZFL-1 promoter in EBV [213,214], and RTA (encoded by gene 50) in KSHV [215-217]. To evaluate whether murine XBP-1s could regulate gene 50/RTA expression in MHV68, we transfected the murine M12 B lymphoma cell line with an expression construct expressing XBP-1s in conjunction with a luciferase reporter construct driven by the proximal gene 50 promoter (PpORF50) (Figure 1). Cells were incubated for 12 hours post-transfection in the presence and absence of treatment with the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) and/or ionomycin (Fig. 1). Notably, expression of XBP-1s alone was capable of inducing a 10-fold increase in PpORF50 activity relative to basal promoter activity. Treatment of the PpORF50 with only TPA or ionomycin alone modestly increased basal activity by 3-5 fold. Interestingly, cells expressing XBP-1s that were treated with either TPA, ionomycin or both, showed synergistic induction of promoter activity up to 50-fold over basal promoter activity (Fig. 1). TPA and ionomycin induce protein kinase C and Ca^{2+} mediated signaling, thus promoting B cell proliferation and mimicking the signals induced by B cell receptor (BCR) crosslinking [227]. Of note, CD40 and Ig crosslinking has been shown to induce MHV68 reactivation from latency [228]. Since XBP-1s reflects a stress signal in a terminally differentiating cell, it is evident that the virus has evolved mechanisms to reactivate from latency when the host cell is at a risk of undergoing cell death. Thus, to ensure continuity of the latency reservoir, the immediate-early gene product Replication and Transcription Activator (RTA) is synthesized to promote viral reactivation from latency. The requirement for BCR crosslinking, co-stimulation (CD40) and plasma cell specific transcription factors [XBP-1s, Blimp-1 [212]] reflects the specificity and timely induction of viral reactivation from latency.

MHV68 infection in XBP-1$^{flox/flox}$ CD19$^{CRE/+}$ results in normal germinal center and plasma cell responses, but reduced levels of immunoglobulin in the periphery.

We next characterized the course of MHV68 infection in mice that were deficient in XBP-1 expression in B cells. Previous work by Todd et al. demonstrated that mice lacking XBP-1 expression in B cells could generate normal germinal center and plasma cell frequencies in response to challenge with a foreign antigen. Interestingly, they determined that XBP-1 expression occurred after B cells up-regulated Syndecan-1 (CD138), a receptor commonly used to identify the plasma cell population [229]. As such, B220^{int} CD138^{+} cell frequencies were comparable to littermate control mice. The only defect observed in these animals was a significant reduction in global levels of IgG and IgM in the periphery [225]. To investigate the role of XBP-1 in MHV68 infection, XBP-1$^{flox/flox}$ CD19$^{CRE/+}$ mice were infected with 1000 PFU of the MHV68-H2bYFP virus [62] via the IN route. Spleen and blood were collected on day 18 post-infection. Upon analysis of splenic subsets, we observed that germinal center responses, measured by the percentage of CD19^{+}/ GL7+ CD95+ cells, were identical in knockout (KO) as well as littermate (LM) controls (Fig. 2A & 2B). Additionally, the frequency of B220^{int} CD138^{+} plasma cells was also comparable in both KOs and LMs (Fig. 2A & 2C).We next evaluated the concentration of total IgM and IgG levels from sera collected at day 18 post-infection (Fig. 3). As reported earlier [225], upon MHV68 infection, XBP-1$^{flox/flox}$ CD19$^{CRE/+}$ animals failed to mount both an IgM and IgG response as compared to LM controls (Fig3 A & 3B) . Taken together this data suggests that XBP-1 expression in B cells is not required for the generation of germinal center and plasma cell responses during an MHV68 infection, but is required for Ig secretion and generation of a virus specific humoral response.

Global frequency of MHV68 infected B cells is unaltered in XBP-1$^{flox/flox}$ CD19$^{CRE/+}$.

Using YFP expression from the MHV68/H2B.YFP virus as an indicator of the frequency of infected B cells [62,230], we evaluated the requirement for XBP-1 expression from B cells during infection and maintenance of latency. Upon evaluation of global levels of YFP expression from the CD3 negative splenic population, we determined that the efficiency of viral infection was equivalent in both KOs and LMs (Fig. 4A & 4B). Collins et al. demonstrated that greater than 75% of YFP positive B cells have a germinal center phenotype. Consistently, we detected close to 70% of YFP+ B cells in an XBP-1$^{flox/flox}$ CD19$^{CRE/+}$ infection with a germinal center phenotype (Fig. 5A & 5B). Interestingly, the frequency of YFP+ B cells with a plasma cell phenotype was significantly lower in XBP-1$^{flox/flox}$ CD19$^{CRE/+}$ mice ($\bar{x}$= 9%) compared to LMs ($\bar{x}$=15%) (Fig. 5A & 5C). As expected, frequency of infected germinal center B cells was comparable (LM $\bar{x}$=7.8% and KO $\bar{x}$=7.4%) (Fig. 6A & 6B). However, when we compared the frequency of plasma cells that were infected with the virus, we noted a modest decrease in frequency of infection in KOs as compared to LM controls that was not statistically significant (LM $\bar{x}$=6.6% and KO $\bar{x}$=4.5%) (Fig. 6A & 6C). Since the efficiency of viral infection of the plasma cell repertoire was not significantly compromised in XBP-1$^{flox/flox}$ CD19$^{CRE/+}$ infections compared to LM controls (Fig. 6C), the defect observed in the frequency of YFP+ cells that have a PC phenotype during a XBP-1$^{flox/flox}$ CD19$^{CRE/+}$ infection (Fig. 5C) cannot be explained by inefficient infection of that cellular subset. It is unclear at this point the basis for this defect. However, this prompted a key question of whether a reduction in the levels of YFP+ cells with a plasma cell phenotype could affect the frequency of cells reactivating virus.

XBP-1s expression in B cells is not essential *in vivo* for viral reactivation or persistence.

Several studies of EBV, KSHV and MHV68 have suggested a strong link between plasma cell differentiation and virus reactivation [166,210,211]. As discussed above, our *in vitro* assays strongly suggested a role for XBP-1s in regulating viral reactivation from latency (Fig. 1). Since we observed a significant decrease in the frequency of YFP+ cells with a plasma cell phenotype in KO versus LM controls (Fig. 5C), we wanted to determine if there was a defect in MHV68 reactivation from latently infected splenocytes, where the vast majority of latently infected cells are B cells. As such, we conducted limiting dilution reactivation assays to evaluate the frequency of cells reactivating from latency at day 18 post-infection. Surprisingly, loss of XBP-1 expression in B cells did not alter the efficiency of viral reactivation from latency in XBP-$1^{flox/flox}$ $CD19^{CRE/+}$ (1: 10,543) compared to LM controls (1: 8,336) (Fig. 7A). As expected, viral genome loads were comparable in both KO (1: 488) and LM (1: 474) animals (Fig. 7B).

IRF4 expression in B cells is required for MHV68 reactivation from latency and maintenance of latency.

The Interferon Response Factor 4 (IRF4) is another key transcription factor involved in plasma cell differentiation. B cells deficient in IRF4 expression have diminished germinal center responses and cannot generate a plasma cell response [231,232]. We recently demonstrated that the unique MHV68 gene product, M2, is capable of inducing IRF4 expression via activation of NFAT, and promotes IL-10 expression from B cells [111]. Infection of $IRF^{fl/fl}$ mice with a recombinant virus that expresses Cre recombinase resulted in a significant defect in the establishment of latency, along with a greater defect in viral reactivation [111]. This novel finding suggested a close link between viral reactivation and a requirement for IRF4 expression. However, since IRF4 is expressed in cell types other than B cells and since the virus can infect

macrophages and dendritic cells, we could not infer that the requirement for reactivation was contingent on IRF4 expression in B cells. Given that XBP-1 does not appear to be involved in controlling MHV68 reactivation from latently infected B cells, along with our previous demonstration that there is only a moderate requirement of Blimp-1 [212], we hypothesized that IRF4 may be a vital player in reactivation associated with plasma cell differentiation upon MHV68 infection of mice.

To address the requirement for IRF4 in a more direct fashion, we used mice in which IRF4 was specifically deleted in B cells. We have recently shown that M2, a viral gene unique to MHV68, is sufficient to drive plasma cell differentiation in a B cell line [166] and to induce IRF4 expression in B cells [111]. Since the phenotype observed with infection of $IRF4^{fl/fl}$ mice with a recombinant MHV68 expressing Cre-recombinase closely resembles that of infection of wild type mice with an M2null virus, we also hypothesized that M2 induction of IRF4 is a critical step in the viral life cycle. As such, $IRF4^{fl/fl}CD19^{+/Cre}$ (IRF4 KO) or $IRF4^{fl/fl}CD19^{+/+}$ littermate control (LM) mice were infected with 1000 PFU of either M2MR.HY or M2stop.HY viruses [described in [111]]. Spleens were harvested on day 16 post-infection and analyzed by flow cytometry. Notably, the percentage of total germinal center B cells was significantly different between the LM and KO mice infected with the M2stop marker rescue virus (MR), suggesting that IRF4 does play a role in the germinal center response to MHV68 infection (Fig. 8C, compare KO-MR and LM-MR infected animals). However, it should be noted that the basal level of germinal center B cells was also substantially lower in IRF4 B cell knockout mice (see Fig. 8C, KO-Naïve). Indeed, the fold increase in splenic gernimal center B cells (naïve mice vs MHV68 infected) was very similar when comparing littermate control and IRF B cell knockout mice (ca. 20-fold for the IRF4 KO vs ca. 15-fold for the littermate control animals) (Fig. 8C). We next assessed the percentage of total plasma cells, defined by $B220^{lo}CD138^{+}$ cells. As expected, there was a significant difference in the percentage of plasma cells in the IRF4 KO animals compared to littermate

controls (Fig. 8D). While in littermate control animals there as a nearly 4-fold increase in the number of splenic plasma cells in marker rescue infected mice, there was only a 1.3-fold increase in the IRF4 KO animals (Fig. 8D).

Given the significant impact of loss of IRF4 expression in B cells on the splenic plasma cell response, we next assessed the impact of loss of IRF4 in B cells on MHV68 reactivation from latency (Fig. 8A). Notably, littermate control animals (LM) infected with M2stop marker rescue virus exhibited the expected frequency of reactivating splenocytes (compared to historical values for wild type MHV68 reactivation from C57Bl/76 splenocytes at day 16 post-infection) (Fig. 8A). In contrast, reactivation of splencytes from IRF4 KO mice infected with the M2stop marker rescue virus exhibited an ca. 30-fold defect (1 in 3,954 cells reactivating virus in littermate controls vs 1 in 115,877 cells in IRF4 KO mice). Importantly, infection of littermate control animals with the M2 null mutant (M2stop) yielded a very similar reactivation defect (Fig. 8A) – consistent with a major component of M2 function being induction of IRF4 expression in latently infected B cells [111] . However, it is also notable that infection of IRF4 knockout mice with the M2 null mutant resulted in a more severe defect in reactivation from latency (1 in 630,957 cells reactivating virus), suggesting that the complete loss of IRF4 is more severe than the loss of IRF4 induction mediated by M2 (i.e., there may be alternatively, M2-independent mechanisms to induce IRF4 expression in B cells during virus infection). Finally, not surprisingly, the amount of preformed infectious virus present in the day 16 samples mirrored the levels of virus reactivation observed – but did not interfere with accurate assessments of the frequency of splenocytes reactivating virus (Fig. 8B).

DISCUSSION

Here we have shown that while XBP-1s can activate the MHV68 immediate-early gene 50 proximal promoter, which drives expression of the essential transcriptional activator RTA, it is not required for MHV68 reactivation from latently infected B cells as assessed by a limiting dilution ex vivo reactivation analysis of latently infected splenocytes. However, it is possible that XBP-1s plays a role in activation of gene 50 transcription in B cells under other conditions (e.g., in response to specific stimuli), or from other infected cell types. Notwithstanding these possibilities, the data presented here brings into question the significance of studies to date that have demonstrated the ability of XBP-1s to activate EBV and KSHV immediate-early gene expression – all of which have exclusively relied on studies in tissue culture models.

Notably, the requirement for IRF4 expression in B cells for efficient MHV68 reactivation from latently infected splenic B cells likely reflects the essential role that IRF4 plays in plasma cell differentiation. This is based on: (i) our earlier studies that demonstrated that the vast majority of MHV68 reactivation from splenocytes arises from the infected splenic plasma cell population [166] ; and (ii) the fact that we have failed to detect IRF4 transactivation of any of the identified gene 50 promoters (data not shown). Taken together with the XBP-1 data, this suggest that there is another plasma cell factor that triggers gene 50 expression upon terminal differentiation of latently infected B cells. One logical possibility is Blimp-1, another cellular factor that is essential for plasma cell. Indeed the phenotype in Blimp-1 B cell knockout mice is similar to that observed in IRF4 B cell knockout animals [212] . It is also possible that the loss of expression of a transcriptional repressor upon terminal differentiation is also involved in the induction of RTA expression. Further studies are required to identify the critical cis-elements and trans-acting factors involved in the induction of MHV68 gene 50 transcription upon plasma cell differentiation.

Interestingly, the reactivation defect in the IRF4$^{flox/flox}$ CD19$^{CRE/+}$ splenocytes is nearly identical to an M2-null virus phenotype. M2 is a unique gene product of MHV68 that is not required for acute viral replication *in vivo*, but has a dose and route specific defect in establishment of latency and reactivation [233]. Consistent with the requirement of M2 in reactivation, it is also required for the differentiation of infected B cells to become plasma cells [166]. Indeed, it was shown that M2 induction of IL-10 from B cells occurs via an NFAT-dependent induction of IRF4 [111]. In fact, when overexpressed in B cells, M2 has been shown to induce up-regulation of IRF4, Blimp1 and XBP-1 transcripts [166]. EBV has been shown to mimic BCR crosslinking and Ca^{2+} signaling via the LMP2a protein product, and was also shown to encode an IL-10 homolog (v-IL10) [113,184]. It is evident that this family of viruses has not only evolved to respond to changes in B cell differentiation state, but they appear also capable of modulating B cell differentiation. This likely reflects the requirement for proper expansion of the B cell reservoir in order to efficiently establish viral latency [234].

Finally, SAP-/- [235] and IL21R-/- (Collins and Speck, manuscript in preparation) mice infected with MHV68 display severe defects in viral reactivation and establishment of latency. Of note, both of these transgenic models show a defective generation or maintenance of the germinal center response, which also negatively impacts the plasma cell repertoire [164,236,237]. Once more, the loss of this cellular subset further supports viral dependency on this host cell for proper regulation of the viral life cycle. Together with our results presented above, it strongly establishes that the virus takes advantage of a healthy host by utilizing the normal B cell differentiation that occurs in response to viral infection. The ability for MHV68 to induce B cell differentiation, as well as respond to plasma cell specific transcription factors, reflects a close link between the viral life cycle and the plasma cell niche. Our data suggests a role for multiple redundant plasma cell specific transcription factors in modulating the viral life cycle. Finally, the

data presented here emphasizes the importance of *in vivo* pathogenesis studies in identifying those host cell factors and cellular reservoirs that are required for gammaherpesvirus infection.

