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The Role of Toll-like Receptor Signaling During the Establishment of and Reactivation from Murine Gammaherpesvirus 68 Latency

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Doctor of Philosophy

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By

Lisa Marie Gargano
B.A., University of Kansas, 2000

Advisor: Samuel H. Speck, Ph.D.

An abstract of
A dissertation submitted to the Faculty of the Graduate School of Emory University
in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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Immunology and Molecular Pathogenesis

2009

Abstract

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Murine gammaherpesvirus 68 (MHV68) establishes a life-long infection in mice and is used as a model system to study the role of viral and host factors in chronic infections. Long-term persistence depends on the ability of MHV68 to establish latency and reactivate from latency to reseed new latency reservoirs. Toll-like receptor (TLR) signaling plays an essential role in the activation of innate immunity by recognizing specific patterns of microbial components. In the first aim, I investigated the role of TLR signaling in MHV68 latency *in vivo*. I found that unlike in $TLR3^{-/-}$ mice, B-cells from $MyD88^{-/-}$ mice displayed a decrease in the frequency of activated, germinal center and class switched B-cells. While acute virus replication in the lungs was unaffected, establishment of latency was decreased in $MyD88^{-/-}$ mice. In mixed bone-marrow chimeric mice, there was a selective failure of the $MyD88^{-/-}$ B-cells to undergo an optimal response. While MHV68 established latency efficiently in the $MyD88^{+/+}$ B-cells, there was a reduction in the frequency of $MyD88^{-/-}$ B-cells harboring latent MHV68. This data supports a model whereby establishment of gammaherpesvirus latency is dependent upon the virus gaining access to the long-lived memory B-cell reservoir. In the second aim I investigated the ability of TLR ligands to induce MHV68 reactivation. I found that stimulation of a latently infected B-cell line with TLR ligands enhanced MHV68 reactivation. *Ex vivo* stimulation of latently infected splenocytes with TLR ligands led to early B-cell activation and proliferation with a concomitant increase in MHV68 reactivation. When LPS or CpG was administered *in vivo* there was an increase in B-cell activation and MHV68 reactivation and also an increase in the $CD8^{+}$ T-cell response. MHV68 reactivation leads to an increase of viral genome positive cells at 14 days post-stimulation. These data suggest that control of gammaherpesvirus latency might be disrupted upon TLR signaling in response to subsequent pathogens *in vivo*. Together findings demonstrate a critical role of TLR signaling during MHV68 infection.

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ACKNOWLEDGMENTS

First, I would like to thank my mentor Dr. Sam Speck. He has allowed me to find myself as an independent scientist and to pursue my own interests. The chance to have such independence as well as the ability to take risks and make mistakes is extremely rare. It has been a fantastic training environment and has given me a confidence that I hope to take with me wherever I go.

I would also express my deep appreciation to past and present members of the Speck lab. A special thanks to Drs. Janice Moser and Laurie Krug who helped me get acclimated to the lab and show me the ropes. Also to Drs. Craig Forrest and Chris Collins who have been patient and wonderful teachers. I especially want to thank the fellow IMP students that I have had the pleasure of working with, Katy Gray, Andrea Siegel, Andrew Evans and Clint Paden. The best thing about working with this group has been the extraordinary amount of talent you find yourself surrounded by.

A special thanks to my committee members, Drs. Andrew Gewirtz, Joshy Jacob, Brian Evavold and Dave Steinhauer. They have always given me their time and support. Also to the late Dr. Kirk Zeigler, who was only on my committee for a short time but always was encouraging.

Throughout my time here at Emory my classmates have always been good friends and colleagues. Especially, Kristen Rosenthal, Rebecca Lynch, Nichole Klatt Brian Norris and Luke Uebelhoer. I hope we will continue to share our struggles and successes.

Importantly, a special thanks to my husband, Jake, while he may not have understood everything I talked about, he always listened, was a shoulder to cry on and was there to celebrate my successes.

Finally, I would like to dedicate this work to my late father William V. Gargano, whom I lost while at Emory. He was endlessly supportive and a source of calm strength and wisdom. I hope to make him proud.

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Chapter I

Introduction

Herpesviruses are characterized by their ability to establish a life-long latent infection in the host, during which there is no production of progeny virus and very limited viral gene transcription. Following the appropriate stimulus, the virus can reactivate from latency and begin producing infectious viral progeny, thus differentiating a bona fide latent infection from an abortive infection. The Herpesviridae are subdivided in to three major classes; alphaherpesviruses, such as the common human Herpes Simplex Viruses (HSVs), are typified by their ability to undergo a latent infection in the sensory ganglia, a wide host-range, a short replication cycle and rapid destruction of infected cells. The betaherpesviruses, such as human cytomegalovirus (HCMV) and murine CMV, are described by their capacity to establish and maintain latency in secretory organs, a long replicative cycle and a restricted host range. Finally, the gammaherpesviruses, such as Epstein-Barr virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV or HHV-8) are characterized by restricted host range, their ability to infect B or T lymphocytes and a tight association with the development of lymphomas and lymphoproliferative diseases. These viruses are not only capable of evading a protective immune response but they are also capable of manipulating the biology of the hematopoetic cells in which they reside. As such, their life cycle is intimately associated with the host immune response. Gammaherpesvirus associated disease is most prevalent among immunocompromised individuals, indicating the degree to which these viruses

have co-evolved with their hosts to maintain a tenuous host-pathogen interaction that normally ensures an asymptomatic infection.

Gammaherpesvirus 68

Gammaherpesviruses are large DNA viruses that are lymphotropic and establish lifelong infection in their host. The gammaherpesvirus subfamily includes both the Lymphocryptoviruses (gamma-1), of which EBV is the prototype, and Rhadinovirus (gamma-2), which consists of KSHV and Herpesvirus saimiri (HVS). EBV infection is associated with the development of Burkitt's lymphomas, Hodgkin's disease and nasopharyngeal carcinoma (28). KSHV infection is closely associated with AIDS-associated Kaposi's sarcoma, multicentric Castleman's disease and primary effusion lymphomas as well as graph rejection in transplant patients (23). Murine gammaherpesvirus 68 (MHV68) shares genomic colinearity and sequence conservation with both EBV and KSHV although nucleotide sequence analysis revealed that MHV68 is more closely related to KSHV and herpesvirus saimiri (HSV) and was placed in the Rhadinovirus (gamma-2) subfamily (105, 109) (Figure 1). Although first isolated from bank voles (*Clethrionomys glareolus*) in Eastern Europe, MHV68 is capable of infecting inbred and outbred stains of laboratory mice and therefore provides a tractable small animal model to study gammaherpesvirus pathogenesis (14, 38, 86, 99, 117, 118). Like other gammaherpesviruses, MHV68 infection is associated with lymphoproliferative disease and long-term infections leading to the development of lymphomas (90, 116).

The MHV68 genome was cloned as a bacterial artificial chromosome (BAC), which allows facile genetic manipulation of the virus by targeting insertion and deletion

of sequences utilizing bacterial genetics (2). Unlike the human gammaherpesviruses, high titer stocks of MHV68 can be grown in the laboratory. Additionally, host determinants of MHV68 pathogenesis can be defined utilizing the numerous transgenic and knockout mouse models. MHV68 facilitates an understanding of viral and host determinants of gammaherpesvirus pathogenesis *in vivo*.

The ability to establish latency infections in the host is a hallmark of all herpesviruses. Acute infection with MHV68 leads to the establishment of long-term latency, which is defined by the absence of lytic virus despite the presence of viral genome and the ability of latent virus to reactivate. Acute infection occurs in the lung epithelium after intranasal inoculation and is dose-dependent (128). From the lungs, infected B cells are required for efficient trafficking of MHV68 to the spleen and establishment of splenic latency (114). Interestingly, route of infection determines these trafficking requirements, as B cells are not required for the establishment of splenic latency following intraperitoneal infection (136). As opposed to acute infection, there is little dose-dependence for the establishment of latency (128). During latency the MHV68 genome is maintained as an episome in the nucleus of the infected cell. During the acute phase of infection, MHV68 infects many tissues and organs and causes acute viremia, which is cleared about two weeks post-infection. $CD8^+$ T cells play a major role in controlling this initial phase of primary MHV68 lytic replication (15, 38, 130). MHV68 infection results in an infectious-mononucleosis-like illness characterized by a CD4-T cell-dependent expansion of splenic B cells and both virus-specific and non-specific hypergammaglobulinemia (112, 113). Virus-specific antibody titers rise steadily over the course of six months (112) and passive transfer of immune serum can prevent splenic

latency (129). Notably, there is a massive expansion of $CD8^+$ T cells characterized by a bias in the repertoire of T cell receptors towards a $V\beta4^+$ phenotype that does not occur till almost one month post-infection (42, 131). These $V\beta4^+$ $CD8^+$ T cells show an activated, functional phenotype and are potent producers of interferon gamma. It has been recently reported that the M1 gene product of MHV68 is responsible for the expansion and that the proposed purpose of the $V\beta4^+$ $CD8^+$ T cells is to suppress MHV68 reactivation *in vivo* (39).

Evidence suggests that MHV68 latency and normal B cell differentiation occur in tandem. Splenic B cells infected with MHV68 undergo proliferation and bear a germinal-center phenotype (40, 41, 88, 106, 142). Notably, isotype-class-switched memory B cells are the predominant long-term reservoir for latent MHV68 infection at late time-points post-infection, a characteristic shared with latent EBV infection (68, 142). Other cell types such as naïve B cells, macrophages, and dendritic cells also harbor latent virus at early times post-infection (40, 41, 136). Thus, MHV68 is a unique system by which the contributions of both the host and the pathogen on viral tropism, latency, reactivation, oncogenesis, immune control and immune evasion can be examined in a controlled manner.

Role of host immune response to MHV68

Investigations of the immune response to MHV68 have yielded a wealth of information regarding host control of lytic and latent phases of infection. Innate immune mechanisms are critical for the control of MHV68 lytic replication in the lungs and inhibit reactivation from latency. $IFN\alpha/\beta R^{-/-}$ mice have faster replication kinetics and

higher peak titers in multiple organs (36). This systemic spread leads to higher incidence of mortality in a dose-dependent manner (8, 36, 137). Studies have shown that natural killer (NK) cells are important in controlling the early stages of infection with alpha- or beta-herpesviruses in both humans and mice. However, the depletion of NK cells had no effect on the control of the acute or latent stages of MHV68 infection, as mice deficient in NK cells controlled the infection in a comparable manner to wild type (132). Mice deficient in B cells ($\mu MT^{-/-}$) are able to control acute replication after an intranasal inoculation and chronic infection is established in the lungs but exhibit an failure to control reactivation (138). Infection in the absence of B cells results in more persistent viral replication in non-B cell subsets, and probably reflects a drastically altered course of infection for a B cell lymphotropic virus. Failure to control replication also indicates a requirement for antibody-mediated control of infection, as passive immunization with serum from MHV68 latently infected mice decreases the establishment of latency in $\mu MT^{-/-}$ mice (47). As mentioned previously, $CD8^+$ T cells play a major role in controlling acute replication; CD8-deficient ($CD8^{-/-}$) and β_2-microglobulin-deficient ($\beta_2M^{-/-}$) mice have increased lytic replication in the lungs in addition to an increased splenic latency and reactivation (15, 130). $CD4^+$ T cells are also critical for the establishment of latency and immune control (18, 20, 42, 133). The cooperative role of both T cell and B cell immunity is perhaps most evident among animals lacking $CD4^+$ T cell help as mice lacking $CD4^+$ T cells ($MHCII^{-/-}$ mice) are able to control acute replication but do not control long-term latency, display increased reactivation, and often succumb to infection despite the presence of a relatively normal $CD8^+$ T cell response (20, 103).

Costimulatory molecules are essential for the development of humoral and cellular immune responses. $CD40^{-/-}$ mice have a persistent infection in the lungs and are unable to control long-term MHV68 infection (141). $CD28^{-/-}$ mice are able to clear MHV68 from the lungs with wild-type kinetics and have slightly lower peak latency but are surprisingly are able to control long-term infection (67, 75, 79). Mice deficient in both CD80 and CD86 ($CD80^{-/-}$/ $CD86^{-/-}$), signaling molecules on the surface of antigen presenting cells that bind CD28 on T cells, exhibit lower peak splenic latency and are unable to maintain long-term control of virus infection (46, 79). Due to the absence of costimulation in both $CD28^{-/-}$ and $CD80/86^{-/-}$ mice, both types of animals have an impaired virus-specific antibody response (46, 67, 79).

Finally, IFNγ plays an interesting role in controlling MHV68. Mice deficient in IFNγ (IFNγ $^{-/-}$) have no obvious defect in their ability to control acute MHV68 infection (102), but by two weeks post-infection demonstrate elevated *ex vivo* reactivation in a tissue-type dependent manner (130). IFNγ appears to be critical for $CD8^+$ T cell mediated control of chronic infection as $IFN\gamma R^{-/-}$ mice exhibit increased reactivation and persistent replication which results in chronic inflammation and multi-organ fibrotic tissue damage (29, 37, 111, 137).

Formation of a B cell response

B cells act as immune effectors, primarily through antigen-specific clonal expansion and plasma cell differentiation. During a B cell response, a mature naïve B cell recognizes its cognate antigen through the B cell receptor (BCR). The activated B cell then migrates to a secondary lymphoid organ, enters the T cell zone (light zone) and

receives help from a $CD4^+$ T cell through CD40-CD40L, co-stimulation and cytokines, and follicular dendritic cells. The B cell then migrates to the B cell zone (dark zone) to establish a germinal center. In the germinal center, cells undergo massive proliferation and differentiation. Proliferating B cells are known as centroblasts. Centroblasts eventually give rise to centrocytes, which are non-dividing B cells that expressing membrane Ig. These centrocytes move back into the light zone where they can make contact with antigen-antibody complexes on the surface of follicular dendritic cells and if their affinity was improved and not a disadvantageous mutation, the B cell is then receives survival signals. In the germinal center B cells under three important differentiation events: affinity maturation, class switching and formation of plasma cells and memory B cells (Figure 2). These processes are key to producing highly specific antibodies and good secondary immune responses.