FIGURES

Figure 1

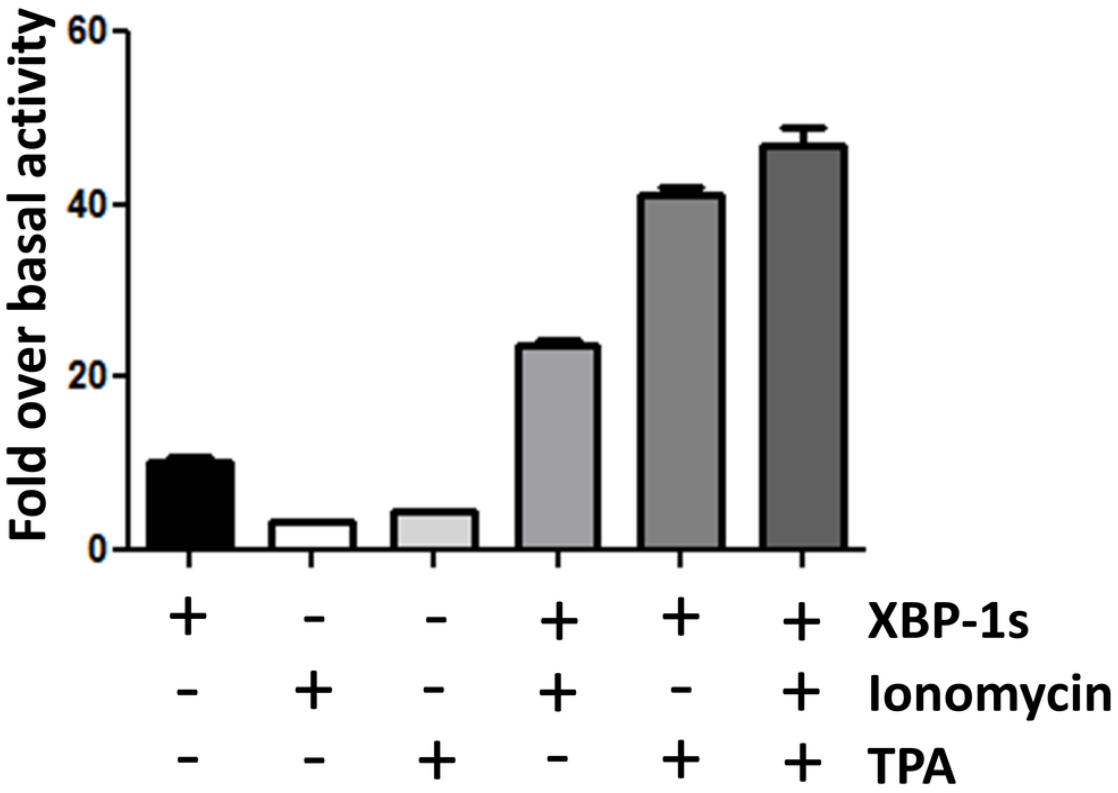

Figure 2

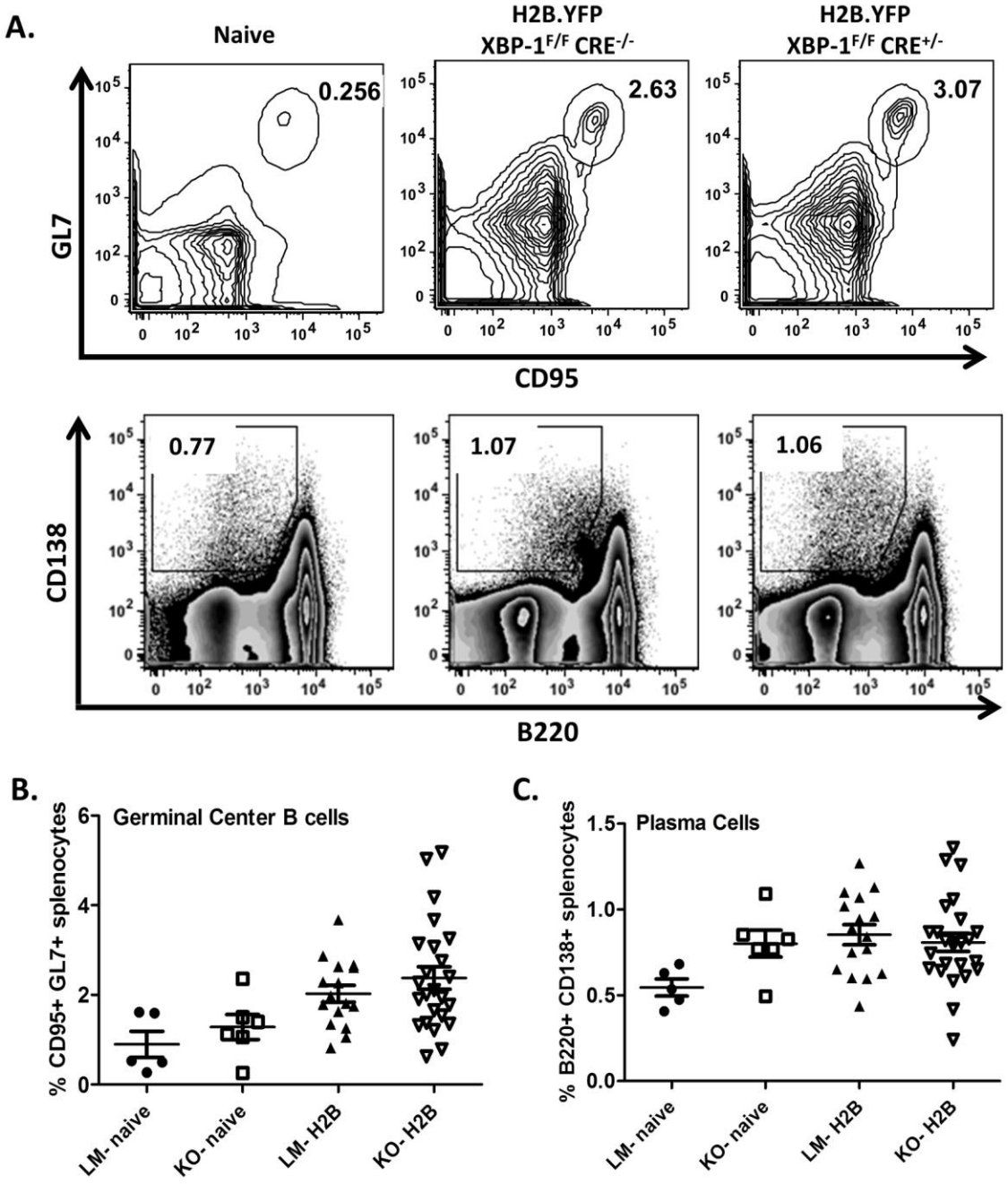
A.
Naive
H2B.YFP
XBP-1F/F CRE-/-
H2B.YFP
XBP-1F/F CRE+/-
0.256
2.63
3.07
GL7
CD95
0.77
1.07
1.06
CD138
B220
B.
Germinal Center B cells
% CD95+ GL7+ splenocytes
LM- naive
KO- naive
LM- H2B
KO- H2B
C.
Plasma Cells
% B220+ CD138+ splenocytes
LM- naive
KO- naive
LM- H2B
KO- H2B

Figure 3

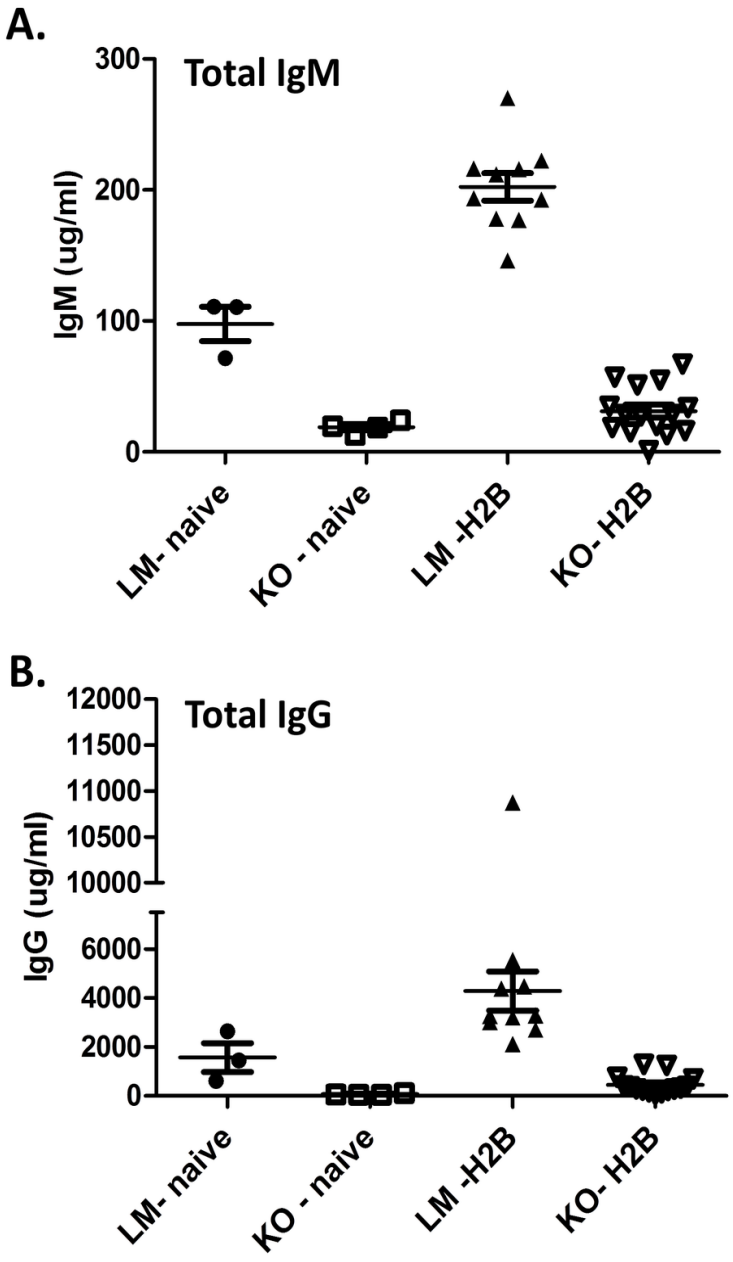
A.
Total IgM
IgM (ug/ml)
300
200
100
0
LM- naive
KO - naive
LM -H2B
KO- H2B
B.
Total IgG
IgG (ug/ml)
12000
11500
11000
10500
10000
6000
4000
2000
0
LM- naive
KO - naive
LM -H2B
KO- H2B

Figure 4

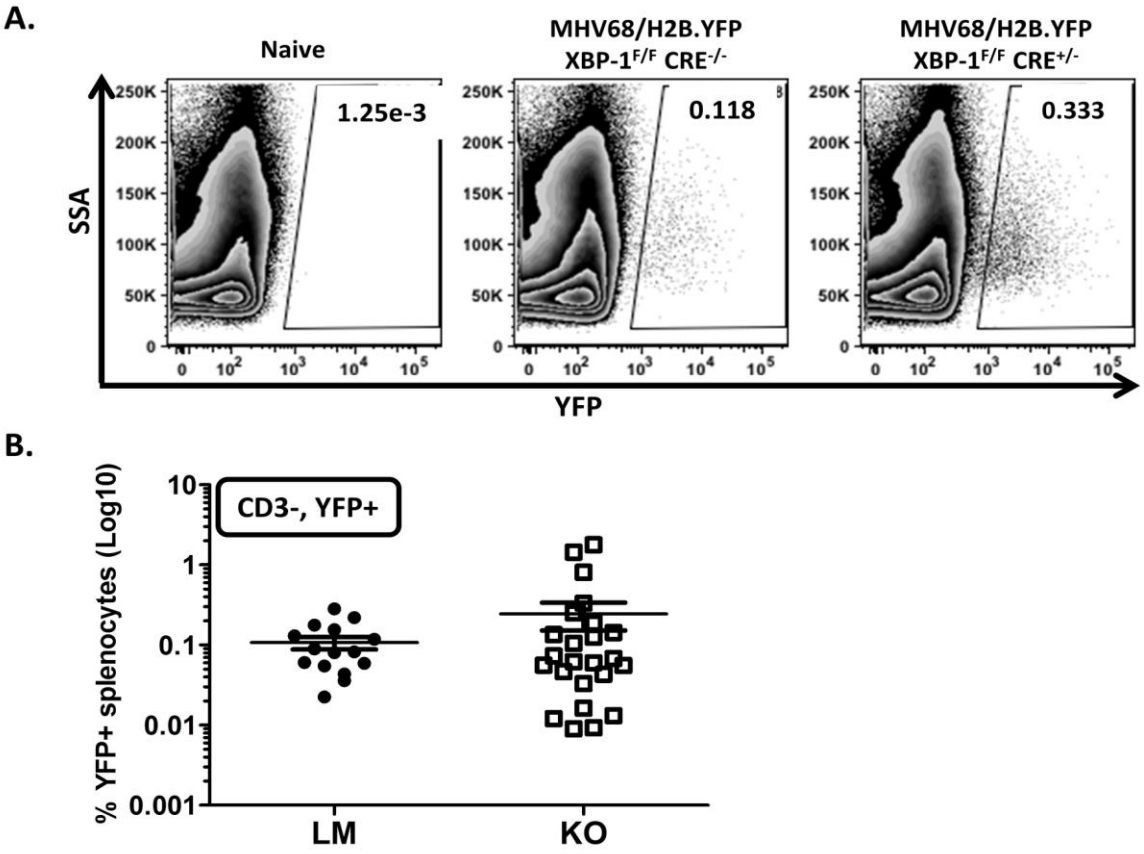
A.
Naive
MHV68/H2B.YFP
XBP-1F/F CRE-/-
MHV68/H2B.YFP
XBP-1F/F CRE+/-
1.25e-3
0.118
0.333
SSA
YFP
B.
CD3-, YFP+
% YFP+ splenocytes (Log10)
LM
KO