There is emerging appreciation for the role of toll-like receptor (TLR) signaling in the activation of B cell responses, including germinal center reactions and antibody production (93). Signaling through the MyD88 pathway has been shown to be required for B cells to optimize responses to foreign antigens, such as human serum albumin or viruses (93). TLRs expressed on B cells may help determine the microbial origin of antigens recognized by the BCR and help direct the response against infectious agents (93). It has been shown that MyD88 via TLR, not IL-1R or IL-18R, signaling is required for the formation of long-term humoral response to polyoma virus (52). During an influenza infection, MyD88 has been shown to influence the class switching of the anti-influenza B cell response (55). EBV up-regulates TLR7 early in infections to enhance B cell proliferation and subsequently modifies the pathway to regulate IRF5 activity (80).

Toll-like receptors

Toll-like receptors (TLRs), a type of pattern recognition receptor (PRR), are an important part of the innate immune system. TLRs sense pathogens by detecting pathogen-associated molecular patterns (PAMPs) (94, 121). There are 12 known mammalian TLRs; with the exception of TLR3, engagement of TLRs by their ligand(s) activates the MyD88-IL-1 associated receptor kinase-TNFR associated factor 6 signaling pathway (MyD88-IRAK-TRAF6), leading to activation of several transcription factors, such as NF-κB, MAPK and interferon regulating factors (IRFs). Engagement of TLRs expressed on antigen-presenting cells (APCs), including dendritic cells (DCs) and macrophages, results in chemokine and cytokine production, increased antigen presentation, and the expression of co-stimulatory molecules (4, 62). These events can initiate an inflammatory response through the chemokine secretion and cellular recruitment (3, 4, 62). TLR engagement mediates the maturation and migration of DCs to lymph nodes that facilitates interaction with T lymphocytes (62, 66). Mature DCs interaction with naïve T cells to differentiate them into T_H1 and T_H2 or T regulatory lymphocytes (81). TLR ligands such as lipopolysaccharide and double stranded RNA are known to act as adjuvant, enhancing the adaptive immune response (59).

TLRs recognize PAMPs from bacteria, fungi and protozoans as well as viruses. TLR 1/2 and 2/6 recognize bacterial component lipoproteins and components from yeast such as zymogen (92, 122-125). TLR4 recognizes LPS (98) and flagellin is recognized by TLR5 (54). There are several TLRs whose ligands are viral PAMPs. TLR3 recognizes double stranded RNA (5), TLR7 and 8 recognize single stranded RNA (33, 56, 57) and TLR9 recognizes CpG DNA (58)**.** These TLRs induce an anti-viral host defense

response, particularly secretion of IFN-α/β. TLRs have been shown to be important in activating the innate immune response to control virus replication during virus infections. MyD88 signaling is important in controlling LCMV infection and the maturation/activation of virus-specific $CD8^+$ T cells (148). TLR signaling has been shown to play a role in several herpesvirus infections. During MSCV infection, TLR9 signaling is required to ensure a rapid antiviral response (32). TLR9 is also involved in MHV68 pathogenesis and contributes to organ-specific immunity (53). Recently it has been demonstrated that certain laboratory and clinical strains of herpes simplex virus activate TLR2 and TLR9 (104). IRF7, a molecule downstream of TLR signaling, is important in regulating KSHV infection by binding to a lytic promoter to repress transcription of the associated gene. There is also evidence of viral evasion of TLR signaling pathways; the NS5A protein of Hepatitis C virus inhibits TLR signaling by binding to MyD88 (1), and infection of endothelial cells with KSHV leads to rapid suppression of TLR4 expression (73). TLRs play an integral role in innate immune responses against microbial pathogens, as well as the subsequent induction of adaptive immune responses.

Gammaherpesvirus Reactivation

Herpesvirus infections cause a mild self-limiting primary infection in immunocompetent host. Like other herpesviruses, gammaherpesviruses then establish latent infection characterized by the absence of infectious virus, until reactivation is induced by various stimuli. During reactivation, latently infected cells regain expression of a variety of lytic cycle genes, linear forms of the genome are produced for packaging,

and viral progeny are released (91, 100, 146). The switch from latent to lytic viral gene expression is crucial for virus spread between cells and hosts (21, 51, 72, 126, 127). Episodes of reactivation are also known to be pathogenic in immunosuppressed individuals, such as AIDS patients or transplant recipients (107, 110). Because of its pathogenic implications, the mechanism by which gammaherpesviruses reactivation occurs has considerable importance. Reactivation of human gammaherpesviruses, EBV and KSHV, has been extensively studied *in vitro*. The EBV lytic cycle can be initiated by several different reagents, including anti-immunoglobulin (anti-Ig), calcium ionophore, sodium butyrate, and tetradecanoyl phorbol acetate (TPA) (108). Phorbol esters and sodium butyrate can also induce KSHV reactivation (22, 85). More physiological relevant stimulants have also been shown to induce human gammaherpesvirus reactivation. CMV superinfection of EBV infected cells stimulates EBV reactivation (7). HIV-1 infection of KSHV infected primary effusion lymphoma (PEL) cells will induce latent KSHV reactivation (84, 135), bacterial end products such as LPS, has been shown to induce KSHV reactivation from infected cell lines (87). Triggers for reactivation are also known for other viruses. LPS can induce murine Cytomegalovirus (MCMV) reactivation *in vivo* (27). TLR signaling has been shown to trigger HIV-1 replication from latently infected mast cells (115)

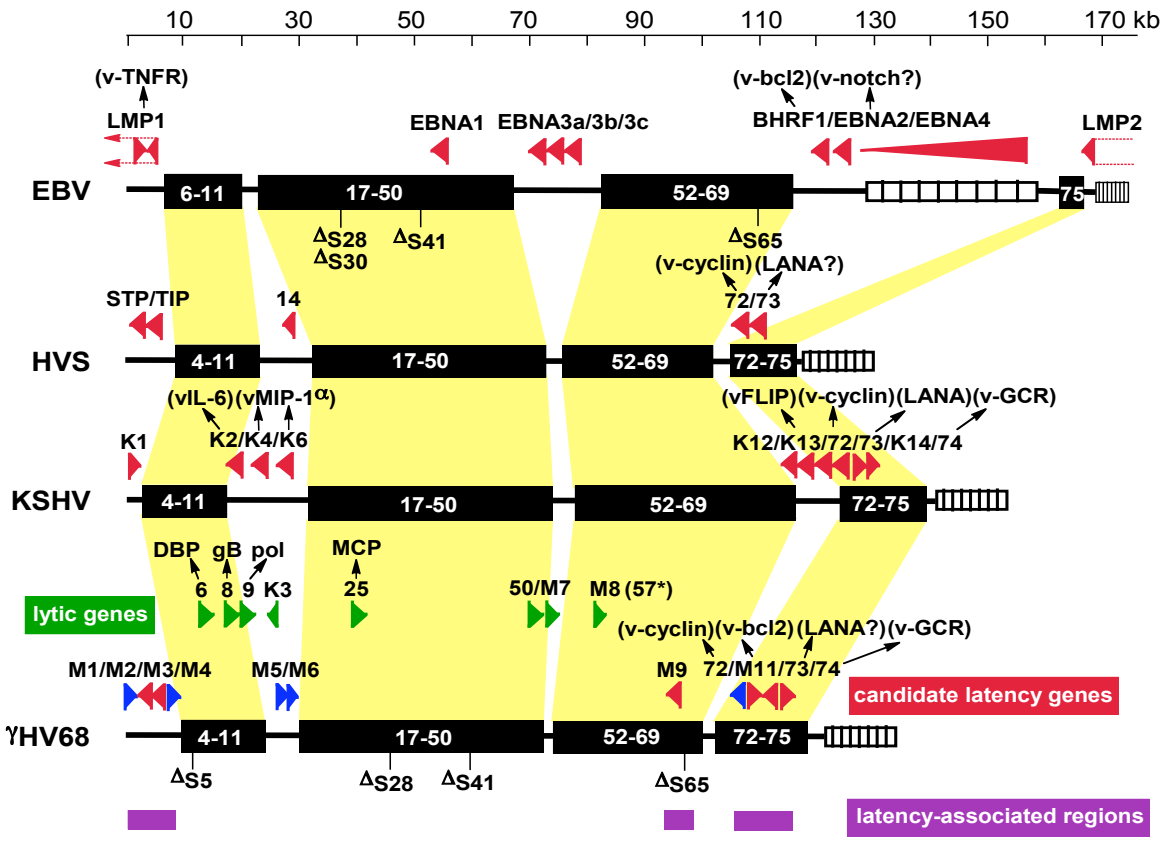
Reactivation studies are problematic in human hosts. Therefore studies of the human gammaherpesviruses reactivation have only been possible *in vitro*, outside the context of the immune system, key to the reactivation process. MHV68 allows use of a model system to answer questions about the steps required for reactivation, and MHV68 infection is well characterized and shares several features of human gammaherpesvirus

infection. Like EBV and KSHV, MHV68 anti-Ig/anti-CD40 and TPA can drive reactivation both *in vitro* and *ex vivo* and correlates with B cell activation and proliferation (89).

Summary

Herpesviruses are ubiquitous throughout the population. The ability to establish latency and reactivate are hallmarks of all herpesviruses. This dissertation is aimed to better understand these two arms of gammaherpesvirus infection and the impact that TLR signaling has on these two characteristics. The first aim of these studies (detailed in chapter II) was to determine the role of TLR signaling during the establishment of MHV68 latency and the role in the formation of the B cell response to MHV68. In the second aim of these studies (detailed in chapter III) I sought to determine the role of TLR ligands as triggers of MHV68 reactivation. These studies were designed to dissect the role of host-pathogen interactions, in particular the role of TLR signaling during MHV68 infection.

Figure 1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17-50
52-69
75
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ΔS30
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STP/TIP
14
HVS
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52-69
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K1
K2/K4/K6
(vFLIP)(v-cyclin)(LANA)(v-GCR)
K12/K13/72/73/K14/74
KSHV
4-11
17-50
52-69
72-75
DBP
gB
pol
MCP
6
8
9
K3
25
50/M7
M8 (57*)
lytic genes
(v-cyclin)(v-bcl2)(LANA?)(v-GCR)
M1/M2/M3/M4
M5/M6
M9
72/M11/73/74
candidate latency genes
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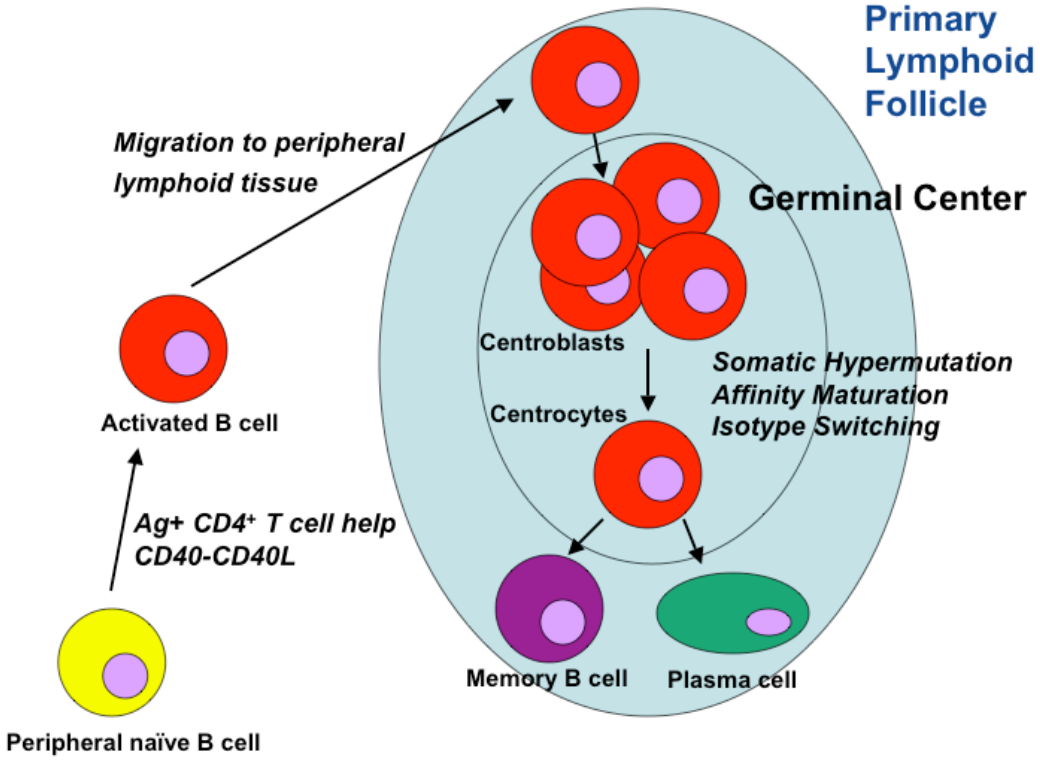
latency-associated regions

Figure 2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Follicle
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Activated B cell
Centroblasts
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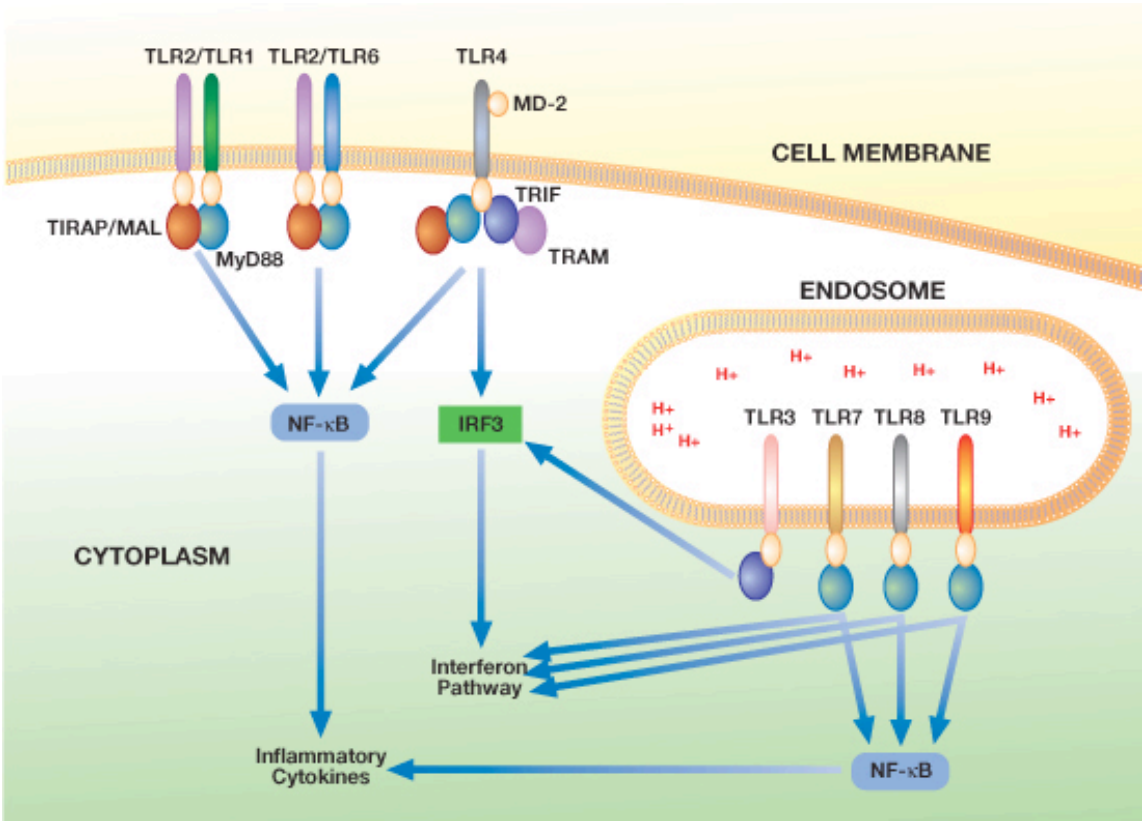
Ag+ CD4+ T cell help
CD40-CD40L
Peripheral naïve B cell
Memory B cell
Plasma cell

Figure 3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TRIF
TIRAP/MAL
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H+
TLR3
TLR7
TLR8
TLR9
NF-κB
IRF3
CYTOPLASM
Interferon
Pathway
Inflammatory
Cytokines
NF-κB

Figure Legends

Figure 1. Alignment of selected collinear gammaherpesvirus genomes. Schematic diagram depicting the genomic arrangement of four gammaherpesvirsues: EBV, HSV, KSHV and MHV68. Highlighted in red are the candidate latency genes that tend to cluster at the either terminus of linear genomes. Genes located at the far left end of each virus are unique to each respective virus. In most cases these factors are non-essential to viral replication, but serve to promote the establishment of latency and/or in vivo infection.

Figure 2. Normal B cell differentiation process in response to a primary antigen. Illustration of the development of a B cell response in a primary lymphoid follicle. B cell enters follicle and undergoes a maturation/differentiation process before exiting and homing to the sight of infection to mount a humoral immune response.

Figure 3. Toll-like receptor signaling pathways. Diagram illustrates how TLR1, TLR2, TLR6 and TLR4 are mostly found on the cell membrane and TLR3, TLR7/8 and TLR9 are usually found in the endosome. Subsequent signaling events upon recognition of ligand lead to induction of innate immune responses. Adapted from Boehme and Compton, 2004. J Virol. 78,7867.

Chapter II

Role of MyD88 Signaling in Murine Gammaherpesvirus 68 Latency

Gargano, LM, Moser, JM, Speck SH. *J. Virol*. 2008; 82(8):3853-63.

ABSTRACT

Toll-like receptors (TLRs) are predominantly known for their role in activating the innate immune response. Recently, TLR signaling via MyD88 has been reported to play an important function in development of a B cell response. As B cells are a major latency reservoir for MHV68, we investigated the role of TLR signaling in the establishment and maintenance of MHV68 latency *in vivo*. Mice deficient in MyD88 (MyD88$^{-/-}$) or TLR3 (TLR3$^{-/-}$) were infected with MHV68. Analysis of splenocytes recovered at day 16 post-infection from MyD88$^{-/-}$ mice compared to wild type control mice revealed a lower frequency of: (i) activated B cells; (ii) germinal center B cells; and (iii) class-switched B cells. Accompanying this substantial defect in the B cell response was an approximately 10-fold decrease in the establishment of splenic latency. In contrast, no defect in viral latency was observed in TLR3$^{-/-}$ mice. Analysis of MHV68-specific antibody responses also demonstrated a substantial decrease in the kinetics of the response in MyD88$^{-/-}$ mice. Analysis of wild type x MyD88$^{-/-}$ mixed bone marrow chimera mice demonstrated that there is a selective failure of the MyD88$^{-/-}$ B cells to participate in germinal center reactions, as well as become activated and class switch. In addition, while MHV68 established latency efficiently in the MyD88-sufficient B cells, there was again a ca. 10-fold reduction in the frequency of MyD88$^{-/-}$ B cells harboring latent

MHV68. This phenotype indicates that MyD88 is important for the establishment of MHV68 latency and is directly related to the role of MyD88 in the generation of a B cell response. Furthermore, the generation of a B cell response to MHV68 was intrinsic to B cells and was independent of the IL-1 receptor, a cytokine receptor that also signals through MyD88. This data provides evidence for a unique role for MyD88 in the establishment of MHV68 latency.

INTRODUCTION

Murine gammaherpesvirus 68 (MHV68) shares genomic colinearity with EBV and KSHV, although it is more closely related to KSHV and herpesvirus saimiri (HSV) (105, 109). It is capable of infecting inbred and outbred stains of laboratory mice and therefore provides a tractable small animal model to study γ-herpesvirus pathogenesis (14, 38, 86, 99, 116-118). Hallmarks of a MHV68 infection include acute viremia that is cleared approximately two weeks post-infection in wild type mice which is accompanied by a massive expansion of immune cells (136). Acute infection leads to the establishment of long-term latency in the memory B cell compartment, although other cell types such as naïve B cells, macrophages, and dendritic cells have been shown to harbor latent virus at early time-points post-infection (40, 41, 136). Like the other γ-herpesviruses, MHV68 has been shown to be associated with lymphoproliferative disease and long-term infections can lead to the development of lymphomas (90, 116). MHV68 facilitates an understanding of viral and host determinants of γ-herpesvirus pathogenesis *in vivo*.

Toll-like receptors (TLRs), a type of pattern recognition receptor (PRR), are an important part of the innate immune system. TLRs recognize pathogens by detecting pathogen-associated molecular patterns (PAMPs) (94, 121). There are 12 known mammalian TLRs and, with the exception of TLR3, the engagement through their ligand(s) activate the MyD88-IL-1 associated receptor kinase-TNFR associated factor 6 signaling pathway (MyD88-IRAK-TRAF6) which then leads to activation of several transcription factors, such as NF-κB, MAPK and interferon regulating factors (IRFs). Engagement of TLRs expressed on antigen-presenting cells (APCs) including dendritic cells (DCs) and macrophages with their ligand(s) results in chemokine and cytokine

production, increased antigen presentation, and the expression of co-stimulatory molecules (4, 62). These events can initiate an inflammatory response through the chemokine secretion and cellular recruitment (3, 4, 62). TLR engagement mediates the maturation and migration of DCs to lymph nodes that facilitates interaction with T lymphocytes (62, 66). TLR ligands such as lipopolysaccharide and double stranded RNA are known to act as adjuvant, enhancing the adaptive immune response (59). DC interactions with naïve T cells differentiate them into T_H1 and T_H2 or T regulatory lymphocytes (81).

There are several TLRs whose ligands are viral PAMPs. TLR3 recognizes double stranded RNA (5), TLR7 and 8 recognize single stranded RNA (33, 56, 57) and TLR9 recognizes CpG DNA (58)**.** These TLRs induce an anti-viral host defense response, especially secretion of IFN-α/β. TLR have been shown to be important to activate the innate immune response to control virus replication during virus infections. MyD88 signaling is important to control LCMV infection and the maturation/activation of virus-specific $CD8^+$ T cells (148). TLR signaling has been shown to play a role in several herpesvirus infections. There is a requirement for TLR9 signaling in sensing murine cytomegalovirus (MCMV) to ensure a rapid antiviral response (32). Recently, it has been demonstrated that certain laboratory and clinical strains of herpes simplex virus activate TLR2 and TLR9 (104). The TLR family plays an instructive role in innate immune responses against microbial pathogens, as well as the subsequent induction of adaptive immune responses.

There is also an emerging appreciation for the role that TLRs play in the activation of B cell responses, including germinal center reactions and antibody

production (93). Signaling through TLRs has been shown to play a role in class switching (63, 93), as well as be necessary for maintenance of neutralizing and long-term antibody responses against infections (52, 147). Signaling through the MyD88 pathway has been shown to be required for B cells to achieve optimal response to foreign antigens (93). This optimization might be due to either direct recognition of the pathogen through the TLR or rather as a tertiary signal following recognition of the antigen through the B cell receptor in conjunction with B cell receptor recognition and T cell help.

In the present study, we explore the role of TLR signaling during a MHV68 infection. We first examined the B cell response during a MHV68 infection in MyD88$^{-/-}$ or TLR3$^{-/-}$ compared to wild type C57Bl/6. We demonstrate that MHV68 infection results in decreased B cell activation, germinal center formation and class switching at early times post-infection in MyD88$^{-/-}$ mice compared to wild type. This decrease is not seen in TLR3$^{-/-}$ mice, showing that the above effects are MyD88 specific. Since MHV68 requires germinal center formation to establish latency (41, 106, 142) we next examined whether MyD88-deficincy had any effect on the ability of MHV68 to establish latency. Corresponding to the decreased B cell response observed in the MyD88$^{-/-}$ mice there was also a decrease in the frequency of viral genome positive cells. Importantly, this deficiency in B cell response is intrinsic to the MyD88$^{-/-}$ B cell as demonstrated by using mixed bone marrow chimeric mice. We also show that it is IL-1 receptor signaling independent. This further illustrates that early establishment of MHV68 latency is dependent on the participation of germinal center reactions to gain entry into the memory B cell compartment.

MATERAILS AND METHODS

Cells, virus and virus culture. MHV68 strain WUMS (ATCC VR1465) was used for all virus infections unless indicated. MHV68-IκBαM.1 and MHV68-IκBαM.MR were kindly donated by Dr. Laurie Krug. Virus passage, maintenance, and titers were preformed as previously described (25). NIH 3T12 cells and mouse embryonic fibroblast (MEF) cells were maintained in Dulbecco's modified Eagle's medium supplemented with 100 U penicillin/ml, 100 mg streptomycin/ml, 10% fetal calf serum, and 2mM L-glutamate (cMEM). Cells were maintained at 37^{o} C in a 5% CO_2 environment. MEF cells were prepared from C57Bl/6 as previously described (97).

Mice, infections and organ harvests. C57Bl/6 (catalog no. 000664; The Jackson Laboratory), MyD88$^{-/-}$ (generous gift from Dr. Andrew Gerwirtz), TLR3$^{-/-}$ (cat. number 005217; The Jackson Laboratory), IL-1R$^{-/-}$ (cat. number 003245; The Jackson Laboratory) and B6.SJL-PtprcaPepb/BoyJ wild-type (Ly5.1) (cat. number 002014; The Jackson Laboratory) mice were housed and bred in the Whitehead Building vivarium in accordance with all federal, university, and facility regulations. Mice were placed under isofluorane anesthesia prior to intranasal inoculation with 1,000 PFU of virus in 20 μl of cMEM or by intraperitoneal of 100 or 1,000 PFU of virus in 500μl of cMEM. Mice were anesthetized with isofluorane prior to sacrifice by cervical dislocation. Spleens were harvested into cMEM homogenized and filtered through a 100-μm-pore-size nylon cell strainer (Becton Dickinson). Erythrocytes were removed with red blood cell lysis buffer (Sigma). Pooled splenocytes from 4 to 10 mice were used in all experiments.

Plaque assays. Plaque assays were performed as previously described (25), with minor modifications. NIH 3T12 cells were plated onto six-well plates 1 day prior to infection at 2.5 x 10^5 cells/well. Organs were subjected to four rounds of mechanical disruption of 1 minute each using 1.0 mm zirconia/silica beads (Biospec Products) in a Mini-Beadbeater-8 (Biospec Products) Serial 10-fold dilutions of organ homogenate were plated into NIH 3T12 monolayers in a 200 μl volume and allowed to absorb for 1 h at 37^o C with rocking every 15 min. Immediately after infection, plates were overlaid with 2% methylcellulose in cMEM. After 7 d, plates were stained with a neutral red overlay, and plaques were scored the next day. The limit of detection for this assay is 50 PFU per organ.