Figure 5

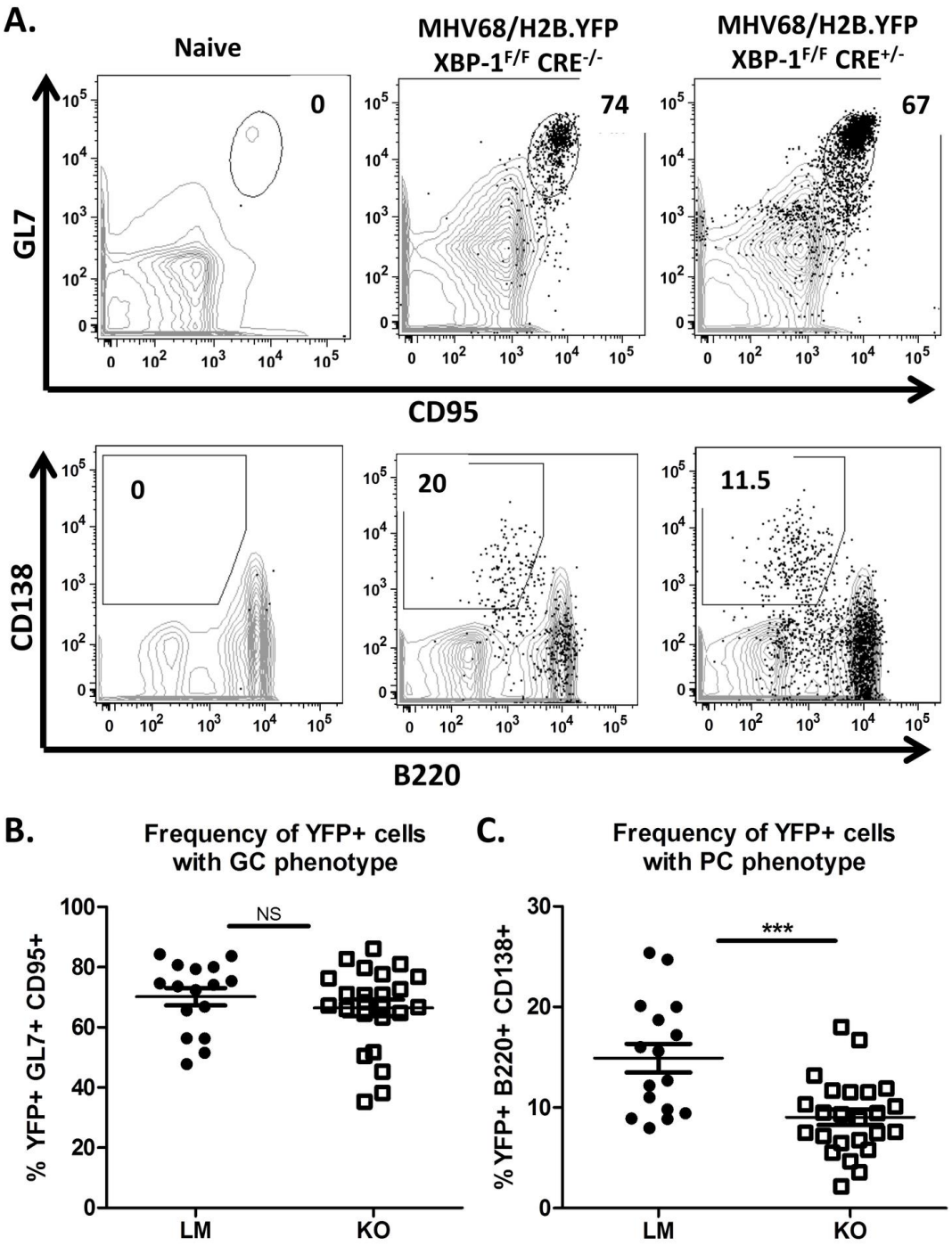
A.
Naive
MHV68/H2B.YFP
XBP-1F/F CRE-/-
MHV68/H2B.YFP
XBP-1F/F CRE+/-
0
74
67
GL7
CD95
0
20
11.5
CD138
B220
B.
Frequency of YFP+ cells
with GC phenotype
NS
% YFP+ GL7+ CD95+
LM
KO
C.
Frequency of YFP+ cells
with PC phenotype
***
% YFP+ B220+ CD138+
LM
KO

Figure 6

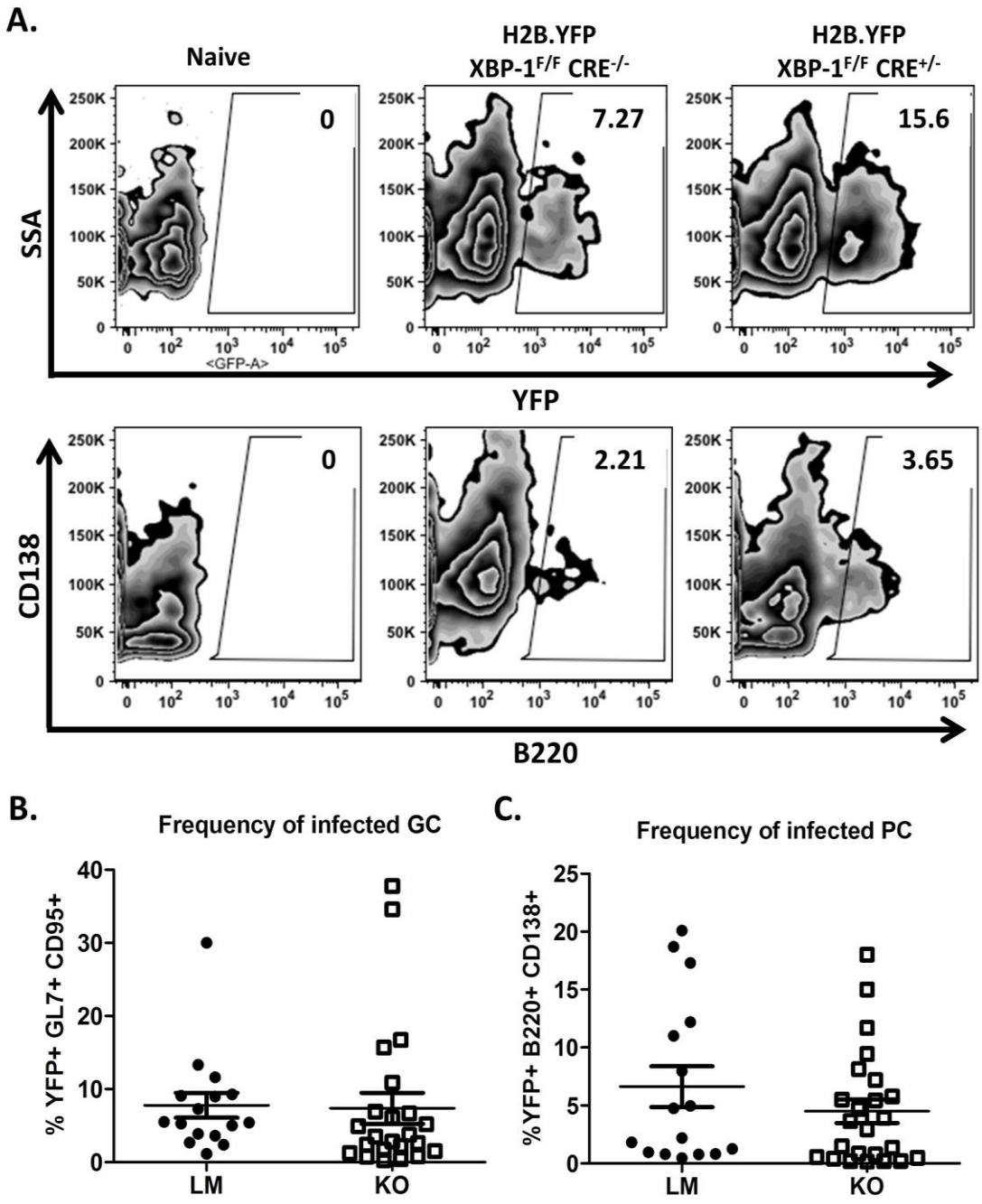
A.
Naive
H2B.YFP
XBP-1F/F CRE-/-
H2B.YFP
XBP-1F/F CRE+/-
0
7.27
15.6
SSA
YFP
<GFP-A>
0
2.21
3.65
CD138
B220
B.
Frequency of infected GC
% YFP+ GL7+ CD95+
LM
KO
C.
Frequency of infected PC
%YFP+ B220+ CD138+
LM
KO

Figure 7

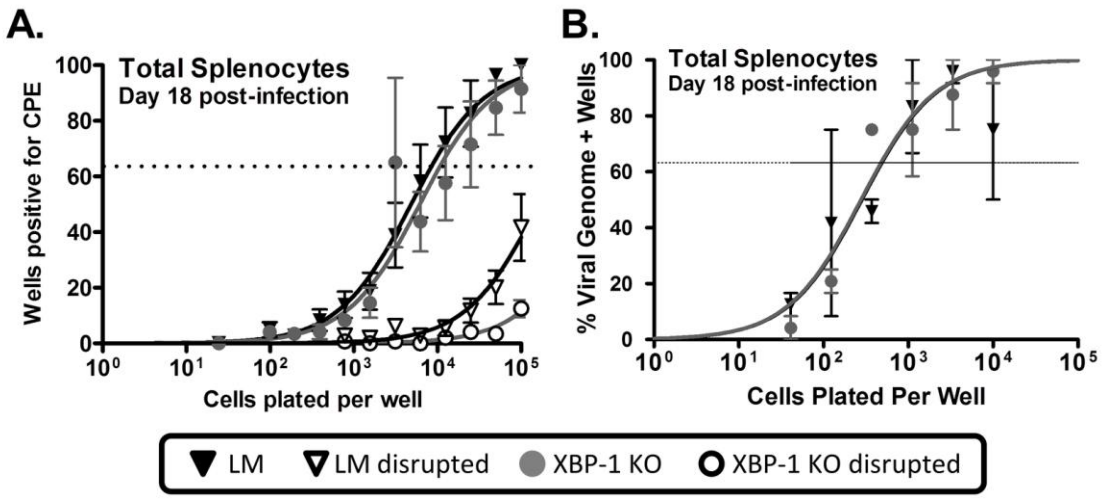
A.
Total Splenocytes
Day 18 post-infection
Wells positive for CPE
Cells plated per well
B.
Total Splenocytes
Day 18 post-infection
% Viral Genome + Wells
Cells Plated Per Well
LM
LM disrupted
XBP-1 KO
XBP-1 KO disrupted

Figure 8

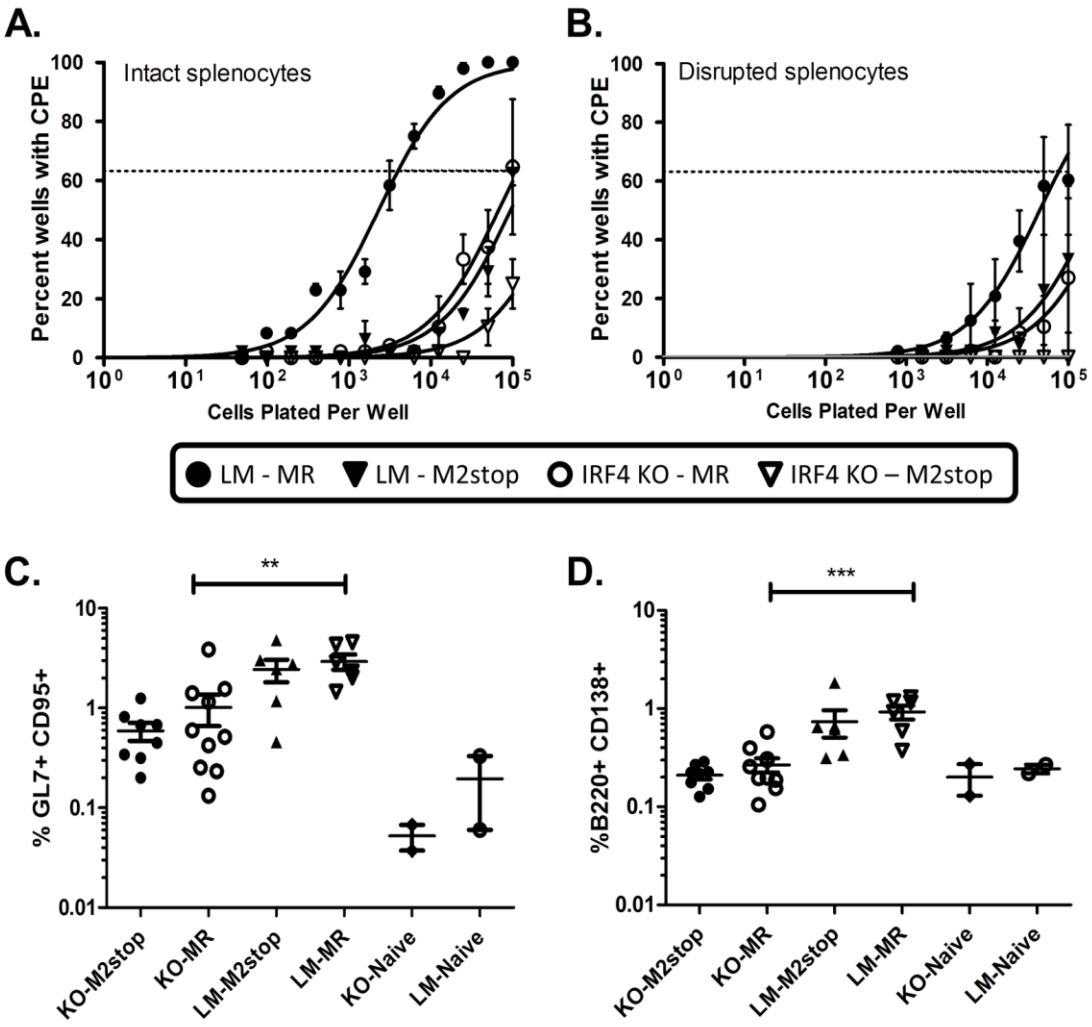
A.
Intact splenocytes
Percent wells with CPE
Cells Plated Per Well
B.
Disrupted splenocytes
Percent wells with CPE
Cells Plated Per Well
LM - MR
LM - M2stop
IRF4 KO - MR
IRF4 KO – M2stop
C.
**
% GL7+ CD95+
KO-M2stop
KO-MR
LM-M2stop
LM-MR
KO-Naive
LM-Naive
D.
***
%B220+ CD138+
KO-M2stop
KO-MR
LM-M2stop
LM-MR
KO-Naive
LM-Naive

FIGURE LEGENDS

Figure 1. XBP-1s can transactivate the MHV68 gene 50 proximal promoter in M12 cells. The murine B cell lymphoma M12 cell line was transfected with an XBP-1s expression vector (pFlag.XBP1p.CMV2) and a luciferase reporter driven by the proximal gene 50 promoter (PpORF50). Where indicated cells were treated at 12 hours post-transfection with TPA and/or ionomycin and allowed to incubate for another 12 hours. Values are reported as fold increase in luciferase activity over basal promoter activity.

Figure 2. MHV68 infection in XBP-1$^{flox/flox}$ CD19$^{CRE/+}$ mice induces normal germinal center and plasma cell responses. Animals were infected with 1000 PFU of the recombinant H2B.YFP MHV68 virus and sacrificed at day 18 post-infection to analyze splenic lymphoid population. (A) Representative flow cytometry plots illustrating the gating strategy employed to identify the splenic germinal center and plasma cell populations. Cells were gated on CD19+ GL7+ CD95+ to identify germinal center B cells, while cells were gated on CD3- B220int CD138+ to identify plasma cells. (B) Frequencies of germinal center populations in XBP-1 knockout (KO) and littermate controls (LM). (C) Frequencies of plasma cell subsets in XBP-1 knockout (KO) and littermate group controls (LM).

Figure 3. XBP-1$^{flox/flox}$ CD19$^{CRE/+}$ mice fail to mount an immunoglobulin response following MHV68 infection. Animals were infected with 1000 PFU of the recombinant H2B.YFP MHV68 virus and terminally bled at day 18 post-infection to collect serum. Total IgM (A) and IgG (B) levels in naïve and MHV68 infected XBP-1$^{flox/flox}$ CD19$^{CRE/+}$ and littermate control animals.

Figure 4. Frequency of virus infected B cells (YFP+) in XBP-1$^{flox/flox}$ CD19$^{CRE/+}$ mice are comparable to littermate controls. Animals were infected with 1000 PFU of the recombinant H2B.YFP MHV68 virus and sacrificed at day 18 post-infection. Splenocytes were collected to evaluate the frequency of virally infected cells. (A) Representative flow cytometry plots are

shown illustrating the gating strategy employed. Cells were gated on the CD3- YFP+ population. (B) Compiled frequencies of virus infected splenocytes (CD3- YFP+) in littermate controls (LM) and XBP-1 knockout (KO) mice. p-value= 0.3.

Figure 5. The frequency of virus infected cells with a plasma cell phenotype is reduced in MHV68 infected XBP-$1^{flox/flox}$ $CD19^{CRE/+}$ animals. Mice were infected with 1000 PFU of the recombinant H2B.YFP MHV68 virus and sacrificed at day 18 post-infection. (A) Representative flow cytometry plots illustrating the gating strategy employed to identify the splenic germinal center and plasma cell populations. Cells were first gated on the CD3- YFP+ population, then gated on either the CD19+ GL7+ CD95+ population to identify germinal center B cells, or on the B220int CD138+ to identify plasma cells. (B) Compiled frequencies of infected cells with a germinal center phenotype in littermate controls (LM) or XBP-1 knockout (KO) mice. NS, p-value= 0.4. (C) Frequencies of the virus infected cells with a plasma cell phenotype in littermate controls (LM) or XBP-1 knockout (KO) mice. *** p-value= 0.003.

Figure 6: Frequency of infected germinal center B cells and plasma cells is similar in XBP-$1^{flox/flox}$ $CD19^{CRE/+}$ and littermate control animals. Mice were infected with 1000 PFU H2B.YFP MHV68 virus and sacrificed at day 18 post infection. (A) Cells were gated on either the CD19+ GL7+ CD95+ germinal center population and subsequent gating on YFP+ cells, or they were gated on the CD3- B330int CD138+ plasma cell population with subsequent gating on YFP+ cells. YFP expression from that subset was then determined. Germinal center cells were then evaluated for YFP expression (B) Frequency of infected germinal center B cells. p-value= 0.9. (C) Frequency of infected plasma cells. p-value= 0.3.

Figure 7: XBP-1 expression is not required for MHV68 reactivation or establishment of latency. (A) Limiting dilution analysis of MHV68 reactivation from total splenocytes isolated at day 18

post-infection. (B) Limiting dilution PCR analyses of MHV68 genome positive splenocytes isolated at day 18 post-infection.

Figure 8: Loss of IRF4 expression in B cells impairs MHV68 reactivation from latency to a similar extent as disruption of the MHV68 M2 gene. $IRF4^{flox/flox}$ $CD19^{CRE/+}$ (IRF4 KO) or littermate controls (LM) mice were infected with 1000 PFU IN of either MHV68/M2MR.HY or MHV68/M2stop.HY viruses. (A & B) Limiting dilution reactivation analyses plating either intact splenocytes (A) and mechanically disrupted splenocytes (B) recovered at day 16 post-infection. (C) Frequencies of germinal center B cells (B220+ GL7+ CD95+) in the spleens of naïve and MHV68 infected animals, as indicated. ** p-value= 0.007.(D) Frequencies of plasma cells (CD3- B220int CD138+) in the spleens of naïve and MHV68 infected animals, as indicated. KO, $IRF4^{flox/flox}$ $CD19^{CRE/+}$; LM, littermate control ($IRF4^{flox/flox}$ $CD19^{+/+}$); M2stop, MHV68/M2stop.HY; MR, MHV68/M2MR.HY. ***p-value= 0.0001.

CHAPTER IV

SUMMARY, FUTURE DIRECTIONS AND CONCLUSIONS

Summary

Plasmodium infections remain a public health burden impacting nearly 200 million individuals and causing nearly 0.5 million deaths annually [1]. Mathematical modeling of disease progression estimated that 1-2 infections with *Plasmodium falciparum* are sufficient to protect children living in areas of high endemicity in Sub-Saharan African from symptoms of non-cerebral severe disease [143]. However, nearly 20% of infected children living in such regions progress onto severe and life threatening disease. Various factors are thought to contribute to the spectrum of disease severity associated with *Plasmodium* infections and include host, parasite and environmental factors [2,4]. One less studied factor that may contribute to disease severity is that of co-infections with other pathogens.

In 1958, Dennis Burkitt described a jaw and abdominal tumor that was prevalent and mostly restricted to areas in Sub-Saharan Africa that were endemic for malaria transmission (termed the "Malaria Belt") [12]. This tumor was later named endemic Burkitt's lymphoma (eBL). eBL is similar to other BL tumors in having a c-myc translocation to the IgH or IgL locus. However, unlike the other forms, eBL shows geographical restriction to Sub-Saharan Africa, Papua New Guinea and some regions of Brazil and has a greater than 90% association with Epstein Barr Virus (EBV) co-infection (reviewed in [123]). EBV is a gammaherpesvirus that infects and maintains latency within the memory B cell compartment of the host [238,239]. Later studies indicated that children latently infected with EBV, and living in endemic regions of malaria transmission, had an increased risk of developing eBL. Studies postulate that repeated *P.*

falciparum infection weakens the EBV specific CD8+ T cell response and results in the outgrowth of EBV transformed B cells [10,13-15,170,171,195].

EBV infection is ubiquitous in the population. Specifically, children living in Sub-Saharan Africa were shown to become seropositive to EBV by 6 months of age [6,7,9]. Thus, primary acquisition of both EBV and *Plasmodium* in a naïve host shows a large temporal overlap. Primary EBV infection in a naïve host, although asymptomatic, has the capacity to modulate host immune responses. One study elegantly demonstrated that a 2.5 year old child asymptomatically infected with EBV showed a transient humoral immune suppression and could not properly respond to secondary antigenic challenge for a duration of 4 weeks post infection [78]. Similar studies in adults experiencing infectious mononucleosis (IM) demonstrated the same transient humoral immune suppression [79]. Another interesting study also demonstrated that EBV, but not cytomegalovirus, could reduce the efficiency of vaccine induced antibody responses [173]. Similarly, mice infected with the murine gammaherpesvirus 68 (MHV68), a murine model of gammaherpesvirus infection, show a marked suppression in the development of the antibody response during secondary antigenic challenge when acutely infected [77].

Humoral responses are protective during *Plasmodium* infection. This has been extensively demonstrated in studies where patients infected with *P. falciparum* could be protected from disease when passively immunized using sera from previously exposed patients [21,151]. Additionally, protection from severe malarial anemia was associated with increased *Plasmodium* specific IgG responses [175]. Since primary asymptomatic EBV infection is capable of suppressing the host's capacity to generate a proper humoral response, we were interested in evaluating the impact of such an infection on disease progression and control of a *Plasmodium* infection. We used the murine model of gammaherpesvirus infection, MHV68, and the two non-lethal murine species; *P. yoelii* XNL and *P. chabaudi* AS.

In Chapter II we show that acute MHV68 co-infection with *P. yoelii* XNL resulted in hyper-parasitemia and severe malarial anemia and ultimately death by day 30 post co-infection. Interestingly, co-infection with *P. chabaudi* AS did not alter the kinetics of parasite infection. MHV68 acute co-infection suppressed total, malaria specific and virus specific IgG responses. Infection of μMT mice (mature B cell deficient mice – lack a humoral response) with either *P. yoelii* XNL or *P. chabaudi* AS indicated that only *P. yoelii* XNL required a humoral response to control the primary peak of parasitemia. This was evident from the observation that all μMT mice infected with *P. yoelii* XNL, but not *P. chabaudi* AS, succumbed to the infection. As such, although parasite specific IgG responses were suppressed during *P. chabaudi* co-infection, there was no impact on control of primary peak of parasitemia.

Evaluation of splenic immune responses revealed that initial germinal center B cell (GC) and plasma cell (PC) responses were comparable in single and co-infected mice. However, by day 12 post co-infection, co-infected animals showed a severe defect in the ability to maintain GC and PC levels as compared to single parasitic infections. Th1 cellular subsets that produce protective IL-10 and IFN-γ responses during *Plasmodium* infection [46,163] and FoxP3+ T regulatory cell frequencies were not altered during co-infection. However, we did notice a significant defect in the maintenance of the T follicular helper (CD4+ CXCR5hi PD1hi) subset in the spleen during co-infection. Previous work demonstrated that chronically infected HIV patients showed defects in the functionality of the Tfh response due to higher levels of PD-L1 expression on GC B cells as a result of chronic antigenic stimulation [182]. PD-L1 ligates the PD-1 receptor which is highly expressed on the Tfh subset and can negatively regulate their activity [240]. Similarly, we observed that PD-L1 expression on GC B cells of animals co-infected with MHV68 and *P. yoelii* XNL was greater as compared to a single parasite infection. More importantly, the immune suppressive effects were dependent on acute but not latent MHV68 infection. Latent infection did

not result in lethality, and showed higher levels of all splenic subsets as well as a reduction in levels of PD-L1 expression on GC B cells.

We were next interested in understanding how the virus was mediating this transient immune suppression of the antibody response in the host. In order to evaluate this, we decided to study the role of the viral gene product M2. M2 is a unique viral gene product that induces IL10 production from B cells [111,112], induces B cell differentiation into a plasma blast phenotype [166] and signals downstream of the BCR [185,186]. It shows functional homology to the EBV LMP-1/2 genes which mimic BCR and CD40 signaling as well as EBV's virally encoded IL-10 [183,184]. IL-10 is a pleiotropic cytokine that has the capacity to induce B cell proliferation as well as suppress pro-inflammatory T cell and APC responses [167]. As such, we wanted to test whether M2 might be mediating this effect. For this purpose, we infected C57BL/6 mice with either the M2.Stop virus or the genetically rescued Marker Rescue (MR) virus. We noted that loss of M2 expression enhanced the anti-viral IgG response by 2 fold at 21 days post infection. When we co-infected mice with M2.Stop and *P. yoelii* XNL, we found a 94% survival rate, 28 fold increase in the parasite specific IgG and complete clearance of peripheral parasitemia. These observations certainly support a role for M2 in contributing to the virally induced immune suppression. However, further work will be required to evaluate whether these observations are IL-10 dependent.

Collectively our work provides the first fully characterized MHV68 and *Plasmodium* co-infection model which implicates acute gammaherpesvirus infection in the development of severe malaria. This certainly provides a strong impetus for carrying out further studies in humans to establish if this correlation is relevant in human co-infections. Additionally, this provides the first evidence that EBV vaccination and anti-virals may be required for treatments when considering specific cases of severe malaria. Thus, efforts towards the development of these drugs should be given precedence in the future.

Future Directions

Our current work prompts many questions regarding the mechanism by which M2 is suppressing the humoral immune response. We are initially interested in evaluating whether M2 is mediating its effect in an IL-10 dependent fashion. In order to address this question, we are breeding mice that are IL-$10^{flox/flox}$ CD19-$Cre^{+/-}$ which we plan to co-infect with MHV68 and *P. yoelii* XNL. If we can recapitulate the rescued phenotype observed during an M2.Stop co-infection, we will have reason to believe that M2 is mediating this effect via IL-10 specifically from the B cell. A more direct manner to evaluate whether IL-10 is playing a role at all would be to either neutralize the cytokine or block the IL-10R *in vivo.* Another important question we are interested in answering is where (what specific B cell subset) M2 is being expressed and whether M2 expression is changing the inherent nature of the B cell it infects. We previously noted that GC B cells in co-infected mice expressed higher levels of PD-L1 expression. Preliminary studies using the inducible M12 cell line that is stably transfected with an M2 expression vector [111] did not show a correlation between M2 expression and PD-L1 expression. It did, however, show an increase in PD-1 surface expression. The same observation was noted in the retrovirally transduced splenic B cell assay that was previously described [212]. Regardless, other surface marker or phenotypic changes induced by M2 expression may explain the observed phenotype. Independent studies are currently underway to identify regulatory B cell subsets that may be induced by M2 expression, in addition to detailed GC tissue sections that evaluate the efficiency of the B-T cell interaction within a follicle in the presence or absence of M2 expression.

Second, we plan on using the non-lethal MHV68 and *P. chabaudi* AS co-infection model to study the long lasting effects of the virus induced humoral suppression. A hallmark of immunity to *Plasmodium* infection is the requirement for constant and long lasting exposure to the parasite in order to develop protective B cell memory responses [3,241,242]. Repeated exposure to the parasite ensures protection from disease associated with infection and attenuates

severity with time of exposure; however, it has never been documented to provide sterile immunity. Various parasitic factors are thought to modulate the host B cell response (reviewed in [243]). The Cysteine-rich Inter-domain region 1 α (CIDR1α) of the *P. falciparum* erythrocyte membrane protein 1 (Pfemp1) was shown to induce polyclonal B cell activation (PBA) of memory B cells. It was shown to induce both MAPK and NF-κB activation as a result and was postulated to cause B cell "exhaustion" with repeated exposure [136,244]. *P. falciparum* was also documented to increase B cell activating factor (BAFF), as evidenced by high levels in the serum of acutely infected children [245]. Persistently high levels of BAFF are thought to reduce BAFF-R levels on B cells which will in turn impact their functionality. Lower levels of BAFF-R on B cells are a hallmark of children acutely infected with *Plasmodium*, where higher BAFF-R correlated with greater IgG and reduced disease severity [245]. The combination of PBA and increased BAFF is thought to collectively impact the B cell repertoire which is marked by increases in transitional B cells in the periphery as well as reductions in the marginal zone B cell populations [246-249]. Another interesting observation is the expansion of a group of B cells called atypical memory B cells that express CD19+ CD21- CD27- CD10- FcRL4+ [250,251]. The role of FcRL4+ B cells in dysfunctional memory B cell responses was first described in chronically infected HIV patients [252]. This subset was refractory to BCR stimulation and activation. It was later observed that this particular subset is largely expanded in *P. falciparum* infected populations, and greater length and rate of exposure positively correlates with higher levels of these cells [253-256]. BCR sequencing analysis has revealed that many of these atypical B cells have indeed class switched and are specific to a large range of *P. falciparum* antigens [257]. Recent work also demonstrated that the BCR V gene composition was identical in both classical and atypical memory B cell subsets, suggesting that they probably arose from the same progenitor cells [258]. However, the correlation and role for atypical memory B cells in accounting for the weak development of the *Plasmodium* specific memory B cell response is not completely clear. Murine models of *Plasmodium* infection have demonstrated efficient induction

of memory B cell responses. *P. chabaudi* AS infection can induce a long lasting and protective memory B cell response within one infection [259,260]. Other infections, such as *P. yoelii* XNL, have been shown to delete memory B cell responses after vaccination [261].