Antibodies for flow cytometry. Cells were resuspended in PBS supplemented with 2% FCS were stained for FACS using a combination of the following antibodies: fluorescein isothiocyanate (FITC) conjugated antibodies to IgA, IgG1, IgG2a/2b, IgG3, IgE, IgD, GL7, CD45.1 (Ly5.1); phycoerythrin (PE) conjugated antibodies to CD19; Peridinin chlorophyll protein (PerCP) conjugated antibodies to CD45.2 (Ly5.2) all purchased from BD Pharmingen and allophycocyanin (APC) conjugated antibody to CD45.1 (Ly5.1) and CD45.2 (Ly5.2) purchased from eBioscience. When necessary, rat anti-mouse CD16/CD32 (Fc block) was used to block Fc receptors prior to staining.

Flow cytometry. For flow cytometry analysis, cells were resuspended at 1 x 10^6 cells/ml in PBS containing 2% FCS and prior to staining they were Fc block for 10 min. then stained with 1:100 dilution of all antibodies except PE-conjugated antibodies and FITC-

GL7 which were used at 1:200, for 20 min on ice in the dark. Cells were then washed with PBS/2% FCS and resuspened in 200 μl. Data were collected on either a FACSCaliber or LSR II (BD Biosciences) and analyzed using FloJo software (TreeStar).

For FACS, cells were resuspended at 2 x 10^7 cells/ml and incubated for 10 min in ice in PBS/1% FCS and Fc block. Cells were then stained with PE-anti-CD19, CD45.1 (Ly5.1), CD45.2 (Ly5.2) at 5 μl per 1 x 10^7 cells for 20 min on ice in the dark. Cells were then washed twice with PBS/1% FCS and resuspended at 1 x 10^8 cells/ml. Stained cell populations were then sorted by FACSVantage (BD Bioscience). Sorted populations were resuspended in cMEM supplemented with 10% dimethyl sulfoxide and stored at -80^o C for limiting-dilution PCR analysis or in cMEM at 4^o C for limiting-dilution *ex vivo* reactivation analyses as described below.

Magnetic cell separation. Murine B cells were isolated by depletion of non-B cells using the B cell isolation kit (Miltenyi Biotec). Briefly, cells were resuspended at 2 x 10^8 cells/ml in 1X PBS/0.5%FCS followed by staining with Fc block (0.125 μg/10^6 cells) on ice for 15 minutes. Cells were then labeled with biotin-antibody cocktail (biotin conjugated antibodies against CD43, CD4 and Ter-119), 10 μl/10^7 cells for 15 min on ice in the dark, followed by staining with anti-biotin microbeads, 20 μl/10^7 cells for 15 min on ice. Cells were washed twice with PBS/0.5% FCS and subjected to magnetic separation using the autoMACS (Miltenyi Biotec). Following separation, stained cell populations were collected as described earlier.

Limiting-dilution ex vivo reactivation analyses. Limiting-dilution analysis to determine the frequency of cells containing virus capable of reactivating from latency was performed as described previously (136, 139). Briefly, bulk splenocytes or sorted cell populations were resuspended in cMEM and plated in serial 2-fold dilutions (starting with 10^6 cells) onto MEF monolayers in 96-well tissue culture plates. Twelve dilutions were plated per sample, and 24 wells were plated per dilution. Wells were scored for cytopathic effect at 21d post planting. To detect preformed infectious virus, parallel samples of mechanically disrupted cells were plated onto MEF monolayers. This process kills more than 99% of live cells, which allows preformed infectious virus to be discerned from virus reactivating from latency infected cells (136, 138, 139). The level of sensitivity of this assay is 0.2 PFU (138). Unless otherwise indicated, significant levels of preformed virus were not detected in these assays.

Limiting-dilution nested-PCR detection of MHV68 genome-positive cells. The frequency of cells harboring the MHV68 genome was determined by a single-copy sensitivity nested PCR assay directed against the MHV68 ORF50 gene sequence as previously described (138, 139). Briefly, bulk splenocytes or sorted cells were thawed, counted, and resuspended in isotonic buffer. A series of six threefold serial dilutions, starting with 10^4 cells/well, were plated in a background of 10^4 uninfected NIH 3T12 cells in 96-well PCR plates. Cells were lysed prior to nested PCR by 6 h treatment at 56^o C in the presence of detergent and proteinase K. Then, 10 μl of round 1 PCR mix was added to each well. Following first round PCR, 10 μl of round 2 PCR buffer was added to each well and samples were subjected to second round of PCR. All cell lysis and PCR

were preformed on a PrimusHT thermal cycler (MWG Biotech). Products were resolved by ethidium bromide staining on 2% agarose gels. Twelve PCRs were preformed for each sample dilution, and a total of six dilutions were preformed for each sample. Every PCR plate contained control reactions (uninfected cells and ten copies, one copy and 0.1 copy of plasmid DNA in a background of 10^4 cells). All of the assays demonstrated approximately single-copy sensitivity with no false positives.

Antibody ELISA. For MHV68-specific immunoglobulin G (IgG) assay, plates were first coated with a 1% final concentration of paraformaldehyde fixed viral antigen for sample wells and standard wells were coated with 2ug/mL concentration of donkey anti-mouse IgG (Jackson ImmunoResearch) in coating buffer (0.1 M Na_2CO_3, 0.2% NaN_3, pH 9.6) and incubated at either 37^{o}C for 2hrs or 4^{o}C overnight, then plates were blocked with 3% BSA in PBS and incubated for 2hrs at37^{o}C. After washing the plates with ELISA wash buffer (200mL 10x PBS, 1mL Tween-20 add H2O to 200mLs), serum samples or mouse IgG at a concentration of 1ug/mL (Jackson ImmunoResearch) were added to each well in a 3-fold dilution in ELISA diluent (BD Bioscience) and then incubated for 3hrs at37^{o}C. Plates were then washed and horseradish peroxidase-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch) was added at 1:5,000 dilution and incubated for 2hrs at37^{o}C. Plates were washed and 100µl of 1:1 developer was added (BD Bioscience). The reaction was stopped after 10 minutes with 50µl stop solution (BD Bioscience) and plates were read on a microplate reader (Bioteck, Synergy HT). The concentration of antibody in serum samples was determined by using a standard curve generated by 3-fold serial dilutions of standard in the same assay. Plates were read using the KC4 program.

Passive antibody transfer. Blood from 3-5 naïve or infected C57Bl/6 or MyD88$^{-/-}$ mice was harvested by sub-mandibular bleed into Microtainer serum separation tubes (Becton Dickinson) at indicated times post-infection, pooled and allowed to clot for 15 minutes at room temperature. Serum was aliquoted and stored at -80^{o}C until used. One day prior to infection and 7 days after infection, 100μl of serum was transferred i.p. to each of 5 naïve C57Bl/6 mice for each group. At sixteen days post-infection splenocytes were harvested and assayed as previously described.

Generation of MyD88-mixed bone marrow chimeric mice. B6.SJL-PtprcaPepb/BoyJ wild-type (Ly5.1) mice (cat. number 002014; The Jackson Laboratory) were lethally irradiated (950 rad) in two doses of 475 rad in a 20 h interval and reconstituted with bone marrow from both MyD88$^{+/+}$ (Ly5.1) and MyD88$^{-/-}$ (Ly5.2) mice. Bone marrow cells were flushed from femurs, depleted of RBC and mixed. Recipient mice (Ly5.1) were anesthetized with 2,2,2-tribromoethanol and a total of 2 x 10^{7} cells in 100 μl PBS were injected intraocular. Mice were rested for 8 weeks to allow for reconstitution, which was confirmed by flow cytometry analysis of peripheral blood lymphocytes, before MHV68 infection. Chimeric mice received neomycin and polymix B (Sigma-Aldrich) in drinking water until reconstitution was confirmed.

Statistical analyses. All data were analyzed by using GraphPad Prism software (GraphPad Prism). Titer data were statistically analyzed with Mann-Whitney nonparametric two-tailed *t*-test. Based on Poisson distribution, the frequency of reactivation and viral genome-positive cells were obtained from nonlinear regression fit

of the data where regression line intersects 63.2%. The frequencies of reactivation and genome-positive cells were statistically analyzed by unpaired two-tailed *t*-test of the log 63.2% effective concentration.

RESULTS

B cells in MyD88$^{-/-}$ mice display a decrease in response. The TLR family plays an instructive role in innate immune response against microbial pathogens, as well as the subsequent induction of an adaptive immune response. As the humoral response is a part of the adaptive immune response we focused on changes in the B cell response in MyD88$^{-/-}$ and TLR3$^{-/-}$ mice during the time when MHV68 is establishing latency, since any alteration in the B cell response during this time might have impact MHV68 latency. MyD88 has been reported to be required for B cells activation, the germinal center reaction and class switching (93). To determine whether any of the same effects could be seen during MHV68 infection, MyD88$^{-/-}$, TLR3$^{-/-}$ and wild-type C57Bl/6 mice were infected intranasally with 1,000 PFU of wild-type MHV68. Spleens were harvested at 16, 42 and 90 dpi and labeled with the indicated phenotypic markers and analyzed by flow cytometry (Fig.1). While total B cell numbers did not differ between any of the mice, (57.9+/- 4.6% for C57Bl/6, 58.5 +/- 4.5% for MyD88$^{-/-}$ and 56.1 +/- 4.9% for TLR3$^{-/-}$), the activation level, as determined by the percentage of cells positive for expression of the activation marker CD69, was significantly reduced in MyD88$^{-/-}$ mice compared to C57Bl/6 and TLR3$^{-/-}$ at 16 dpi (Fig. 1A & 1B and Table 1).

To ascertain if MyD88$^{-/-}$ B cells were able to undergo a germinal center reaction, we assessed the presence of the germinal center markers, GL7 and CD95 (Fas), on CD19^{+} B cells. At 16 dpi MyD88$^{-/-}$ mice had a decreased frequency of germinal center B cells compared to the other mice (Fig. 1C & 1D and Table 1). Since immunoglobulin

isotype switching is associated with the development of germinal center and transition through germinal centers, we examined the frequency of class switched B cells using a cocktail of antibodies to assess surface expression of IgG, IgA and IgE. Notably, we observed that at 16 dpi MyD88$^{-/-}$ mice had a statistically significant decrease in the ability to undergo class switching compared to MHV68 infected C57Bl/6 and TLR 3$^{-/-}$ mice (Fig. 1E & 1F and Table 1). Taken together, this data demonstrates that MyD88 signaling is required for an optimal B cell response to MHV68 infection. It should be noted that this phenotype is not due to the absence of IL-1 receptor signaling, a pro-inflammatory cytokine that has MyD88 dependent signaling. Analyses of MHV68 infected IL-1R$^{-/-}$ mice at 16 dpi demonstrated that B cell activation; germinal center formation and isotype switching were similar to infected C57Bl/6 mice (Data not shown).

Development of an antibody response to MHV68 is delayed in MyD88$^{-/-}$ mice.

The observed decrease in isotype switched B cells in MHV68 infected MyD88$^{-/-}$ mice suggested a defect in antibody production. To determine the impact on the MHV68 specific antibody response, the levels of MHV68 antibody specific IgG in the serum of C57Bl/6 and MyD88$^{-/-}$ infected mice was determined by enzyme-linked immunosorbent assay (ELISA). In agreement with a published report (112) anti-MHV68 antibody was not detected during the first 20 days post-infection from either strain of mouse. However, by day 30 a MHV68-specific IgG response could be detected (Fig. 2A). As expected, at days 30 and 42 post-infection there was a significant reduction in the amount of MHV68 specific IgG in the MyD88$^{-/-}$ mice as compared to wild type C57Bl/6 (Fig. 2A). However, by day 60 and later post-infection both C57Bl/6 and MyD88$^{-/-}$ mice exhibited

similar levels of MHV68 specific IgG (Fig. 2A), indicating that the defect in the humoral immune response is transient in MyD88$^{-/-}$ mice. This data correlates with the decrease in isotype switched B cells seen in the MyD88$^{-/-}$ mice at early times post infection (Fig. 1E & 1F).

While the ELISA showed a decrease in quantity of MHV68 specific IgG, we also wondered if there was a difference in quality of MHV68 specific antibody between C57Bl/6 and MyD88$^{-/-}$ mice. To investigate whether there was a difference in the ability of C57Bl/6 and MyD88$^{-/-}$ mice antibody to give protection against a challenge infection (*in vivo* antibody neutralization assay), we injected naïve C57Bl/6 mice with serum from either C57Bl/6 or MyD88$^{-/-}$ infected harvested at days 42-45 post-infection [the time at which we observed the greatest difference in the antibody response (see Fig. 2A)] or naive mice, followed by MHV68 infection one day later. Mice were treated again with serum at day 8 post-infection. Immune serum from C57Bl/6 mice reduced the level of latency to an undetectable level in the spleen following an iintraperitoneal infection, while immune serum from MyD88$^{-/-}$ mice provided only partial protection (Fig. 2B). Naïve serum from either strain of mouse had no effect on the ability of MHV68 to establish latency (Fig. 2B). Thus, the decreased levels of MHV68 specific antibody observed in MyD88$^{-/-}$ mice at days 42-45 post-infection directly correlate with a failure to provide full protection from MHV68 infection. In contrast, serum from MyD88$^{-/-}$ and C57Bl/6 mice harvested at day 180 post-infection displayed similar abilities to protect against a challenge MHV68 infection (Fig. 2C), which was consistent with ELISA data demonstrating comparable levels of MHV68 specific IgG at this time point (Fig. 2A).