Collectively, it appears that various factors contribute to the defective development of protective *Plasmodium* specific memory B cell responses. However, no work has been done to understand how other infections may impact this generation. EBV infection is ubiquitous in the Sub-Saharan community and circulating loads of EBV are high in patients that are acutely infected with *Plasmodium* [10]. Additionally, our findings show that acute MHV68 infection can alter responses to a secondary *Plasmodium* infection. Using our non-lethal model of co-infection with *P. chabaudi* AS, we are interested in understanding whether the observed decreases in early parasite specific B cell responses will also translate into deficient memory B cell recall responses. For this purpose, we have infected mice with either the MR or M2.Stop virus at day 0 and challenged with *P. chabaudi* AS at day 7. We sacrificed animals at day 30 or 60 post co-infection and collected bone marrow and spleen for analysis. In a separate group, we re-challenged the animals at day 60 post co-infection with *P. chabaudi* and waited 20 days after re-challenge to assess re-call memory responses in the bone marrow and spleen. Infections are being monitored through smears and blood collections that will be used to detect sub-patent parasitemia using qPCR techniques. We will extract RNA from the BM and spleen to generate cDNA. This will then be used to amplify the IgH of the BCR. All IgG isotypes and IgM will be evaluated. We are using MiSeq next generation sequencing to look at the clonal diversity generated after co-infection with MHV68, M2.Stop or a single *Plasmodium* infection. This will help us determine whether acute EBV infection could alter the capacity of the B cell to class switch and hypermutate when responding to *Plasmodium* infection. These studies will be instrumental in identifying a role for EBV co-infection on long term immune modulation of the host that may subsequently impact *Plasmodium* memory B cell responses. If there is a correlation between the

two, this once more implicates EBV in malarial disease and once more supports our hypothesis that control of the EBV infection will be required for proper clearance of the parasitic infection.

CONCLUDING REMARKS

In conclusion, our work has provided compelling evidence to implicate acute gammaherpesvirus infection in modulation of the host immune response and altering control of secondary infections. This data provides premise for evaluating other mechanisms of controlling and treating malarial disease. More importantly, these studies highlight the importance of acknowledging the impact of various secondary infections in disease prognosis and treatment of any infectious disease. Properly designed and comprehensive human studies will be required to fully appreciate the importance and complexity of multiple infections in significantly altering disease outcome.

REFERENCES

1. World Health Organization. Global Malaria Programme. (2012) World malaria report 2012. Geneva: World Health Organization. xxxiv, 249 p. p.
2. Miller LH, Baruch DI, Marsh K, Doumbo OK (2002) The pathogenic basis of malaria. Nature 415: 673-679.
3. Langhorne J, Ndungu FM, Sponaas AM, Marsh K (2008) Immunity to malaria: more questions than answers. Nat Immunol 9: 725-732.
4. Greenwood B, Marsh K, Snow R (1991) Why do some African children develop severe malaria? Parasitol Today 7: 277-281.
5. Riley EM, Stewart VA (2013) Immune mechanisms in malaria: new insights in vaccine development. Nature medicine 19: 168-178.
6. Biggar RJ, Henle W, Fleisher G, Bocker J, Lennette ET, et al. (1978) Primary Epstein-Barr virus infections in African infants. I. Decline of maternal antibodies and time of infection. Int J Cancer 22: 239-243.
7. Biggar RJ, Henle G, Bocker J, Lennette ET, Fleisher G, et al. (1978) Primary Epstein-Barr virus infections in African infants. II. Clinical and serological observations during seroconversion. Int J Cancer 22: 244-250.
8. Henle G, Henle W (1970) Observations on childhood infections with the Epstein-Barr virus. J Infect Dis 121: 303-310.
9. Piriou E, Asito AS, Sumba PO, Fiore N, Middeldorp JM, et al. (2012) Early age at time of primary Epstein-Barr virus infection results in poorly controlled viral infection in infants from Western Kenya: clues to the etiology of endemic Burkitt lymphoma. J Infect Dis 205: 906-913.
10. Moormann AM, Chelimo K, Sumba OP, Lutzke ML, Ploutz-Snyder R, et al. (2005) Exposure to holoendemic malaria results in elevated Epstein-Barr virus loads in children. J Infect Dis 191: 1233-1238.
11. Moormann AM, Snider CJ, Chelimo K (2011) The company malaria keeps: how co-infection with Epstein-Barr virus leads to endemic Burkitt lymphoma. Curr Opin Infect Dis 24: 435-441.
12. Burkitt D (1958) A sarcoma involving the jaws in African children. Br J Surg 46: 218-223.
13. Moormann AM, Chelimo K, Sumba PO, Tisch DJ, Rochford R, et al. (2007) Exposure to holoendemic malaria results in suppression of Epstein-Barr virus-specific T cell immunosurveillance in Kenyan children. J Infect Dis 195: 799-808.
14. Moormann AM, Heller KN, Chelimo K, Embury P, Ploutz-Snyder R, et al. (2009) Children with endemic Burkitt lymphoma are deficient in EBNA1-specific IFN-gamma T cell responses. Int J Cancer 124: 1721-1726.
15. Whittle HC, Brown J, Marsh K, Greenwood BM, Seidelin P, et al. (1984) T-cell control of Epstein-Barr virus-infected B cells is lost during P. falciparum malaria. Nature 312: 449-450.
16. Njie R, Bell AI, Jia H, Croom-Carter D, Chaganti S, et al. (2009) The effects of acute malaria on Epstein-Barr virus (EBV) load and EBV-specific T cell immunity in Gambian children. J Infect Dis 199: 31-38.
17. Snider CJ, Cole SR, Chelimo K, Sumba PO, Macdonald PD, et al. (2012) Recurrent Plasmodium falciparum malaria infections in Kenyan children diminish T-cell immunity to Epstein Barr virus lytic but not latent antigens. PLoS One 7: e31753.
18. Chene A, Donati D, Guerreiro-Cacais AO, Levitsky V, Chen Q, et al. (2007) A molecular link between malaria and Epstein-Barr virus reactivation. PLoS Pathog 3: e80.

19. Chene A, Nylen S, Donati D, Bejarano MT, Kironde F, et al. (2011) Effect of acute Plasmodium falciparum malaria on reactivation and shedding of the eight human herpes viruses. PLoS One 6: e26266.
20. Piriou E, Kimmel R, Chelimo K, Middeldorp JM, Odada PS, et al. (2009) Serological evidence for long-term Epstein-Barr virus reactivation in children living in a holoendemic malaria region of Kenya. J Med Virol 81: 1088-1093.
21. Cohen S, Mc GI, Carrington S (1961) Gamma-globulin and acquired immunity to human malaria. Nature 192: 733-737.
22. Sabchareon A, Burnouf T, Ouattara D, Attanath P, Bouharoun-Tayoun H, et al. (1991) Parasitologic and clinical human response to immunoglobulin administration in falciparum malaria. The American journal of tropical medicine and hygiene 45: 297-308.
23. Yone CL, Kube D, Kremsner PG, Luty AJ (2006) Persistent Epstein-Barr viral reactivation in young African children with a history of severe Plasmodium falciparum malaria. Trans R Soc Trop Med Hyg 100: 669-676.
24. Mueller I, Schoepflin S, Smith TA, Benton KL, Bretscher MT, et al. (2012) Force of infection is key to understanding the epidemiology of Plasmodium falciparum malaria in Papua New Guinean children. Proceedings of the National Academy of Sciences of the United States of America 109: 10030-10035.
25. Smith T, Beck HP, Kitua A, Mwankusye S, Felger I, et al. (1999) Age dependence of the multiplicity of Plasmodium falciparum infections and of other malariological indices in an area of high endemicity. Transactions of the Royal Society of Tropical Medicine and Hygiene 93 Suppl 1: 15-20.
26. Piriou E, Asito AS, Sumba PO, Fiore N, Middeldorp JM, et al. (2012) Early age at time of primary Epstein-Barr virus infection results in poorly controlled viral infection in infants from Western Kenya: clues to the etiology of endemic Burkitt lymphoma. The Journal of infectious diseases 205: 906-913.
27. Chene A, Donati D, Guerreiro-Cacais AO, Levitsky V, Chen Q, et al. (2007) A molecular link between malaria and Epstein-Barr virus reactivation. PLoS pathogens 3: e80.
28. Rochford R, Cannon MJ, Moormann AM (2005) Endemic Burkitt's lymphoma: a polymicrobial disease? Nature reviews Microbiology 3: 182-187.
29. Killick-Kendrick R, Peters W (1978) Rodent malaria. London: Academic Press.
30. Perkins DJ, Weinberg JB, Kremsner PG (2000) Reduced interleukin-12 and transforming growth factor-beta1 in severe childhood malaria: relationship of cytokine balance with disease severity. J Infect Dis 182: 988-992.
31. Malaguarnera L, Imbesi RM, Pignatelli S, Simpore J, Malaguarnera M, et al. (2002) Increased levels of interleukin-12 in Plasmodium falciparum malaria: correlation with the severity of disease. Parasite Immunol 24: 387-389.
32. Luty AJ, Perkins DJ, Lell B, Schmidt-Ott R, Lehman LG, et al. (2000) Low interleukin-12 activity in severe Plasmodium falciparum malaria. Infect Immun 68: 3909-3915.
33. Stevenson MM, Tam MF, Wolf SF, Sher A (1995) IL-12-induced protection against blood-stage Plasmodium chabaudi AS requires IFN-gamma and TNF-alpha and occurs via a nitric oxide-dependent mechanism. J Immunol 155: 2545-2556.
34. Urban BC, Ferguson DJ, Pain A, Willcox N, Plebanski M, et al. (1999) Plasmodium falciparum-infected erythrocytes modulate the maturation of dendritic cells. Nature 400: 73-77.
35. Giusti P, Urban BC, Frascaroli G, Albrecht L, Tinti A, et al. (2011) Plasmodium falciparum-infected erythrocytes and beta-hematin induce partial maturation of human dendritic cells and increase their migratory ability in response to lymphoid chemokines. Infection and immunity 79: 2727-2736.

36. Mukherjee P, Chauhan VS (2008) Plasmodium falciparum-free merozoites and infected RBCs distinctly affect soluble CD40 ligand-mediated maturation of immature monocyte-derived dendritic cells. Journal of leukocyte biology 84: 244-254.
37. Elliott SR, Spurck TP, Dodin JM, Maier AG, Voss TS, et al. (2007) Inhibition of dendritic cell maturation by malaria is dose dependent and does not require Plasmodium falciparum erythrocyte membrane protein 1. Infection and immunity 75: 3621-3632.
38. Ing R, Segura M, Thawani N, Tam M, Stevenson MM (2006) Interaction of mouse dendritic cells and malaria-infected erythrocytes: uptake, maturation, and antigen presentation. Journal of immunology 176: 441-450.
39. Ruedl C, Rieser C, Bock G, Wick G, Wolf H (1996) Phenotypic and functional characterization of CD11c+ dendritic cell population in mouse Peyer's patches. Eur J Immunol 26: 1801-1806.
40. deWalick S, Amante FH, McSweeney KA, Randall LM, Stanley AC, et al. (2007) Cutting edge: conventional dendritic cells are the critical APC required for the induction of experimental cerebral malaria. Journal of immunology 178: 6033-6037.
41. Perry JA, Rush A, Wilson RJ, Olver CS, Avery AC (2004) Dendritic cells from malaria-infected mice are fully functional APC. J Immunol 172: 475-482.
42. Artavanis-Tsakonas K, Eleme K, McQueen KL, Cheng NW, Parham P, et al. (2003) Activation of a subset of human NK cells upon contact with Plasmodium falciparum-infected erythrocytes. J Immunol 171: 5396-5405.
43. Chelimo K, Embury PB, Sumba PO, Vulule J, Ofulla AV, et al. (2011) Age-related differences in naturally acquired T cell memory to Plasmodium falciparum merozoite surface protein 1. PLoS One 6: e24852.
44. D'Ombrain MC, Hansen DS, Simpson KM, Schofield L (2007) gammadelta-T cells expressing NK receptors predominate over NK cells and conventional T cells in the innate IFN-gamma response to Plasmodium falciparum malaria. Eur J Immunol 37: 1864-1873.
45. Troye-Blomberg M, Worku S, Tangteerawatana P, Jamshaid R, Soderstrom K, et al. (1999) Human gamma delta T cells that inhibit the in vitro growth of the asexual blood stages of the Plasmodium falciparum parasite express cytolytic and proinflammatory molecules. Scand J Immunol 50: 642-650.
46. Freitas do Rosario AP, Lamb T, Spence P, Stephens R, Lang A, et al. (2012) IL-27 promotes IL-10 production by effector Th1 CD4+ T cells: a critical mechanism for protection from severe immunopathology during malaria infection. J Immunol 188: 1178-1190.
47. D'Ombrain MC, Robinson LJ, Stanisic DI, Taraika J, Bernard N, et al. (2008) Association of early interferon-gamma production with immunity to clinical malaria: a longitudinal study among Papua New Guinean children. Clin Infect Dis 47: 1380-1387.
48. Imai T, Shen J, Chou B, Duan X, Tu L, et al. (2010) Involvement of CD8+ T cells in protective immunity against murine blood-stage infection with Plasmodium yoelii 17XL strain. Eur J Immunol 40: 1053-1061.
49. Ing R, Stevenson MM (2009) Dendritic cell and NK cell reciprocal cross talk promotes gamma interferon-dependent immunity to blood-stage Plasmodium chabaudi AS infection in mice. Infect Immun 77: 770-782.
50. Lundie RJ, de Koning-Ward TF, Davey GM, Nie CQ, Hansen DS, et al. (2008) Blood-stage Plasmodium infection induces CD8+ T lymphocytes to parasite-expressed antigens, largely regulated by CD8alpha+ dendritic cells. Proc Natl Acad Sci U S A 105: 14509-14514.
51. Seixas EM, Langhorne J (1999) gammadelta T cells contribute to control of chronic parasitemia in Plasmodium chabaudi infections in mice. J Immunol 162: 2837-2841.