TLR signaling is dispensable for MHV68 acute replication in vivo. To evaluate the affects of TLR signaling on acute MHV68 replication, we infected MyD88$^{-/-}$, TLR3$^{-/-}$ and wild-type C57Bl/6 mice intranasally with 1,000 PFU of wild-type MHV68. At days 4 and 9 post-infection virus lung titers were determined by plaque assay. Titers in the lungs at both time points were comparable between all three types of mice (Fig. 3). Additionally, both MyD88$^{-/-}$ and TLR3$^{-/-}$ mice displayed no signs of illness after intranasal infection with 1 x 10^6 PFU for >6 months (data not shown). Despite the precedence for TLRs to be used to control productive viral infections (32, 61, 104, 148), these finding indicate that TLR signaling is dispensable for control of acute MHV68 replication of *in vivo*.

Loss of MyD88 signaling results in route of inoculation specific defects in MHV68 latency. Previous studies have shown that MHV68 latency is preferentially established in activated germinal center B cells that allow access to memory B cells (41, 106, 142). Since a decrease in this B cell population was seen in MyD88$^{-/-}$ mice, but not in C57Bl/6 or TLR3$^{-/-}$ mice, we examined if this had any effect on the ability of MHV68 to establish latency. To evaluate this, we determined the frequency of splenocytes harboring MHV68 genome employing a limiting-dilution PCR assay. Spleens from infected mice were harvested at days 16, 42 and 90 post-infection. At 16 dpi there is an ca. 10-fold reduction in the frequency of splenocytes harboring viral genome (1 in 690 for MyD88$^{-/-}$ compared to 1 in 68 for C57Bl/6 and 1 in 110 for TLR3$^{-/-}$ mice) (Figure 4A). In addition, we also analyzed the frequency of latently infected cells that are capable of reactivating from latency *ex vivo*. In MyD88$^{-/-}$ there was also an ca. 10-fold

decrease in this frequency (1 in 13,500) compared to C57Bl/6 (1 in 1,800) and TLR3$^{-/-}$ mice (1 in 2,600) (Figure 4B). This decrease in reactivation reflects the decrease in the frequency of viral genome positive cells, and thus following intranasal inoculation there does not appear to be any contribution of MyD88 to virus reactivation. Notably, MyD88$^{-/-}$ mice exhibit lower levels of latent infection despite a less effective humoral response against MHV68. Importantly, no defect in MHV68 latency was observed IL-1R$^{-/-}$ mice (Data not shown).

The defect in MHV68 latency in MyD88$^{-/-}$ mice was maintained at day 42 post-infection, with the frequency of latently infected cells dropping in wt, TLR3$^{-/-}$ and MyD88$^{-/-}$ mice (1 in 6,500 vs. 1 in 1340 in C57Bl/6 mice and 1 in 880 in TLR3$^{-/-}$ mice) (Fig. 4C). However, by 3 months post-infection all three strains of mice exhibited comparable frequencies of splenocytes harboring MHV68 genome (1 in 5,000 for C57Bl/6, 1 in 4,050 for MyD88$^{-/-}$ and 1 in 3,300 for TLR3$^{-/-}$) (Fig. 4D). The latency defect in MyD88$^{-/-}$ mice correlates with the loss in B cell activation and germinal center formation observed at the peak of MHV68 latency (see Fig. 1).

To examine whether a more permissive route of infection would alter the phenotype of MHV68 in MyD88$^{-/-}$ mice, we infected MyD88$^{-/-}$ and C57Bl/6 control mice with 1,000 PFU of virus via intraperitoneal inoculation. We assessed latency at day 18 post-infection in both peritoneal exudate cells (PECs) and splenocytes. Limiting dilution PCR analyses to determine the frequency of cells harboring viral genome revealed no defect in the establishment of MHV68 in either PECs or splenocytes (Fig. 5A & 5C). However, limiting dilution *ex vivo* reactivation analyses did reveal distinct reactivation phenotypes in PECs and splenocytes recovered from MyD88$^{-/-}$ mice. Notably, PECs

from MyD88$^{-/-}$ mice exhibited a mild hyper-reactivation phenotype (Fig. 5B), suggesting that MyD88 may play a role in suppressing virus reactivation from latently infected peritoneal macrophages. In contrast, we observed a significant defect in virus reactivation from latently infected splenocytes recovered from MyD88$^{-/-}$ mice (Fig. 5D). This is particularly notable since we did not observe an additional defect in reactivation following MHV68 infection via intranasal inoculation of MyD88$^{-/-}$ mice – only a defect in establishment of latency (see Fig. 4). This may reflect route specific differences in the splenic B cell populations harboring virus at day 16, and points out the potential importance of assessing multiple routes of infection in the characterization of MHV68 pathogenesis.

Inhibition of NF-κB signaling in MHV68 infected MyD88$^{-/-}$ mice leads to a further reduction in viral latency. NF-κB is a key component in TLR signaling; many genes induced by TLRs are NF-κB dependent. To discern if the defect in establishing latency in MyD88$^{-/-}$ splenic B cells can be attributed solely to the absence of NF-κB signaling modulated by MyD88, we infected MyD88$^{-/-}$ mice with a recombinant MHV68 virus expressing a mutant form of IκBα (IκBαM), that functions as a constitutive repressor of NF-κB activation (17, 71). As we previously observed in normal immunocompetent mice (71), upon infection with 1,000 PFU via intranasal inoculation we observed that acute virus replication in the lungs was unaffected by inhibiting NF-κB activation (Fig. 6A). However, when we assessed the frequency of latently infected splenocytes at day 16 post-infection we observed that inhibiting NF-κB activation further exacerbated the latency phenotype in MyD88$^{-/-}$ mice. MyD88$^{-/-}$ mice infected with the

control marker rescue virus (MHV68-IκBαM.MR) exhibited the expected frequency of latently infected cells (1 in 760), while MyD88$^{-/-}$ mice infected with the recombinant MHV68 expressing the IκBαM super-repressor (MHV68-IκBαM.1) had a frequency of viral genome positive cells of <1 in 10,000 (Fig. 6B). Thus, while we cannot rule out a role for NF-κB activation driven by MyD88 playing a role in the phenotype of MHV68 in B cells, there are clearly other pathways involved in activating NF-κB during the establishment of latency that are critical for MHV68 latency.

MyD88$^{+/+}$/MyD88$^{-/-}$ mixed bone marrow chimeric mice are able to control acute MHV68 replication, but MyD88-/- B cells in these mice retain the defects in B cell differentiation and establishment of MHV68 latency. To determine if the defect in B cell response is intrinsic to MyD88 signaling in B cells or other cell types, we generated MyD88$^{+/+}$/MyD88$^{-/-}$ mixed bone marrow chimeric mice. Lethally irradiated C57Bl/6 (Ly5.1) mice were constituted with a mixture of wild-type (Ly5.1) and MyD88$^{-/-}$ (Ly5.2) bone marrow cells. Eight weeks after generating the chimeras, and prior to MHV68 infection, chimerism was verified by FACS analyses of peripheral blood (Fig. 7A). As expected, based on the replication of MHV68 in MyD88$^{-/-}$ mice, the chimeric mice were able to control acute replication with comparable kinetics to MyD88$^{+/+}$ wild type and Ly5.2 MyD88$^{-/-}$ mice after a 1,000 PFU intranasal infection (Fig. 7B). Importantly, this demonstrated that the bone marrow transplants had appropriately reconstituted these chimeric mice.

We subsequently assessed whether the defective B cell response observed in the MyD88$^{-/-}$ mice also occurred in the MyD88$^{-/-}$ B cells of the mixed bone marrow chimeras.

The ability of the MyD88$^{+/+}$ B cells and MyD88$^{-/-}$ B cells to respond to infection was analyzed by flow cytometry at days 16 and 42 post-infection. Notably, MyD88$^{-/-}$ B cells from the chimeric mice exhibited the previously observed defect in their ability to respond to virus infection (Fig. 8). At 16 dpi they displayed a decrease in the frequency of MyD88$^{-/-}$ B cells that were activated when compared to MyD88$^{+/+}$ B cells (Fig. 8A and Table 2). The ability to undergo a germinal center reaction was also diminished for MyD88$^{-/-}$ B cells (Fig. 8B and Table 2). Finally, the ability of MyD88$^{-/-}$ B cells to class switch was also decreased compared to MyD88$^{+/+}$ B cells (Fig. 8C and Table 2). These results are entirely consistent with the B cell responses observed following MHV68 infection of MyD88$^{-/-}$ mice, giving support to the hypothesis that the observed defect is intrinsic to the MyD88$^{-/-}$ B cells and not the consequence of some other defect in the MyD88$^{-/-}$ mice.

The observed defect in the MyD88$^{-/-}$ B cell response in the MyD88$^{+/+}$/MyD88$^{-/-}$ mixed bone marrow chimeric mice strongly suggested that there would also be a defect in MHV68 latency in the MyD88-null B cells. To assess the impact of MyD88 on B cell latency in the chimeric mice, we separated splenocytes by magnetic separation into B cell and non-B cell populations [purity of the B cell population was >94% (data not shown)], followed by flow cytometry to further sort the purified B cell population into Ly5.1 and Ly5.2 populations [>97% purity for each population (data not shown)]. The MyD88$^{+/+}$ B cells populations harbored the expected frequency of latently infected cells at both days 16 and 42 post-infection (Fig. 9A & 9C). In contrast, the MyD88$^{-/-}$ B cells exhibited an ca. 10-fold lower frequency of latently infected cells at day 16 and an ca. 5-fold lower frequency of at day 42 post-infection (Fig. 9A & 9C). Finally, the expected decrease in

the frequency of B cells reactivating virus was observed in the purified MyD88$^{-/-}$ B cells – which could be accounted for by the decreased frequency of latently infected cells (Fig. 9B). These results very closely recapitulate the results obtained in MHV68 infected MyD88$^{-/-}$ mice, and furthermore are consistent with the analyses of B cell responses in the MyD88$^{+/+}$/MyD88$^{-/-}$ chimeric mice (see Fig 8). Therefore, this data shows that MyD88 plays a role in enhancing the ability of MHV68 to establish latency in the memory B cell compartment.

DISCUSSION

Here we report several findings regarding a novel role for MyD88 signaling during MHV68 infection: (i) TLR signaling is dispensable during acute MHV68 replication; (ii) MyD88 signaling is required for the generation of an optimal B cell response during MHV68 infection, which correlated with defects in B cell latency; (iii) MyD88$^{-/-}$ mice displayed a significant delay in the appearance of a robust MHV68-specific IgG response; (iv) the MHV68 latency phenotype observed in the MyD88$^{-/-}$ mice does not appear to be solely due to the loss of NF-κB signaling, as the phenotype of a recombinant MHV68 expressing a super-repressor of NF-κB was substantially different in MyD88-sufficient and MyD88-deficient mice; and (v) using mixed bone marrow chimeras we have shown that the defective B cell response to MHV68 infection, as well as the defects observed in MHV68 latency, are intrinsic to the MyD88$^{-/-}$ B cells. Taken together, these data demonstrate that MyD88 signaling in B cells plays an important role in the formation of a B cell response during MHV68 infection, as well as in the establishment of MHV68 latency – although both defects are transient, being ameliorated by 3 months post-infection.

The absence of a role for TLR signaling in controlling acute MHV68 replication contrasts with results obtained with some other viruses (e.g., MCMV and LCMV) where TLR signaling has been reported to play an important role (32, 148). During MHV68 infection other pathways, most notably type I interferons (8, 36), may be the primary mechanisms involved in controlling acute replication. This does not rule out an as yet

undiscovered PRR or other innate immune mechanisms being involved in controlling MHV68 replication.

There is accumulating, albeit controversial, evidence that MyD88-dependent TLR signaling directly on B cells is critical for their full activation (49, 93). MyD88$^{-/-}$ B cells during MHV68 infection display decreased activation, germinal center participation and, as a consequence of this, a decrease in isotype switching. However, as we have noted, this impairment in the B cell response appears to be transient - a phenotype that is so far is unique to MHV68. MyD88 signaling is required for the formation of a long-term humoral response to polyoma virus (52). The latter is not due to a lack of IL-1 or IL-18 receptor signaling, which signal through MyD88, as IL-1R$^{-/-}$ and IL-18R$^{-/-}$ mice did not display a defect (52). During an influenza infection, MyD88 via TLR7 signaling fine-tunes the anti-influenza B cell response (55). These reports, in conjunction with our studies, highlight a role for MyD88 signaling in the formation of an optimal B cell response to viral infections.

Early in MHV68 infection, latency is primarily found in proliferating B cells bearing markers characteristic of B cells participating in germinal center reactions (41, 106, 142). Characterization of splenocytes recovered from MHV68 infected MyD88$^{-/-}$ mice, following intranasal inoculation, revealed a decrease in latency at 16 and 42 dpi. This was not seen in TLR3$^{-/-}$ mice which signaling through TRIF not MyD88, illustrating that this is a MyD88-dependent phenomenon. Notably, the latency defect in MyD88$^{-/-}$ mice was not absolute, indicating that other pathways that activate B cells, such as CD40-CD40L interaction, direct activation through the BCR, or a direct infection of memory B cells (a pathway that may be independent of B cell activation) may contribute to the pool

of latently infected splenocytes. The reduction in splenic latency in MyD88$^{-/-}$ mice could reflect, in part, a role for this host cell factor in mediating inflammatory cytokine production or up-regulation of homing molecules to the spleen by infected cells that could lead to alterations in the recruitment, activation, and/or subsequent trafficking of infected cells to the spleen. MyD88 signaling may also be required for proliferation or survival signals upon entering the germinal center.

Following intraperitoneal inoculation, MHV68 did not require MyD88 signaling for the establishment of latency in either PECs or the spleen. However, unlike the intranasal route of inoculation, virus reactivation from splenocytes was significantly impaired in MyD88-deficient mice following intraperitoneal inoculation, suggesting route-specific differences in the latently infected cell populations and/or their activation state. At this point, little is known about how latency is seeded to the spleen following intranasal inoculation, although studies in B cell-deficient mice clearly demonstrate a critical role for B cells. With respect to latency in PECs, macrophages are the major cell type harboring latent MHV68 in PECs. As such, the observed hyper-reactivation phenotype suggests that MyD88 signaling plays a role in suppressing MHV68 reactivation from latently infected macrophages.