52. Osier FH, Fegan G, Polley SD, Murungi L, Verra F, et al. (2008) Breadth and magnitude of antibody responses to multiple Plasmodium falciparum merozoite antigens are associated with protection from clinical malaria. Infect Immun 76: 2240-2248.
53. van der Heyde HC, Huszar D, Woodhouse C, Manning DD, Weidanz WP (1994) The resolution of acute malaria in a definitive model of B cell deficiency, the JHD mouse. J Immunol 152: 4557-4562.
54. Ndungu FM, Cadman ET, Coulcher J, Nduati E, Couper E, et al. (2009) Functional memory B cells and long-lived plasma cells are generated after a single Plasmodium chabaudi infection in mice. PLoS pathogens 5: e1000690.
55. Stephens R, Ndungu FM, Langhorne J (2009) Germinal centre and marginal zone B cells expand quickly in a second Plasmodium chabaudi malaria infection producing mature plasma cells. Parasite Immunol 31: 20-31.
56. von der Weid T, Honarvar N, Langhorne J (1996) Gene-targeted mice lacking B cells are unable to eliminate a blood stage malaria infection. J Immunol 156: 2510-2516.
57. Francois S, Vidick S, Sarlet M, Desmecht D, Drion P, et al. (2013) Illumination of murine gammaherpesvirus-68 cycle reveals a sexual transmission route from females to males in laboratory mice. PLoS Pathog 9: e1003292.
58. Isaacs R (1948) Chronic infectious mononucleosis. Blood 3: 858-861.
59. Sixbey JW, Lemon SM, Pagano JS (1986) A second site for Epstein-Barr virus shedding: the uterine cervix. Lancet 2: 1122-1124.
60. Israele V, Shirley P, Sixbey JW (1991) Excretion of the Epstein-Barr virus from the genital tract of men. J Infect Dis 163: 1341-1343.
61. Damania B, Pipas JM (2009) DNA tumor viruses. New York: Springer Science + Business Media. xxvi, 794 p., 794 p. of plates p.
62. Collins CM, Speck SH (2012) Tracking murine gammaherpesvirus 68 infection of germinal center B cells in vivo. PLoS One 7: e33230.
63. Willer DO, Speck SH (2003) Long-term latent murine Gammaherpesvirus 68 infection is preferentially found within the surface immunoglobulin D-negative subset of splenic B cells in vivo. J Virol 77: 8310-8321.
64. Weck KE, Kim SS, Virgin HI, Speck SH (1999) B cells regulate murine gammaherpesvirus 68 latency. J Virol 73: 4651-4661.
65. Schaade L, Kleines M, Hausler M (2001) Application of virus-specific immunoglobulin M (IgM), IgG, and IgA antibody detection with a polyantigenic enzyme-linked immunosorbent assay for diagnosis of Epstein-Barr virus infections in childhood. J Clin Microbiol 39: 3902-3905.
66. Cardin RD, Brooks JW, Sarawar SR, Doherty PC (1996) Progressive loss of CD8+ T cell-mediated control of a gamma-herpesvirus in the absence of CD4+ T cells. J Exp Med 184: 863-871.
67. Christensen JP, Cardin RD, Branum KC, Doherty PC (1999) CD4(+) T cell-mediated control of a gamma-herpesvirus in B cell-deficient mice is mediated by IFN-gamma. Proc Natl Acad Sci U S A 96: 5135-5140.
68. Dias P, Giannoni F, Lee LN, Han D, Yoon S, et al. (2010) CD4 T-cell help programs a change in CD8 T-cell function enabling effective long-term control of murine gammaherpesvirus 68: role of PD-1-PD-L1 interactions. J Virol 84: 8241-8249.
69. McClellan KB, Gangappa S, Speck SH, Virgin HWt (2006) Antibody-independent control of gamma-herpesvirus latency via B cell induction of anti-viral T cell responses. PLoS Pathog 2: e58.
70. Sangster MY, Topham DJ, D'Costa S, Cardin RD, Marion TN, et al. (2000) Analysis of the virus-specific and nonspecific B cell response to a persistent B-lymphotropic gammaherpesvirus. J Immunol 164: 1820-1828.

71. Barton ES, Lutzke ML, Rochford R, Virgin HWt (2005) Alpha/beta interferons regulate murine gammaherpesvirus latent gene expression and reactivation from latency. J Virol 79: 14149-14160.
72. Guggemoos S, Hangel D, Hamm S, Heit A, Bauer S, et al. (2008) TLR9 contributes to antiviral immunity during gammaherpesvirus infection. J Immunol 180: 438-443.
73. Gargano LM, Moser JM, Speck SH (2008) Role for MyD88 signaling in murine gammaherpesvirus 68 latency. J Virol 82: 3853-3863.
74. Pasare C, Medzhitov R (2005) Control of B-cell responses by Toll-like receptors. Nature 438: 364-368.
75. Mandal P, Krueger BE, Oldenburg D, Andry KA, Beard RS, et al. (2011) A gammaherpesvirus cooperates with interferon-alpha/beta-induced IRF2 to halt viral replication, control reactivation, and minimize host lethality. PLoS Pathog 7: e1002371.
76. Haque A, Rachinel N, Quddus MR, Haque S, Kasper LH, et al. (2004) Co-infection of malaria and gamma-herpesvirus: exacerbated lung inflammation or cross-protection depends on the stage of viral infection. Clin Exp Immunol 138: 396-404.
77. Getahun A, Smith MJ, Kogut I, van Dyk LF, Cambier JC (2012) Retention of anergy and inhibition of antibody responses during acute gamma herpesvirus 68 infection. J Immunol 189: 2965-2974.
78. Bowen TJ, Wedgwood RJ, Ochs HD, Henle W (1983) Transient immunodeficiency during asymptomatic Epstein-Barr virus infection. Pediatrics 71: 964-967.
79. Junker AK, Ochs HD, Clark EA, Puterman ML, Wedgwood RJ (1986) Transient immune deficiency in patients with acute Epstein-Barr virus infection. Clin Immunol Immunopathol 40: 436-446.
80. Yager EJ, Szaba FM, Kummer LW, Lanzer KG, Burkum CE, et al. (2009) gamma-Herpesvirus-induced protection against bacterial infection is transient. Viral Immunol 22: 67-72.
81. Barton ES, White DW, Cathelyn JS, Brett-McClellan KA, Engle M, et al. (2007) Herpesvirus latency confers symbiotic protection from bacterial infection. Nature 447: 326-329.
82. Sponaas AM, Freitas do Rosario AP, Voisine C, Mastelic B, Thompson J, et al. (2009) Migrating monocytes recruited to the spleen play an important role in control of blood stage malaria. Blood 114: 5522-5531.
83. Stevenson MM, Huang DY, Podoba JE, Nowotarski ME (1992) Macrophage activation during Plasmodium chabaudi AS infection in resistant C57BL/6 and susceptible A/J mice. Infect Immun 60: 1193-1201.
84. Cunnington AJ, Riley EM, Walther M (2013) Stuck in a rut? Reconsidering the role of parasite sequestration in severe malaria syndromes. Trends Parasitol 29: 585-592.
85. Paludan SR, Bowie AG, Horan KA, Fitzgerald KA (2011) Recognition of herpesviruses by the innate immune system. Nat Rev Immunol 11: 143-154.
86. Hwang S, Kim KS, Flano E, Wu TT, Tong LM, et al. (2009) Conserved herpesviral kinase promotes viral persistence by inhibiting the IRF-3-mediated type I interferon response. Cell Host Microbe 5: 166-178.
87. Rodrigues L, Filipe J, Seldon MP, Fonseca L, Anrather J, et al. (2009) Termination of NF-kappaB activity through a gammaherpesvirus protein that assembles an EC5S ubiquitin-ligase. EMBO J 28: 1283-1295.
88. Dong X, Feng P (2011) Murine gamma herpesvirus 68 hijacks MAVS and IKKbeta to abrogate NFkappaB activation and antiviral cytokine production. PLoS Pathog 7: e1002336.
89. Zimring JC, Goodbourn S, Offermann MK (1998) Human herpesvirus 8 encodes an interferon regulatory factor (IRF) homolog that represses IRF-1-mediated transcription. J Virol 72: 701-707.

90. Gao SJ, Boshoff C, Jayachandra S, Weiss RA, Chang Y, et al. (1997) KSHV ORF K9 (vIRF) is an oncogene which inhibits the interferon signaling pathway. Oncogene 15: 1979-1985.
91. Chatterjee M, Osborne J, Bestetti G, Chang Y, Moore PS (2002) Viral IL-6-induced cell proliferation and immune evasion of interferon activity. Science 298: 1432-1435.
92. Wies E, Hahn AS, Schmidt K, Viebahn C, Rohland N, et al. (2009) The Kaposi's Sarcoma-associated Herpesvirus-encoded vIRF-3 Inhibits Cellular IRF-5. J Biol Chem 284: 8525-8538.
93. Hahn AM, Huye LE, Ning S, Webster-Cyriaque J, Pagano JS (2005) Interferon regulatory factor 7 is negatively regulated by the Epstein-Barr virus immediate-early gene, BZLF-1. J Virol 79: 10040-10052.
94. Wang JT, Doong SL, Teng SC, Lee CP, Tsai CH, et al. (2009) Epstein-Barr virus BGLF4 kinase suppresses the interferon regulatory factor 3 signaling pathway. J Virol 83: 1856-1869.
95. Barton E, Mandal P, Speck SH (2011) Pathogenesis and host control of gammaherpesviruses: lessons from the mouse. Annu Rev Immunol 29: 351-397.
96. Parroche P, Lauw FN, Goutagny N, Latz E, Monks BG, et al. (2007) Malaria hemozoin is immunologically inert but radically enhances innate responses by presenting malaria DNA to Toll-like receptor 9. Proc Natl Acad Sci U S A 104: 1919-1924.
97. Wu X, Gowda NM, Kumar S, Gowda DC (2010) Protein-DNA complex is the exclusive malaria parasite component that activates dendritic cells and triggers innate immune responses. J Immunol 184: 4338-4348.
98. Haque A, Best SE, Ammerdorffer A, Desbarrieres L, de Oca MM, et al. (2011) Type I interferons suppress CD4(+) T-cell-dependent parasite control during blood-stage Plasmodium infection. Eur J Immunol 41: 2688-2698.
99. Voisine C, Mastelic B, Sponaas AM, Langhorne J (2010) Classical CD11c+ dendritic cells, not plasmacytoid dendritic cells, induce T cell responses to Plasmodium chabaudi malaria. Int J Parasitol 40: 711-719.
100. Tarumi T, Sawada K, Sato N, Kobayashi S, Takano H, et al. (1995) Interferon-alpha-induced apoptosis in human erythroid progenitors. Exp Hematol 23: 1310-1318.
101. Vigario AM, Belnoue E, Gruner AC, Mauduit M, Kayibanda M, et al. (2007) Recombinant human IFN-alpha inhibits cerebral malaria and reduces parasite burden in mice. J Immunol 178: 6416-6425.
102. Essers MA, Offner S, Blanco-Bose WE, Waibler Z, Kalinke U, et al. (2009) IFNalpha activates dormant haematopoietic stem cells in vivo. Nature 458: 904-908.
103. Sato T, Onai N, Yoshihara H, Arai F, Suda T, et al. (2009) Interferon regulatory factor-2 protects quiescent hematopoietic stem cells from type I interferon-dependent exhaustion. Nat Med 15: 696-700.
104. Binder D, Fehr J, Hengartner H, Zinkernagel RM (1997) Virus-induced transient bone marrow aplasia: major role of interferon-alpha/beta during acute infection with the noncytopathic lymphocytic choriomeningitis virus. J Exp Med 185: 517-530.
105. Lamikanra AA, Brown D, Potocnik A, Casals-Pascual C, Langhorne J, et al. (2007) Malarial anemia: of mice and men. Blood 110: 18-28.
106. Hardy GA, Sieg S, Rodriguez B, Anthony D, Asaad R, et al. (2013) Interferon-alpha is the primary plasma type-I IFN in HIV-1 infection and correlates with immune activation and disease markers. PLoS One 8: e56527.
107. Davenport GC, Hittner JB, Were T, Ong'echa JM, Perkins DJ (2012) Relationship between inflammatory mediator patterns and anemia in HIV-1 positive and exposed children with Plasmodium falciparum malaria. Am J Hematol 87: 652-658.

108. Orish VN, Onyeabor OS, Boampong JN, Acquah S, Sanyaolu AO, et al. (2013) The effects of malaria and HIV co-infection on hemoglobin levels among pregnant women in Sekondi-Takoradi, Ghana. Int J Gynaecol Obstet 120: 236-239.
109. Smith CM, Gill MB, May JS, Stevenson PG (2007) Murine gammaherpesvirus-68 inhibits antigen presentation by dendritic cells. PLoS One 2: e1048.
110. Li L, Liu D, Hutt-Fletcher L, Morgan A, Masucci MG, et al. (2002) Epstein-Barr virus inhibits the development of dendritic cells by promoting apoptosis of their monocyte precursors in the presence of granulocyte macrophage-colony-stimulating factor and interleukin-4. Blood 99: 3725-3734.
111. Rangaswamy US, Speck SH (2014) Murine Gammaherpesvirus M2 Protein Induction of IRF4 via the NFAT Pathway Leads to IL-10 Expression in B Cells. PLoS Pathog 10: e1003858.
112. Siegel AM, Herskowitz JH, Speck SH (2008) The MHV68 M2 protein drives IL-10 dependent B cell proliferation and differentiation. PLoS Pathog 4: e1000039.
113. Miyazaki I, Cheung RK, Dosch HM (1993) Viral interleukin 10 is critical for the induction of B cell growth transformation by Epstein-Barr virus. J Exp Med 178: 439-447.
114. Saraiva M, O'Garra A (2010) The regulation of IL-10 production by immune cells. Nat Rev Immunol 10: 170-181.
115. Huang L, Li L, Klonowski KD, Tompkins SM, Tripp RA, et al. (2013) Induction and role of indoleamine 2,3 dioxygenase in mouse models of influenza a virus infection. PLoS One 8: e66546.
116. Munn DH, Mellor AL (2013) Indoleamine 2,3 dioxygenase and metabolic control of immune responses. Trends Immunol 34: 137-143.
117. Makala LH, Baban B, Lemos H, El-Awady AR, Chandler PR, et al. (2011) Leishmania major attenuates host immunity by stimulating local indoleamine 2,3-dioxygenase expression. J Infect Dis 203: 715-725.
118. Divanovic S, Sawtell NM, Trompette A, Warning JI, Dias A, et al. (2012) Opposing biological functions of tryptophan catabolizing enzymes during intracellular infection. J Infect Dis 205: 152-161.
119. van der Sluijs KF, Nijhuis M, Levels JH, Florquin S, Mellor AL, et al. (2006) Influenza-induced expression of indoleamine 2,3-dioxygenase enhances interleukin-10 production and bacterial outgrowth during secondary pneumococcal pneumonia. J Infect Dis 193: 214-222.
120. Tetsutani K, To H, Torii M, Hisaeda H, Himeno K (2007) Malaria parasite induces tryptophan-related immune suppression in mice. Parasitology 134: 923-930.
121. Kim SK, Welsh RM (2004) Comprehensive early and lasting loss of memory CD8 T cells and functional memory during acute and persistent viral infections. J Immunol 172: 3139-3150.
122. Liu H, Andreansky S, Diaz G, Turner SJ, Wodarz D, et al. (2003) Quantitative analysis of long-term virus-specific CD8+-T-cell memory in mice challenged with unrelated pathogens. J Virol 77: 7756-7763.
123. Rochford R, Cannon MJ, Moormann AM (2005) Endemic Burkitt's lymphoma: a polymicrobial disease? Nat Rev Microbiol 3: 182-187.
124. Guermonprez P, Helft J, Claser C, Deroubaix S, Karanje H, et al. (2013) Inflammatory Flt3l is essential to mobilize dendritic cells and for T cell responses during Plasmodium infection. Nat Med 19: 730-738.
125. Imai T, Shen J, Chou B, Duan X, Tu L, et al. (2010) Involvement of CD8+ T cells in protective immunity against murine blood-stage infection with Plasmodium yoelii 17XL strain. European journal of immunology 40: 1053-1061.