MyD88$^{-/-}$ mice also displayed slower kinetics in the development of a MHV68-specific IgG response. Notably, the slow appearance of a robust IgG response correlated with decreased MHV68 latency at days 16 and 42 post-infection post-intranasal inoculation, suggesting that MyD88 signaling early in MHV68 infection is involved in activating B cells which is both beneficial to the humoral immune response as well as establishment of MHV68 latency. Consistent with this observation, serum recovered

from MyD88$^{-/-}$ mice at day 42 post-infection also displayed a decrease in ability to protect against establishment of MHV68 latency following challenge with wt MHV68 compared to serum harvested from MHV68 C57Bl/6 at the same time point. On the other hand, serum recovered at day 180 post-infection, a time point at which similar levels of MHV68-specific IgG was present in both strains of mice, was equally efficient in neutralizing establishment of MHV68 latency *in vivo*. This result underscores the transient nature of the defective B cell response in MyD88$^{-/-}$ mice, indicating that ultimately other activation pathways over-come the loss MyD88 function. NF-κB signaling is one of the major activation pathways downstream of TLRs signaling. Notably, we have previously demonstrated that NF-κB activation is required for proper establishment of MHV68 latency *in vivo* (71). As such, we investigated whether the primary requirement for MyD88 involved NF-κB activation using a recombinant MHV68 harboring a dominant inhibitor of NF-κB activation. Somewhat unexpectedly this analysis revealed no amelioration of the phenotype observed with the MHV68-IκBαM recombinant virus (compared to the marker rescue virus) in MyD88$^{-/-}$ mice. This suggests that either the required MyD88 activation of NF-κB in B cells occurs prior to MHV68 infection and expression of the NF-κB super-repressor, or that an alternative activation pathway driven by MyD88 is involved in modulating MHV68 latency.

Experiments with mixed bone marrow chimeric mice provide a direct demonstration that the decrease in the response of MyD88$^{-/-}$ B cells is intrinsic to these B cells. The defect in B cell response was preserved in these mice in that there is a selective failure of MyD88$^{-/-}$ B cells to become activated and enter the germinal centers. When presented with both MyD88$^{+/+}$ and MyD88$^{-/-}$ B cells in the same mouse, MHV68

preferentially establishes latency in the MyD88$^{+/+}$ B cells. It is known that MHV68 infection results in polyclonal activation of B cells, characterized by an up-regulation of the CD69 activation marker and an increase in nonspecific antibody production - a process that is dependent on CD4+ T cells (82, 101, 113). A reduction in CD69 activation has been correlated to both a decrease in splenomegaly and splenic latency (10, 16, 31, 71). This phenomenon was seen in both the global MyD88 knockout mice, as well as in the MyD88$^{+/+}$/MyD88$^{-/-}$ mixed bone marrow chimera mice. Nonspecific B cell activation might represent a mechanism by which the virus drives B cell participation in germinal center reactions to increase the likelihood of gaining access to the long-lived memory B cell reservoir, and MyD88 signaling may play a role in the activation and subsequent proliferation of B cells. In a mixed CD40$^{+/+}$/CD40$^{-/-}$ bone marrow chimeric mice both CD40-deficient and CD40-sufficient B cells were found to harbor viral genome at 14 dpi at similar frequencies, although ultimately latency in the CD40-deficient B cells waned at later times post-infection (68). In contrast, the MyD88$^{+/+}$/MyD88$^{-/-}$ mixed bone marrow chimera mice exhibit a defect in the establishment of latency at 16 dpi, suggesting that activation of MyD88 signaling plays an important role in addition to, and perhaps prior to, the engagement of infected cells with CD40L in germinal center reactions.

The events involved in the activation of B cells, the transition of activated B cells into germinal center cells and succeeding formation of memory B cells are not completely understood. This requirement of MyD88 in the establishment of MHV68 latency may define a unique differentiation step in the process of activation and transition of B cells into the germinal center and then to memory B cells. Alternatively, MyD88

may be required for the expansion, survival or migration of B cells during an immune response. Ultimately, MHV68 may exploit MyD88 signaling to gain access to memory B cells, its long-term latency reservoir (141).

MyD88 is not only involved in TLR signaling, but also in both IL-1 and IL-18 receptor signaling. While IL-1R mediated signaling is essential during mycobacterium tuberculosis infection (45), as well as playing a role in controlling H5N1 influenza infection (119), neither IL-1R nor IL-18R signaling is necessary to maintain long-term antibody production to polyoma virus (52). We show here that IL-1R$^{-/-}$ mice display no defect in B cell response to MHV68, nor any detectable changes in the frequency of MHV68 latently infected cells. Thus, it does not appear that IL-1R signaling is involved in the observed processes. However, it remains a formal possibility that several MyD88 dependent receptors are involved in the generation of the B cell response to MHV68, and as such a defect in only one may not impair formation of the humoral response.

In summary, we have shown here that MyD88 signaling is critical in the formation of a B cell response to MHV68, as well as efficient establishment of viral latency. Our studies demonstrate that during MHV68 infection, B cells lacking MyD88 have a defective early B cell response and that this leads to a decrease in the early establishment of latency. Bone marrow chimeras illustrate that the role for MyD88 signaling is an intrinsic requirement of B cells. Finally, our study provides supporting evidence to the hypothesis that MHV68 benefits from a normal B cell response to infection to gain access to the long-lived memory B cell compartment and establishment of long-term latency.

Acknowledgements

This work was supported by NIH R01 CA95318. SHS is also supported by NIH R01 grants CA52004, CA58524, CA87650, and AI58057.

Much thanks to Andrea Siegel for help with the generation of the bone marrow chimeric mice, members of the Speck lab for all of their helpful discussions, Robert Karaffa for flow cytometry, Andrew Gerwirtz for the gift of the MyD88$^{-/-}$ mice and Shivaprakash Gangappa for help with the MHV68-specific ELISA.

Figure 1

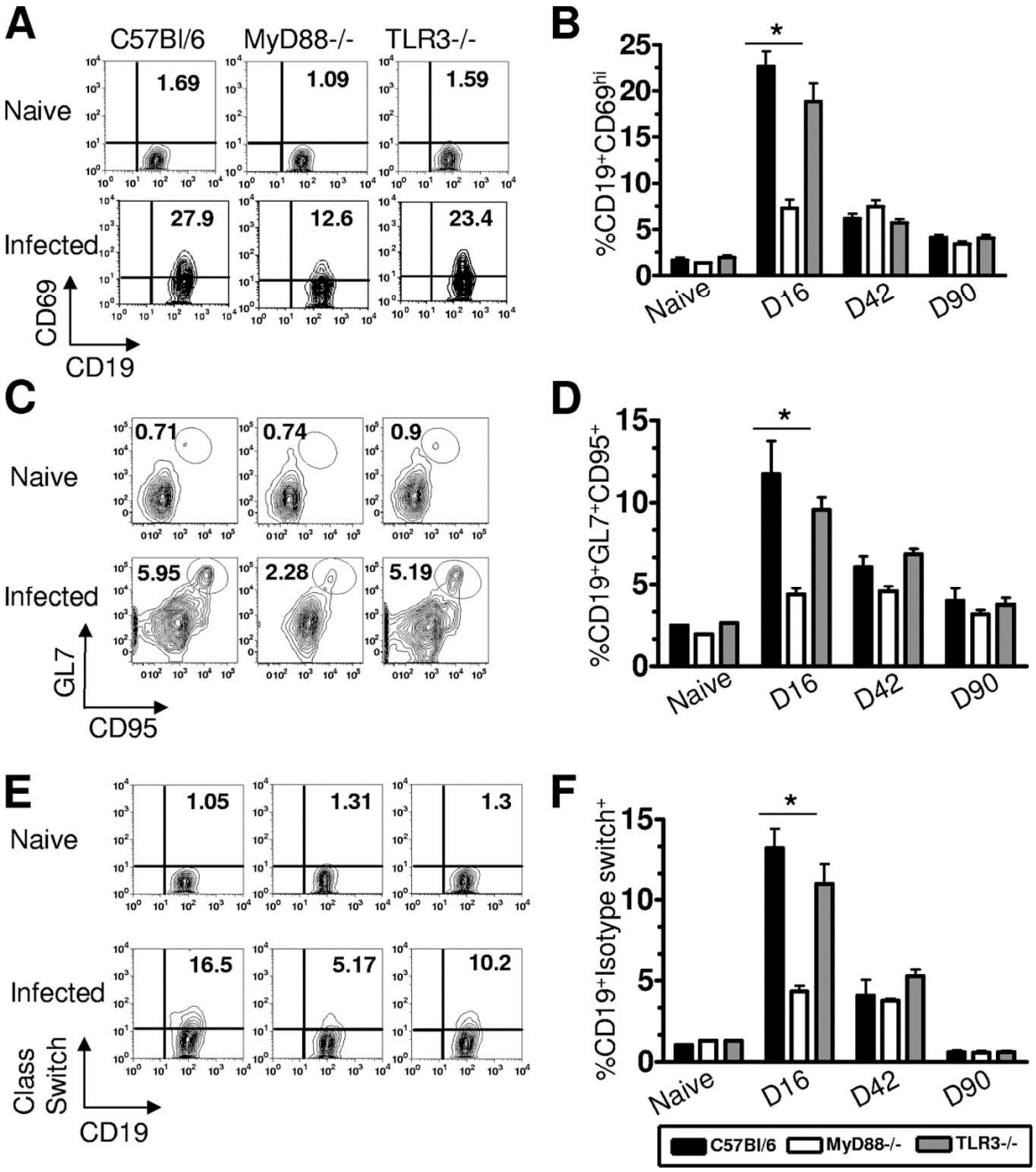

A
C57Bl/6
MyD88-/-
TLR3-/-
Naive
Infected
1.69
1.09
1.59
27.9
12.6
23.4
CD69
CD19
B
%CD19+CD69hi
Naive
D16
D42
D90
C
0.71
0.74
0.9
5.95
2.28
5.19
GL7
CD95
D
%CD19+GL7+CD95+
E
1.05
1.31
1.3
16.5
5.17
10.2
Class Switch
F
%CD19+Isotype switch+
C57Bl/6
MyD88-/-
TLR3-/-

Figure 2

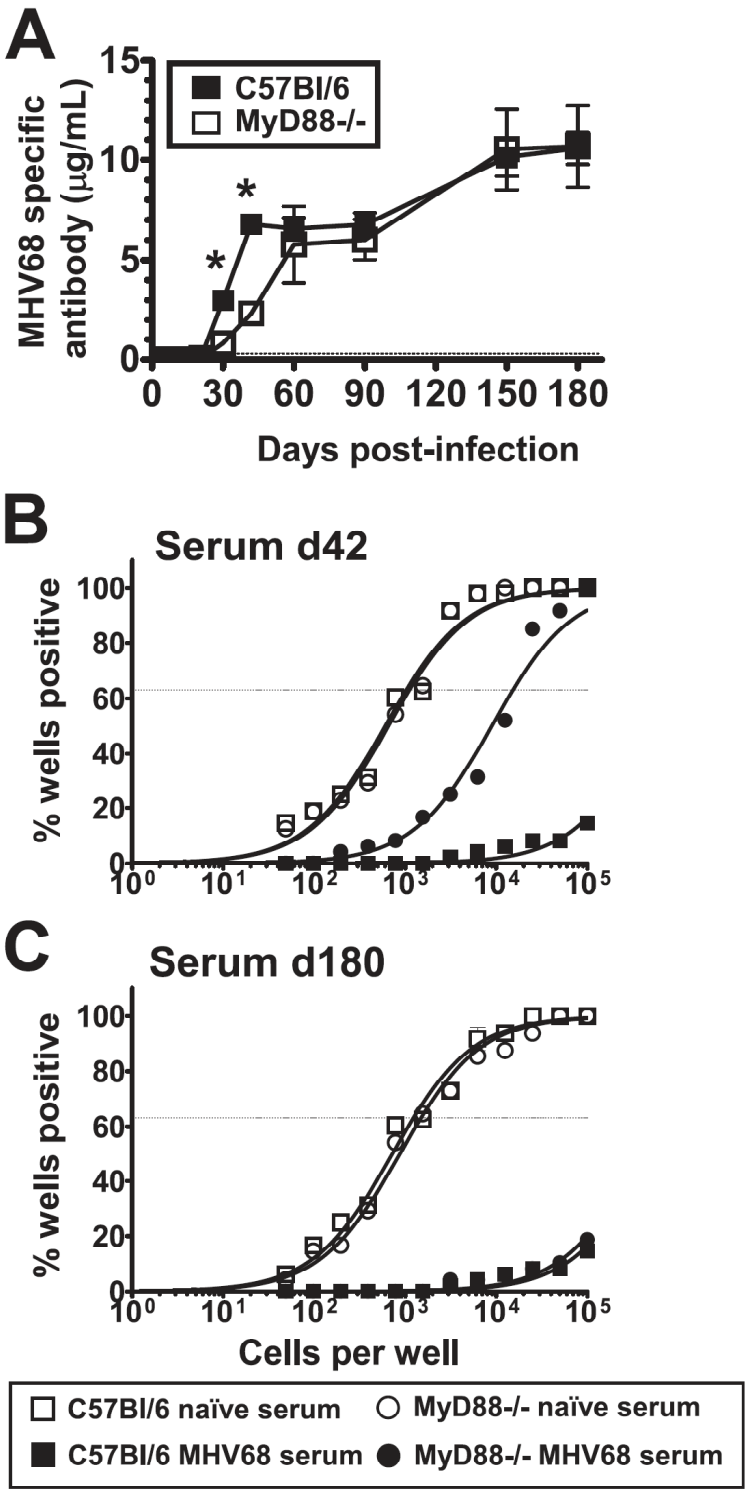

A
MHV68 specific antibody (μg/mL)
C57Bl/6
MyD88-/-
*
*
Days post-infection
B
Serum d42
% wells positive
C
Serum d180
% wells positive
Cells per well
C57Bl/6 naïve serum
MyD88-/- naïve serum
C57Bl/6 MHV68 serum
MyD88-/- MHV68 serum