126. Belnoue E, Potter SM, Rosa DS, Mauduit M, Gruner AC, et al. (2008) Control of pathogenic CD8+ T cell migration to the brain by IFN-gamma during experimental cerebral malaria. Parasite Immunol 30: 544-553.
127. Villegas-Mendez A, Greig R, Shaw TN, de Souza JB, Gwyer Findlay E, et al. (2012) IFN-gamma-producing CD4+ T cells promote experimental cerebral malaria by modulating CD8+ T cell accumulation within the brain. J Immunol 189: 968-979.
128. Chang WL, Jones SP, Lefer DJ, Welbourne T, Sun G, et al. (2001) CD8(+)-T-cell depletion ameliorates circulatory shock in Plasmodium berghei-infected mice. Infection and immunity 69: 7341-7348.
129. Van den Steen PE, Geurts N, Deroost K, Van Aelst I, Verhenne S, et al. (2010) Immunopathology and dexamethasone therapy in a new model for malaria-associated acute respiratory distress syndrome. American journal of respiratory and critical care medicine 181: 957-968.
130. Haque A, Best SE, Amante FH, Ammerdorffer A, de Labastida F, et al. (2011) High parasite burdens cause liver damage in mice following Plasmodium berghei ANKA infection independently of CD8(+) T cell-mediated immune pathology. Infect Immun 79: 1882-1888.
131. Mayor A, Bir N, Sawhney R, Singh S, Pattnaik P, et al. (2005) Receptor-binding residues lie in central regions of Duffy-binding-like domains involved in red cell invasion and cytoadherence by malaria parasites. Blood 105: 2557-2563.
132. Mo M, Lee HC, Kotaka M, Niang M, Gao X, et al. (2008) The C-terminal segment of the cysteine-rich interdomain of Plasmodium falciparum erythrocyte membrane protein 1 determines CD36 binding and elicits antibodies that inhibit adhesion of parasite-infected erythrocytes. Infect Immun 76: 1837-1847.
133. Belnoue E, Kayibanda M, Vigario AM, Deschemin JC, van Rooijen N, et al. (2002) On the pathogenic role of brain-sequestered alphabeta CD8+ T cells in experimental cerebral malaria. J Immunol 169: 6369-6375.
134. deWalick S, Amante FH, McSweeney KA, Randall LM, Stanley AC, et al. (2007) Cutting edge: conventional dendritic cells are the critical APC required for the induction of experimental cerebral malaria. J Immunol 178: 6033-6037.
135. Piva L, Tetlak P, Claser C, Karjalainen K, Renia L, et al. (2012) Cutting edge: Clec9A+ dendritic cells mediate the development of experimental cerebral malaria. J Immunol 189: 1128-1132.
136. Donati D, Zhang LP, Chene A, Chen Q, Flick K, et al. (2004) Identification of a polyclonal B-cell activator in Plasmodium falciparum. Infect Immun 72: 5412-5418.
137. Pichyangkul S, Yongvanitchit K, Kum-arb U, Hemmi H, Akira S, et al. (2004) Malaria blood stage parasites activate human plasmacytoid dendritic cells and murine dendritic cells through a Toll-like receptor 9-dependent pathway. J Immunol 172: 4926-4933.
138. Gabrielsen AA, Jr., Jensen JB (1982) Mitogenic activity of extracts from continuous cultures of Plasmodium falciparum. Am J Trop Med Hyg 31: 441-448.
139. Greenwood BM (1974) Possible role of a B-cell mitogen in hypergammaglobulinaemia in malaria and trypanosomiasis. Lancet 1: 435-436.
140. Ishikawa H, Barber GN (2008) STING is an endoplasmic reticulum adaptor that facilitates innate immune signalling. Nature 455: 674-678.
141. Bouharoun-Tayoun H, Attanath P, Sabchareon A, Chongsuphajaisiddhi T, Druilhe P (1990) Antibodies that protect humans against Plasmodium falciparum blood stages do not on their own inhibit parasite growth and invasion in vitro, but act in cooperation with monocytes. J Exp Med 172: 1633-1641.
142. Rovira-Vallbona E, Moncunill G, Bassat Q, Aguilar R, Machevo S, et al. (2012) Low antibodies against Plasmodium falciparum and imbalanced pro-inflammatory cytokines

are associated with severe malaria in Mozambican children: a case-control study. Malar J 11: 181.
143. Gupta S, Snow RW, Donnelly CA, Marsh K, Newbold C (1999) Immunity to non-cerebral severe malaria is acquired after one or two infections. Nat Med 5: 340-343.
144. Vinetz JM, Kumar S, Good MF, Fowlkes BJ, Berzofsky JA, et al. (1990) Adoptive transfer of CD8+ T cells from immune animals does not transfer immunity to blood stage Plasmodium yoelii malaria. J Immunol 144: 1069-1074.
145. Langhorne J, Simon-Haarhaus B, Meding SJ (1990) The role of CD4+ T cells in the protective immune response to Plasmodium chabaudi in vivo. Immunol Lett 25: 101-107.
146. van der Heyde HC, Manning DD, Roopenian DC, Weidanz WP (1993) Resolution of blood-stage malarial infections in CD8+ cell-deficient beta 2-m0/0 mice. J Immunol 151: 3187-3191.
147. Yanez DM, Manning DD, Cooley AJ, Weidanz WP, van der Heyde HC (1996) Participation of lymphocyte subpopulations in the pathogenesis of experimental murine cerebral malaria. J Immunol 157: 1620-1624.
148. Claser C, Malleret B, Gun SY, Wong AY, Chang ZW, et al. (2011) CD8+ T cells and IFN-gamma mediate the time-dependent accumulation of infected red blood cells in deep organs during experimental cerebral malaria. PLoS One 6: e18720.
149. Nunes JK, Starnbach MN, Wirth DF (2009) Secreted antibody is required for immunity to Plasmodium berghei. Infect Immun 77: 414-418.
150. Speck SH, Ganem D (2010) Viral latency and its regulation: lessons from the gamma-herpesviruses. Cell Host Microbe 8: 100-115.
151. Sabchareon A, Burnouf T, Ouattara D, Attanath P, Bouharoun-Tayoun H, et al. (1991) Parasitologic and clinical human response to immunoglobulin administration in falciparum malaria. Am J Trop Med Hyg 45: 297-308.
152. Rono J, Osier FH, Olsson D, Montgomery S, Mhoja L, et al. (2013) Breadth of anti-merozoite antibody responses is associated with the genetic diversity of asymptomatic Plasmodium falciparum infections and protection against clinical malaria. Clin Infect Dis 57: 1409-1416.
153. Dobano C, Rogerson SJ, Mackinnon MJ, Cavanagh DR, Taylor TE, et al. (2008) Differential antibody responses to Plasmodium falciparum merozoite proteins in Malawian children with severe malaria. J Infect Dis 197: 766-774.
154. Provisor AJ, Iacuone JJ, Chilcote RR, Neiburger RG, Crussi FG, et al. (1975) Acquired agammaglobulinemia after a life threatening illness with clinical and laboratory features of Infectious Mononucleosis in three related male children. The New England Journal of Medicine 293: 62-65.
155. Spence PJ, Jarra W, Levy P, Reid AJ, Chappell L, et al. (2013) Vector transmission regulates immune control of Plasmodium virulence. Nature 498: 228-231.
156. Krug LT, Evans AG, Gargano LM, Paden CR, Speck SH (2013) The absence of M1 leads to increased establishment of murine gammaherpesvirus 68 latency in IgD-negative B cells. J Virol 87: 3597-3604.
157. Cardiff RD, Miller CH, Munn RJ (2014) Manual hematoxylin and eosin staining of mouse tissue sections. Cold Spring Harb Protoc 2014: 655-658.
158. Johnston RJ, Poholek AC, DiToro D, Yusuf I, Eto D, et al. (2009) Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation. Science 325: 1006-1010.
159. Harris JV, Bohr TM, Stracener C, Landmesser ME, Torres V, et al. (2012) Sequential Plasmodium chabaudi and Plasmodium berghei infections provide a novel model of severe malarial anemia. Infect Immun 80: 2997-3007.
160. Grun JL, Weidanz WP (1983) Antibody-independent immunity to reinfection malaria in B-cell-deficient mice. Infect Immun 41: 1197-1204.

161. Crotty S (2011) Follicular helper CD4 T cells (TFH). Annu Rev Immunol 29: 621-663.
162. Belkaid Y, Rouse BT (2005) Natural regulatory T cells in infectious disease. Nat Immunol 6: 353-360.
163. Couper KN, Blount DG, Wilson MS, Hafalla JC, Belkaid Y, et al. (2008) IL-10 from CD4CD25Foxp3CD127 adaptive regulatory T cells modulates parasite clearance and pathology during malaria infection. PLoS Pathog 4: e1000004.
164. Rasheed MA, Latner DR, Aubert RD, Gourley T, Spolski R, et al. (2013) Interleukin-21 is a critical cytokine for the generation of virus-specific long-lived plasma cells. J Virol 87: 7737-7746.
165. Keir ME, Butte MJ, Freeman GJ, Sharpe AH (2008) PD-1 and its ligands in tolerance and immunity. Annu Rev Immunol 26: 677-704.
166. Liang X, Collins CM, Mendel JB, Iwakoshi NN, Speck SH (2009) Gammaherpesvirus-driven plasma cell differentiation regulates virus reactivation from latently infected B lymphocytes. PLoS Pathog 5: e1000677.
167. Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A (2001) Interleukin-10 and the interleukin-10 receptor. Annu Rev Immunol 19: 683-765.
168. de Waal Malefyt R, Haanen J, Spits H, Roncarolo MG, te Velde A, et al. (1991) Interleukin 10 (IL-10) and viral IL-10 strongly reduce antigen-specific human T cell proliferation by diminishing the antigen-presenting capacity of monocytes via downregulation of class II major histocompatibility complex expression. J Exp Med 174: 915-924.
169. Jacoby MA, Virgin HWt, Speck SH (2002) Disruption of the M2 gene of murine gammaherpesvirus 68 alters splenic latency following intranasal, but not intraperitoneal, inoculation. J Virol 76: 1790-1801.
170. Moss DJ, Burrows SR, Castelino DJ, Kane RG, Pope JH, et al. (1983) A comparison of Epstein-Barr virus-specific T-cell immunity in malaria-endemic and -nonendemic regions of Papua New Guinea. Int J Cancer 31: 727-732.
171. Whittle HC, Brown J, Marsh K, Blackman M, Jobe O, et al. (1990) The effects of Plasmodium falciparum malaria on immune control of B lymphocytes in Gambian children. Clin Exp Immunol 80: 213-218.
172. Lam KM, Syed N, Whittle H, Crawford DH (1991) Circulating Epstein-Barr virus-carrying B cells in acute malaria. Lancet 337: 876-878.
173. Holder B, Miles DJ, Kaye S, Crozier S, Mohammed NI, et al. (2010) Epstein-Barr virus but not cytomegalovirus is associated with reduced vaccine antibody responses in Gambian infants. PLoS One 5: e14013.
174. Wedderburn N, Davies DR, Mitchell GH, Desgranges C, de The G (1988) Glomerulonephritis in common marmosets infected with Plasmodium brasilianum and Epstein-Barr virus. J Infect Dis 158: 789-794.
175. Leoratti FM, Durlacher RR, Lacerda MV, Alecrim MG, Ferreira AW, et al. (2008) Pattern of humoral immune response to Plasmodium falciparum blood stages in individuals presenting different clinical expressions of malaria. Malar J 7: 186.
176. Su Z, Stevenson MM (2000) Central role of endogenous gamma interferon in protective immunity against blood-stage Plasmodium chabaudi AS infection. Infect Immun 68: 4399-4406.
177. De Souza JB, Williamson KH, Otani T, Playfair JH (1997) Early gamma interferon responses in lethal and nonlethal murine blood-stage malaria. Infect Immun 65: 1593-1598.
178. Langhorne J, Mombaerts P, Tonegawa S (1995) alpha beta and gamma delta T cells in the immune response to the erythrocytic stages of malaria in mice. Int Immunol 7: 1005-1011.

179. Sayles PC, Rakhmilevich L (1996) Exacerbation of Plasmodium chabaudi malaria in mice by depletion of TCR alpha beta+ T cells, but not TCR gamma delta+ T cells. Immunology 87: 29-33.
180. Nutt SL, Tarlinton DM (2011) Germinal center B and follicular helper T cells: siblings, cousins or just good friends? Nat Immunol 12: 472-477.
181. Vinuesa CG, Linterman MA, Goodnow CC, Randall KL (2010) T cells and follicular dendritic cells in germinal center B-cell formation and selection. Immunol Rev 237: 72-89.
182. Cubas RA, Mudd JC, Savoye AL, Perreau M, van Grevenynghe J, et al. (2013) Inadequate T follicular cell help impairs B cell immunity during HIV infection. Nat Med 19: 494-499.
183. Uchida J, Yasui T, Takaoka-Shichijo Y, Muraoka M, Kulwichit W, et al. (1999) Mimicry of CD40 signals by Epstein-Barr virus LMP1 in B lymphocyte responses. Science 286: 300-303.
184. Caldwell RG, Wilson JB, Anderson SJ, Longnecker R (1998) Epstein-Barr virus LMP2A drives B cell development and survival in the absence of normal B cell receptor signals. Immunity 9: 405-411.
185. Pires de Miranda M, Alenquer M, Marques S, Rodrigues L, Lopes F, et al. (2008) The Gammaherpesvirus m2 protein manipulates the Fyn/Vav pathway through a multidocking mechanism of assembly. PLoS One 3: e1654.
186. Pires de Miranda M, Lopes FB, McVey CE, Bustelo XR, Simas JP (2013) Role of Src homology domain binding in signaling complexes assembled by the murid gamma-herpesvirus M2 protein. J Biol Chem 288: 3858-3870.
187. Hausler M, Sellhaus B, Scheithauer S, Engler M, Alberg E, et al. (2005) Murine gammaherpesvirus-68 infection of mice: A new model for human cerebral Epstein-Barr virus infection. Ann Neurol 57: 600-603.
188. Hausler M, Sellhaus B, Scheithauer S, Gaida B, Kuropka S, et al. (2007) Myocarditis in newborn wild-type BALB/c mice infected with the murine gamma herpesvirus MHV-68. Cardiovasc Res 76: 323-330.
189. Graham AL, Lamb TJ, Read AF, Allen JE (2005) Malaria-filaria coinfection in mice makes malarial disease more severe unless filarial infection achieves patency. J Infect Dis 191: 410-421.
190. Brooker S, Akhwale W, Pullan R, Estambale B, Clarke SE, et al. (2007) Epidemiology of plasmodium-helminth co-infection in Africa: populations at risk, potential impact on anemia, and prospects for combining control. Am J Trop Med Hyg 77: 88-98.
191. Hasang W, Dembo EG, Wijesinghe R, Molyneux ME, Kublin JG, et al. (2014) HIV-1 infection and antibodies to Plasmodium falciparum in adults. J Infect Dis 210: 1407-1414.
192. Thursz MR, Kwiatkowski D, Torok ME, Allsopp CE, Greenwood BM, et al. (1995) Association of hepatitis B surface antigen carriage with severe malaria in Gambian children. Nat Med 1: 374-375.
193. Berkley JA, Bejon P, Mwangi T, Gwer S, Maitland K, et al. (2009) HIV infection, malnutrition, and invasive bacterial infection among children with severe malaria. Clin Infect Dis 49: 336-343.
194. Otieno RO, Ouma C, Ong'echa JM, Keller CC, Were T, et al. (2006) Increased severe anemia in HIV-1-exposed and HIV-1-positive infants and children during acute malaria. AIDS 20: 275-280.
195. Henle G, Henle W, Diehl V (1968) Relation of Burkitt's tumor-associated herpes-ytpe virus to infectious mononucleosis. Proc Natl Acad Sci U S A 59: 94-101.
196. Johansson B, Klein G, Henle W, Henle G (1970) Epstein-Barr virus (EBV)-associated antibody patterns in malignant lymphoma and leukemia. I. Hodgkin's disease. Int J Cancer 6: 450-462.