Figure 3

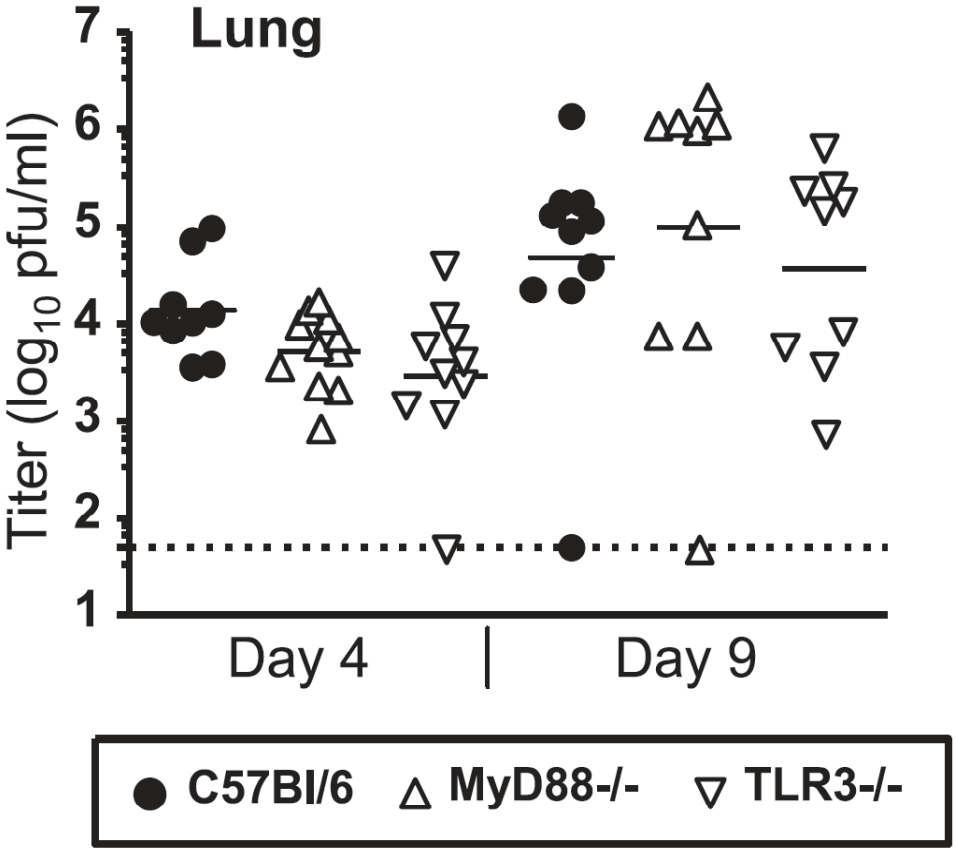


Figure 4

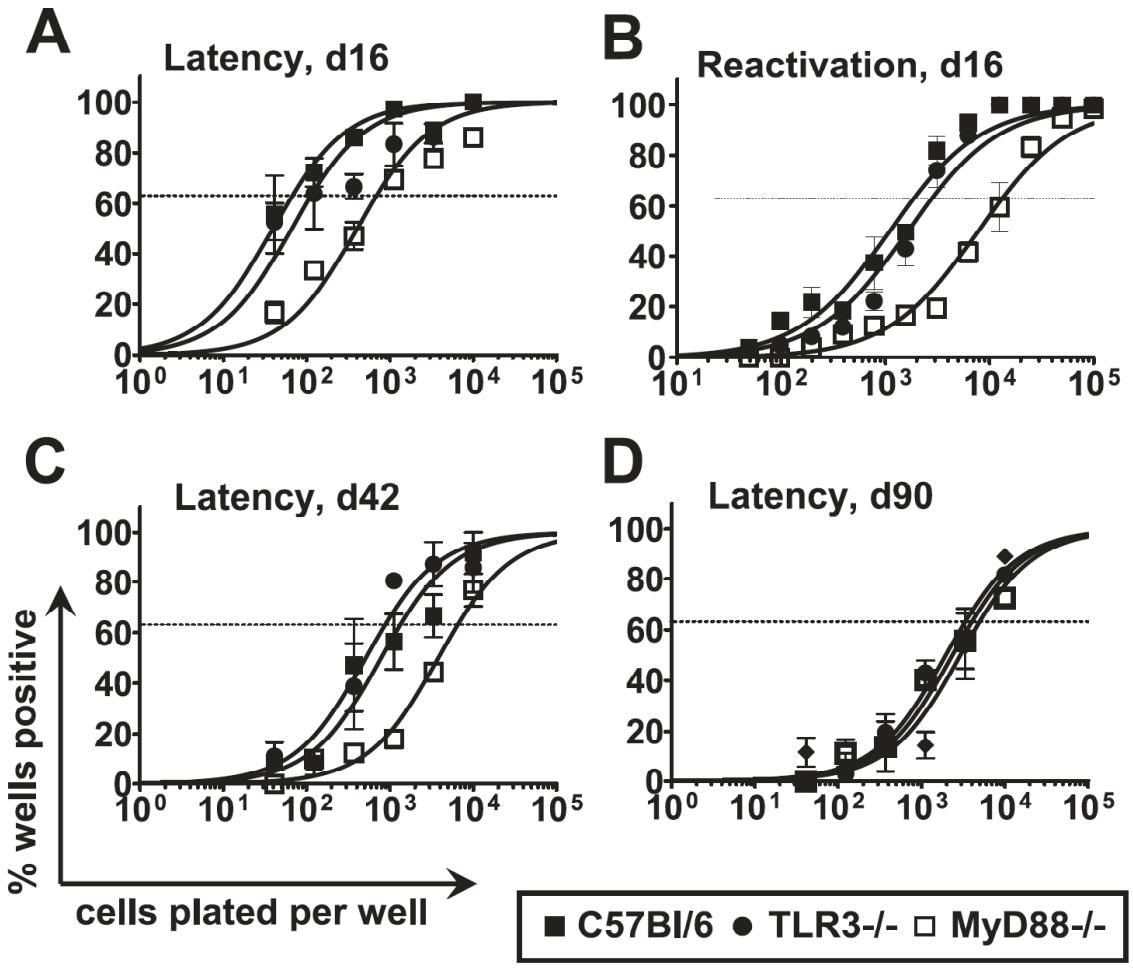

A
Latency, d16
B
Reactivation, d16
C
Latency, d42
D
Latency, d90
% wells positive
cells plated per well
C57Bl/6
TLR3-/-
MyD88-/-

Figure 5

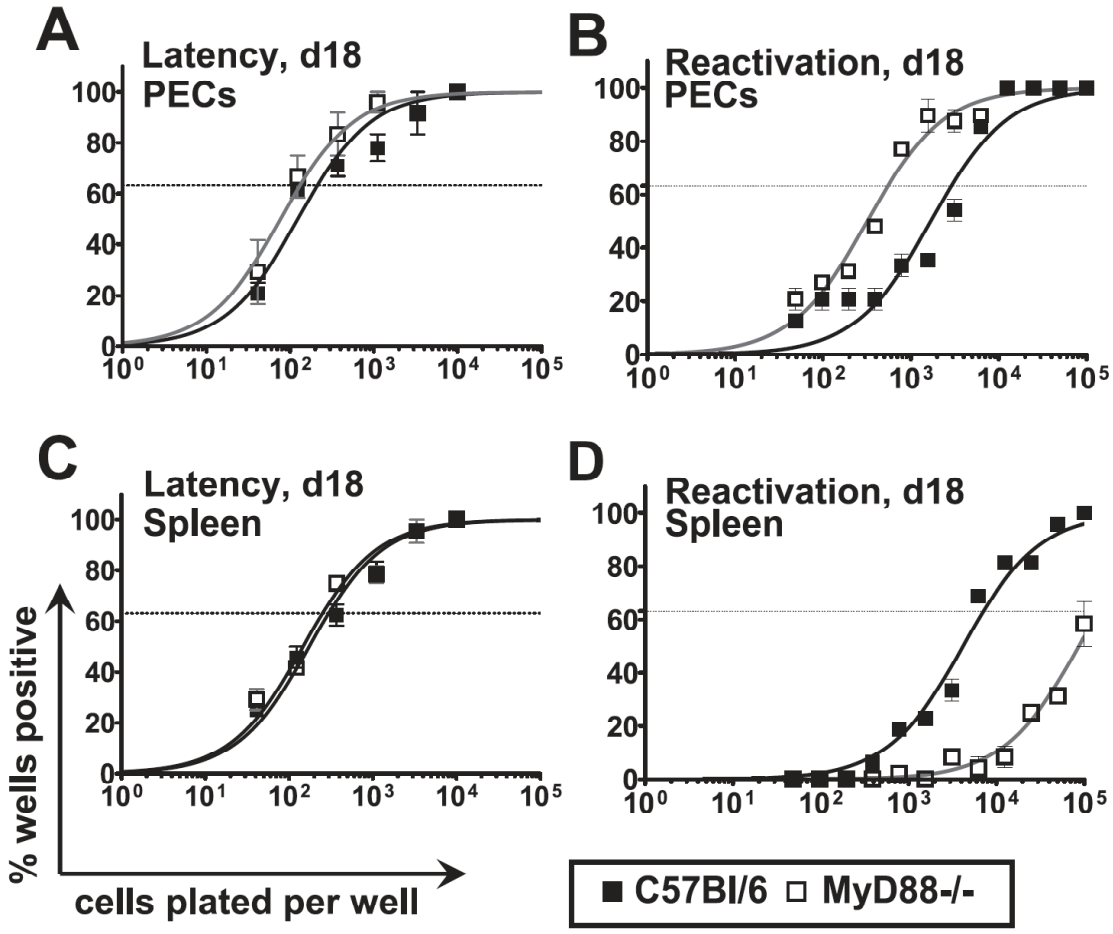

A
Latency, d18
PECs
B
Reactivation, d18
PECs
C
Latency, d18
Spleen
D
Reactivation, d18
Spleen
% wells positive
cells plated per well
C57Bl/6
MyD88-/-

Figure 6

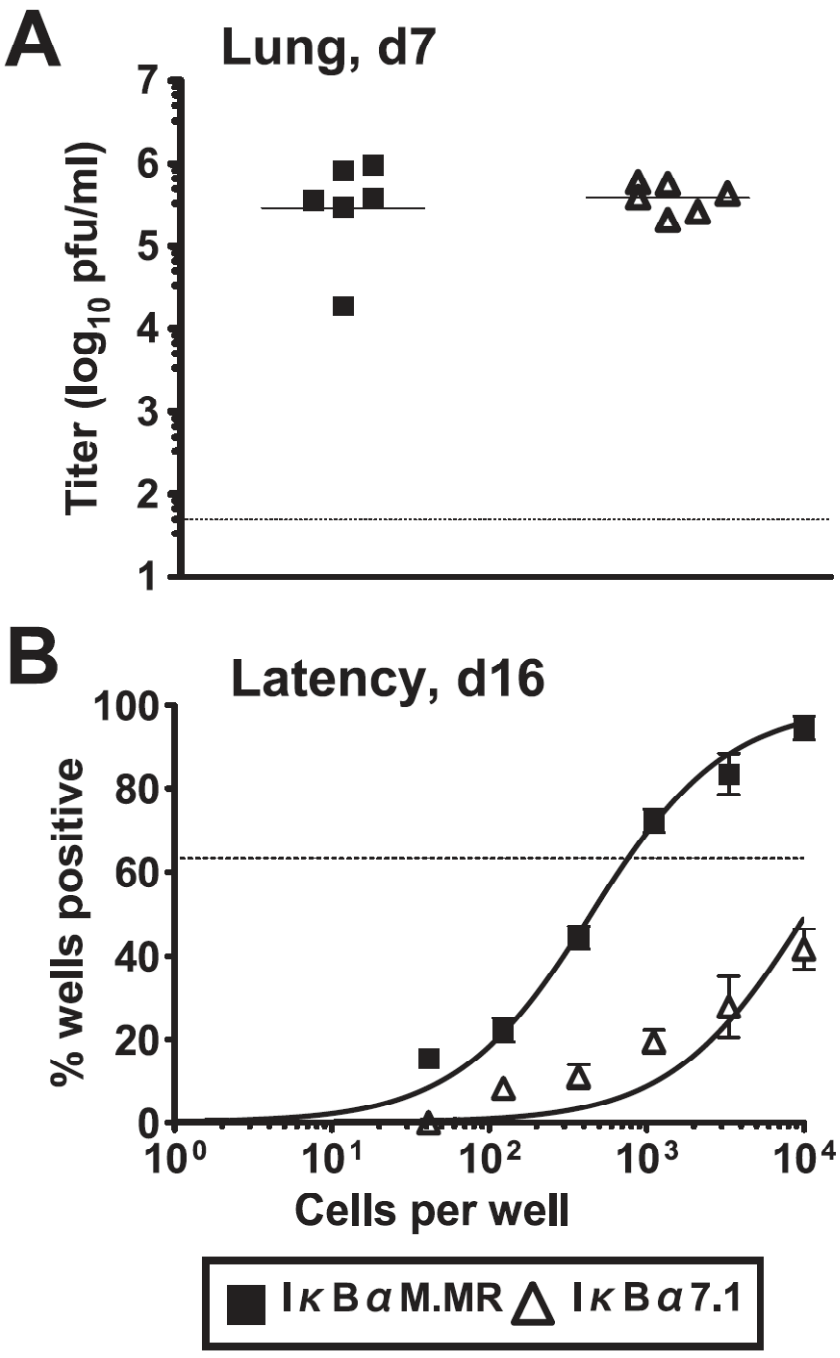

A
Lung, d7
Titer (log10 pfu/ml)
7
6
5
4
3
2
1
B
Latency, d16
% wells positive
100
80
60
40
20
0
10^0
10^1
10^2
10^3
10^4
Cells per well
IκBαM.MR
IκBα7.1