197. Nonoyama M, Huang CH, Pagano JS, Klein G, Singh S (1973) DNA of Epstein-Barr virus detected in tissue of Burkitt's lymphoma and nasopharyngeal carcinoma. Proc Natl Acad Sci U S A 70: 3265-3268.
198. Andersson J (2006) Epstein-Barr virus and Hodgkin's lymphoma. Herpes 13: 12-16.
199. Greenspan JS, Greenspan D, Lennette ET, Abrams DI, Conant MA, et al. (1985) Replication of Epstein-Barr virus within the epithelial cells of oral "hairy" leukoplakia, an AIDS-associated lesion. N Engl J Med 313: 1564-1571.
200. Raab-Traub N, Flynn K, Pearson G, Huang A, Levine P, et al. (1987) The differentiated form of nasopharyngeal carcinoma contains Epstein-Barr virus DNA. Int J Cancer 39: 25-29.
201. Collandre H, Ferris S, Grau O, Montagnier L, Blanchard A (1995) Kaposi's sarcoma and new herpesvirus. Lancet 345: 1043.
202. Chang Y, Cesarman E, Pessin MS, Lee F, Culpepper J, et al. (1994) Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. Science 266: 1865-1869.
203. Chen KT (1984) Multicentric Castleman's disease and Kaposi's sarcoma. Am J Surg Pathol 8: 287-293.
204. Fan W, Bubman D, Chadburn A, Harrington WJ, Jr., Cesarman E, et al. (2005) Distinct subsets of primary effusion lymphoma can be identified based on their cellular gene expression profile and viral association. J Virol 79: 1244-1251.
205. Tarakanova VL, Suarez F, Tibbetts SA, Jacoby MA, Weck KE, et al. (2005) Murine gammaherpesvirus 68 infection is associated with lymphoproliferative disease and lymphoma in BALB beta2 microglobulin-deficient mice. J Virol 79: 14668-14679.
206. Sunil-Chandra NP, Arno J, Fazakerley J, Nash AA (1994) Lymphoproliferative disease in mice infected with murine gammaherpesvirus 68. Am J Pathol 145: 818-826.
207. Liang X, Paden CR, Morales FM, Powers RP, Jacob J, et al. (2011) Murine gamma-herpesvirus immortalization of fetal liver-derived B cells requires both the viral cyclin D homolog and latency-associated nuclear antigen. PLoS Pathog 7: e1002220.
208. Klein G, Svedmyr E, Jondal M, Persson PO (1976) EBV-determined nuclear antigen (EBNA)-positive cells in the peripheral blood of infectious mononucleosis patients. Int J Cancer 17: 21-26.
209. Mesri EA, Cesarman E, Arvanitakis L, Rafii S, Moore MA, et al. (1996) Human herpesvirus-8/Kaposi's sarcoma-associated herpesvirus is a new transmissible virus that infects B cells. J Exp Med 183: 2385-2390.
210. Crawford DH, Ando I (1986) EB virus induction is associated with B-cell maturation. Immunology 59: 405-409.
211. Laichalk LL, Thorley-Lawson DA (2005) Terminal differentiation into plasma cells initiates the replicative cycle of Epstein-Barr virus in vivo. J Virol 79: 1296-1307.
212. Siegel AM, Rangaswamy US, Napier RJ, Speck SH (2010) Blimp-1-dependent plasma cell differentiation is required for efficient maintenance of murine gammaherpesvirus latency and antiviral antibody responses. J Virol 84: 674-685.
213. Bhende PM, Dickerson SJ, Sun X, Feng WH, Kenney SC (2007) X-box-binding protein 1 activates lytic Epstein-Barr virus gene expression in combination with protein kinase D. J Virol 81: 7363-7370.
214. Sun CC, Thorley-Lawson DA (2007) Plasma cell-specific transcription factor XBP-1s binds to and transactivates the Epstein-Barr virus BZLF1 promoter. J Virol 81: 13566-13577.
215. Dalton-Griffin L, Wilson SJ, Kellam P (2009) X-box binding protein 1 contributes to induction of the Kaposi's sarcoma-associated herpesvirus lytic cycle under hypoxic conditions. J Virol 83: 7202-7209.
216. Wilson SJ, Tsao EH, Webb BL, Ye H, Dalton-Griffin L, et al. (2007) X box binding protein XBP-1s transactivates the Kaposi's sarcoma-associated herpesvirus (KSHV) ORF50

promoter, linking plasma cell differentiation to KSHV reactivation from latency. J Virol 81: 13578-13586.
217. Yu F, Feng J, Harada JN, Chanda SK, Kenney SC, et al. (2007) B cell terminal differentiation factor XBP-1 induces reactivation of Kaposi's sarcoma-associated herpesvirus. FEBS Lett 581: 3485-3488.
218. Liou HC, Boothby MR, Finn PW, Davidon R, Nabavi N, et al. (1990) A new member of the leucine zipper class of proteins that binds to the HLA DR alpha promoter. Science 247: 1581-1584.
219. Reimold AM, Iwakoshi NN, Manis J, Vallabhajosyula P, Szomolanyi-Tsuda E, et al. (2001) Plasma cell differentiation requires the transcription factor XBP-1. Nature 412: 300-307.
220. Iwakoshi NN, Lee AH, Vallabhajosyula P, Otipoby KL, Rajewsky K, et al. (2003) Plasma cell differentiation and the unfolded protein response intersect at the transcription factor XBP-1. Nat Immunol 4: 321-329.
221. Welihinda AA, Kaufman RJ (1996) The unfolded protein response pathway in Saccharomyces cerevisiae. Oligomerization and trans-phosphorylation of Ire1p (Ern1p) are required for kinase activation. J Biol Chem 271: 18181-18187.
222. Sidrauski C, Walter P (1997) The transmembrane kinase Ire1p is a site-specific endonuclease that initiates mRNA splicing in the unfolded protein response. Cell 90: 1031-1039.
223. Shamu CE, Walter P (1996) Oligomerization and phosphorylation of the Ire1p kinase during intracellular signaling from the endoplasmic reticulum to the nucleus. EMBO J 15: 3028-3039.
224. Gass JN, Gifford NM, Brewer JW (2002) Activation of an unfolded protein response during differentiation of antibody-secreting B cells. J Biol Chem 277: 49047-49054.
225. Todd DJ, McHeyzer-Williams LJ, Kowal C, Lee AH, Volpe BT, et al. (2009) XBP1 governs late events in plasma cell differentiation and is not required for antigen-specific memory B cell development. J Exp Med 206: 2151-2159.
226. Xu D, Zhao L, Del Valle L, Miklossy J, Zhang L (2008) Interferon regulatory factor 4 is involved in Epstein-Barr virus-mediated transformation of human B lymphocytes. J Virol 82: 6251-6258.
227. Kim KM, Ishigami T, Hata D, Yamaoka K, Mayumi M, et al. (1992) Regulation of cell division of mature B cells by ionomycin and phorbol ester. J Immunol 148: 1797-1803.
228. Moser JM, Upton JW, Gray KS, Speck SH (2005) Ex vivo stimulation of B cells latently infected with gammaherpesvirus 68 triggers reactivation from latency. J Virol 79: 5227-5231.
229. Rawstron AC (2006) Immunophenotyping of plasma cells. Curr Protoc Cytom Chapter 6: Unit6 23.
230. Collins CM, Boss JM, Speck SH (2009) Identification of infected B-cell populations by using a recombinant murine gammaherpesvirus 68 expressing a fluorescent protein. J Virol 83: 6484-6493.
231. Sciammas R, Shaffer AL, Schatz JH, Zhao H, Staudt LM, et al. (2006) Graded expression of interferon regulatory factor-4 coordinates isotype switching with plasma cell differentiation. Immunity 25: 225-236.
232. Klein U, Casola S, Cattoretti G, Shen Q, Lia M, et al. (2006) Transcription factor IRF4 controls plasma cell differentiation and class-switch recombination. Nat Immunol 7: 773-782.
233. Herskowitz JH, Jacoby MA, Speck SH (2005) The murine gammaherpesvirus 68 M2 gene is required for efficient reactivation from latently infected B cells. J Virol 79: 2261-2273.
234. Moser JM, Upton JW, Allen RD, 3rd, Wilson CB, Speck SH (2005) Role of B-cell proliferation in the establishment of gammaherpesvirus latency. J Virol 79: 9480-9491.

235. Collins CM, Speck SH (2014) Expansion of murine gammaherpesvirus latently infected B cells requires T follicular help. PLoS Pathog 10: e1004106.
236. Al-Alem U, Li C, Forey N, Relouzat F, Fondaneche MC, et al. (2005) Impaired Ig class switch in mice deficient for the X-linked lymphoproliferative disease gene Sap. Blood 106: 2069-2075.
237. Yin L, Al-Alem U, Liang J, Tong WM, Li C, et al. (2003) Mice deficient in the X-linked lymphoproliferative disease gene sap exhibit increased susceptibility to murine gammaherpesvirus-68 and hypo-gammaglobulinemia. J Med Virol 71: 446-455.
238. Babcock GJ, Decker LL, Volk M, Thorley-Lawson DA (1998) EBV persistence in memory B cells in vivo. Immunity 9: 395-404.
239. Joseph AM, Babcock GJ, Thorley-Lawson DA (2000) EBV persistence involves strict selection of latently infected B cells. J Immunol 165: 2975-2981.
240. Latchman YE, Liang SC, Wu Y, Chernova T, Sobel RA, et al. (2004) PD-L1-deficient mice show that PD-L1 on T cells, antigen-presenting cells, and host tissues negatively regulates T cells. Proc Natl Acad Sci U S A 101: 10691-10696.
241. Dorfman JR, Bejon P, Ndungu FM, Langhorne J, Kortok MM, et al. (2005) B cell memory to 3 Plasmodium falciparum blood-stage antigens in a malaria-endemic area. J Infect Dis 191: 1623-1630.
242. Struik SS, Riley EM (2004) Does malaria suffer from lack of memory? Immunol Rev 201: 268-290.
243. Scholzen A, Sauerwein RW (2013) How malaria modulates memory: activation and dysregulation of B cells in Plasmodium infection. Trends Parasitol 29: 252-262.
244. Donati D, Mok B, Chene A, Xu H, Thangarajh M, et al. (2006) Increased B cell survival and preferential activation of the memory compartment by a malaria polyclonal B cell activator. J Immunol 177: 3035-3044.
245. Nduati E, Gwela A, Karanja H, Mugyenyi C, Langhorne J, et al. (2011) The plasma concentration of the B cell activating factor is increased in children with acute malaria. J Infect Dis 204: 962-970.
246. Asito AS, Moormann AM, Kiprotich C, Ng'ang'a ZW, Ploutz-Snyder R, et al. (2008) Alterations on peripheral B cell subsets following an acute uncomplicated clinical malaria infection in children. Malar J 7: 238.
247. Asito AS, Piriou E, Jura WG, Ouma C, Odada PS, et al. (2011) Suppression of circulating IgD+CD27+ memory B cells in infants living in a malaria-endemic region of Kenya. Malar J 10: 362.
248. Rowland SL, Leahy KF, Halverson R, Torres RM, Pelanda R (2010) BAFF receptor signaling aids the differentiation of immature B cells into transitional B cells following tonic BCR signaling. J Immunol 185: 4570-4581.
249. Vugmeyster Y, Seshasayee D, Chang W, Storn A, Howell K, et al. (2006) A soluble BAFF antagonist, BR3-Fc, decreases peripheral blood B cells and lymphoid tissue marginal zone and follicular B cells in cynomolgus monkeys. Am J Pathol 168: 476-489.
250. Ehrhardt GR, Hijikata A, Kitamura H, Ohara O, Wang JY, et al. (2008) Discriminating gene expression profiles of memory B cell subpopulations. J Exp Med 205: 1807-1817.
251. Ehrhardt GR, Hsu JT, Gartland L, Leu CM, Zhang S, et al. (2005) Expression of the immunoregulatory molecule FcRH4 defines a distinctive tissue-based population of memory B cells. J Exp Med 202: 783-791.
252. Moir S, Ho J, Malaspina A, Wang W, DiPoto AC, et al. (2008) Evidence for HIV-associated B cell exhaustion in a dysfunctional memory B cell compartment in HIV-infected viremic individuals. J Exp Med 205: 1797-1805.
253. Weiss GE, Crompton PD, Li S, Walsh LA, Moir S, et al. (2009) Atypical memory B cells are greatly expanded in individuals living in a malaria-endemic area. J Immunol 183: 2176-2182.

254. Weiss GE, Clark EH, Li S, Traore B, Kayentao K, et al. (2011) A positive correlation between atypical memory B cells and Plasmodium falciparum transmission intensity in cross-sectional studies in Peru and Mali. PLoS One 6: e15983.
255. Weiss GE, Traore B, Kayentao K, Ongoiba A, Doumbo S, et al. (2010) The Plasmodium falciparum-specific human memory B cell compartment expands gradually with repeated malaria infections. PLoS Pathog 6: e1000912.
256. Illingworth J, Butler NS, Roetynck S, Mwacharo J, Pierce SK, et al. (2013) Chronic exposure to Plasmodium falciparum is associated with phenotypic evidence of B and T cell exhaustion. J Immunol 190: 1038-1047.
257. Muellenbeck MF, Ueberheide B, Amulic B, Epp A, Fenyo D, et al. (2013) Atypical and classical memory B cells produce Plasmodium falciparum neutralizing antibodies. J Exp Med 210: 389-399.
258. Zinocker S, Schindler CE, Skinner J, Rogosch T, Waisberg M, et al. (2015) The v gene repertoires of classical and atypical memory B cells in malaria-susceptible west african children. J Immunol 194: 929-939.
259. Ndungu FM, Cadman ET, Coulcher J, Nduati E, Couper E, et al. (2009) Functional memory B cells and long-lived plasma cells are generated after a single Plasmodium chabaudi infection in mice. PLoS Pathog 5: e1000690.
260. Nduati EW, Ng DH, Ndungu FM, Gardner P, Urban BC, et al. (2010) Distinct kinetics of memory B-cell and plasma-cell responses in peripheral blood following a blood-stage Plasmodium chabaudi infection in mice. PLoS One 5: e15007.
261. Wykes MN, Zhou YH, Liu XQ, Good MF (2005) Plasmodium yoelii can ablate vaccine-induced long-term protection in mice. J Immunol 175: 2510-2516.