Figure 7

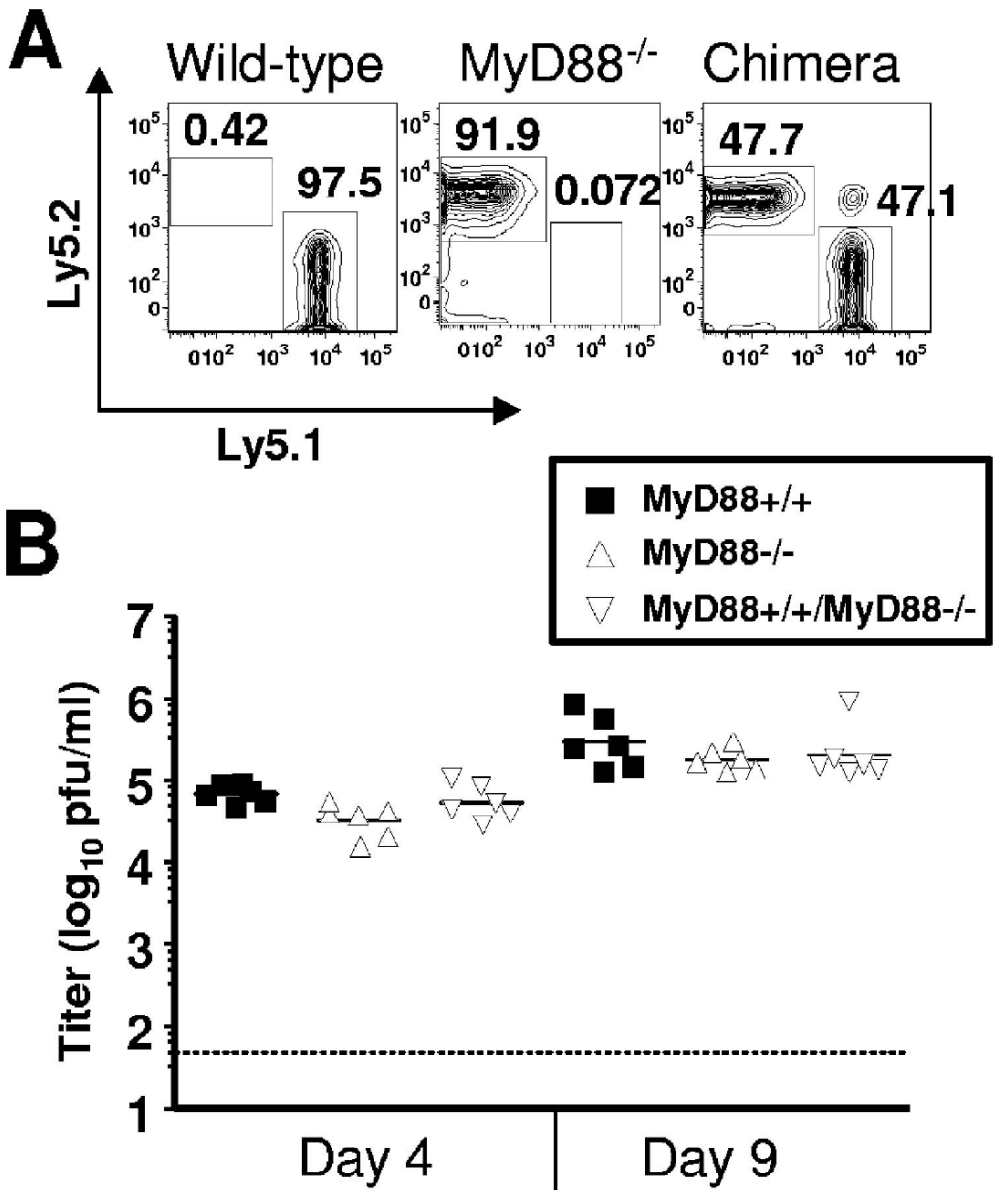

A
Wild-type
MyD88-/-
Chimera
0.42
97.5
91.9
0.072
47.7
47.1
Ly5.2
Ly5.1
B
MyD88+/+
MyD88-/-
MyD88+/+/MyD88-/-
Titer (log10 pfu/ml)
Day 4
Day 9

Figure 8

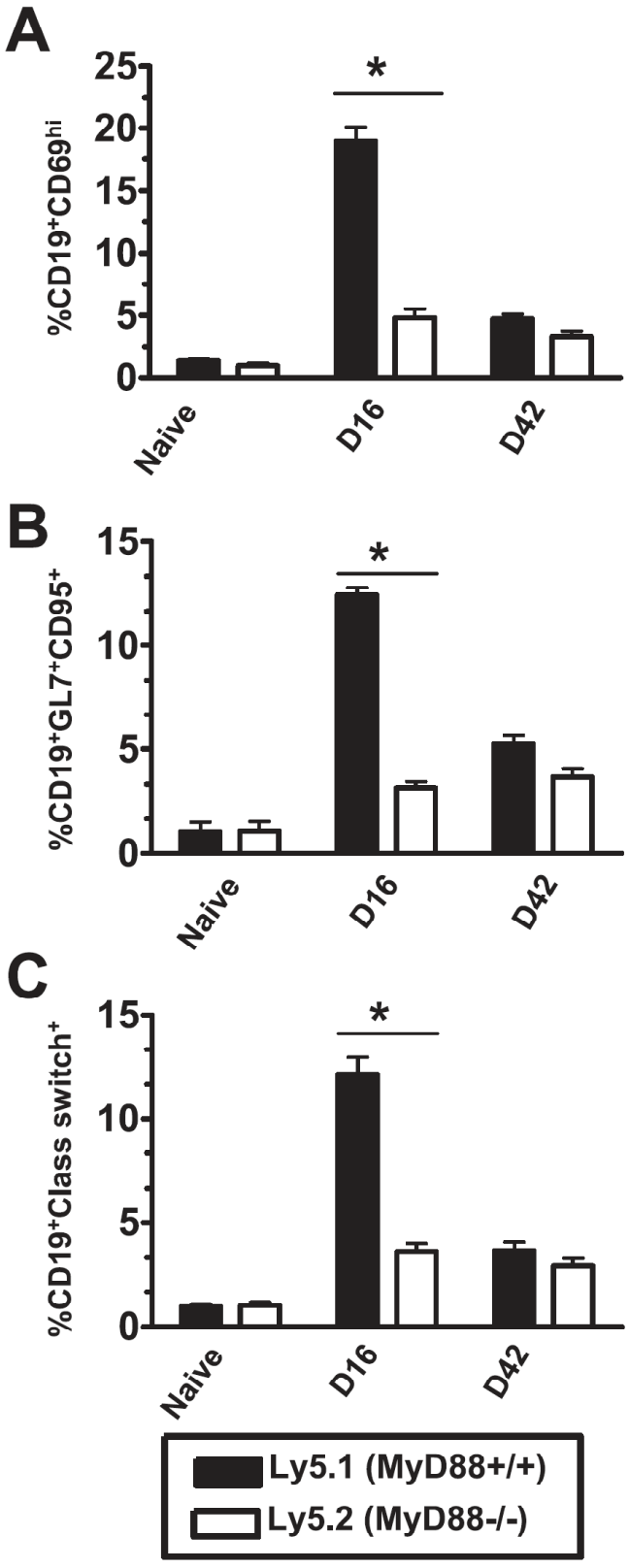

A
%CD19+CD69hi
25
20
15
10
5
0
*
Naive
D16
D42
B
%CD19+GL7+CD95+
15
10
5
0
*
Naive
D16
D42
C
%CD19+Class switch+
15
10
5
0
*
Naive
D16
D42
Ly5.1 (MyD88+/+)
Ly5.2 (MyD88-/-)

Figure 9

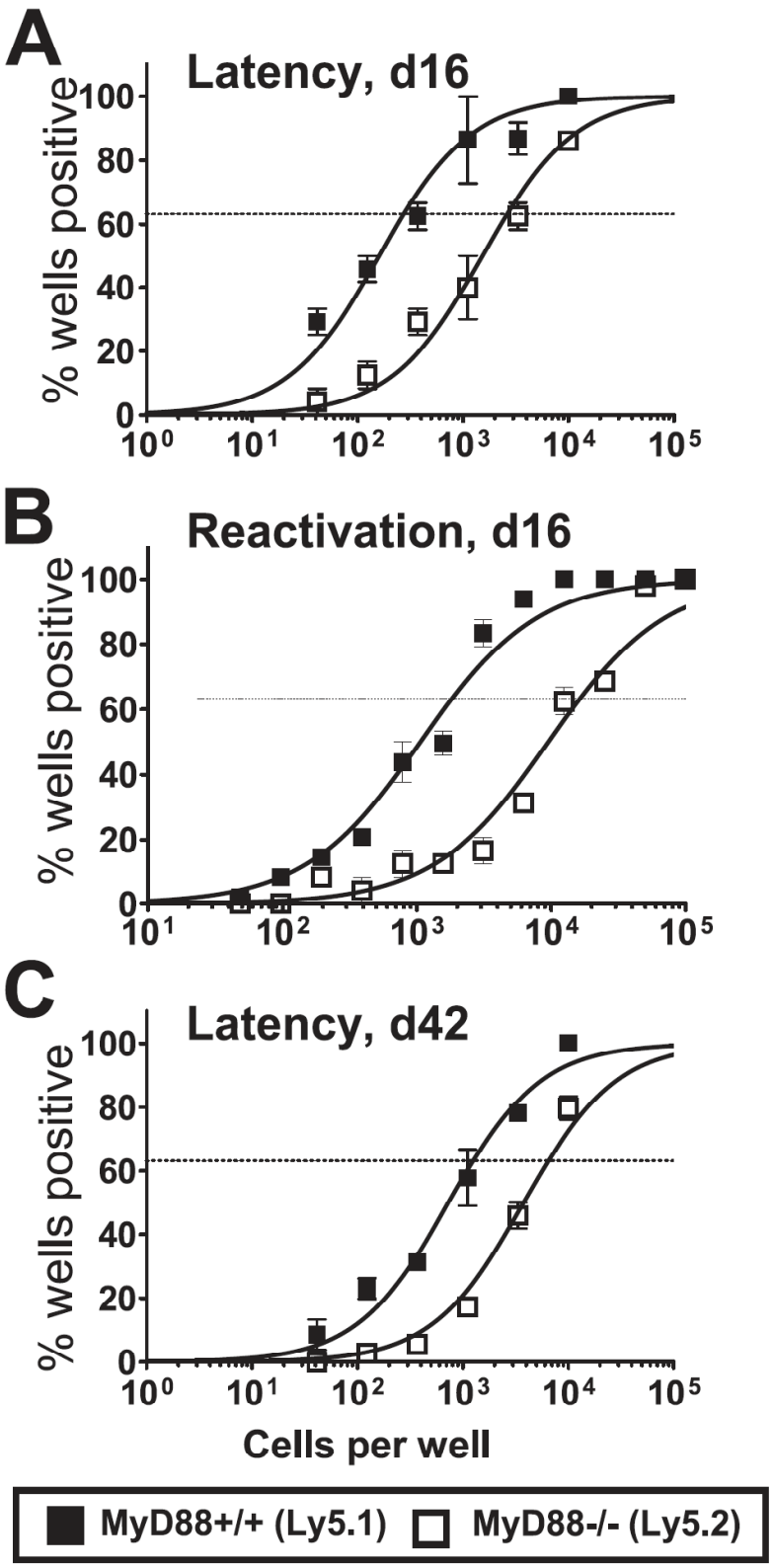

A
Latency, d16
% wells positive
100
80
60
40
20
0
10^0
10^1
10^2
10^3
10^4
10^5
B
Reactivation, d16
% wells positive
100
80
60
40
20
0
10^1
10^2
10^3
10^4
10^5
C
Latency, d42
% wells positive
100
80
60
40
20
0
10^0
10^1
10^2
10^3
10^4
10^5
Cells per well
MyD88+/+ (Ly5.1)
MyD88-/- (Ly5.2)

TABLE 1. Analysis of B-cell Phenotype by Surface Antigen Markers in Infected Mice

Mouse strain [a]	dpi	$CD19^{+b,c}$	$CD19^{+}CD69^{hi}$	$CD19^{+}$ $GL7^{+}CD95^{+}$	$CD19^{+}$ $IsotypeSwitch^{+}$
C57BL/6	16	57.9 (4.6)	22.7 (4.6)	11.7 (4.5)	13.2 (2.9)
$MyD88^{-/-}$	16	58.5 (4.5)	7.3 (3.3)	4.4 (1.1)	4.7 (0.9)
$TLR3^{-/-}$	16	56.1 (4.9)	18.8 (6.4)	9.6 (2.0)	11.0 (3.6)
C57BL/6	42	50.5 (2.1)	6.2 (1.8)	6.1 (1.2)	4.1 (1.7)
$MyD88^{-/-}$	42	52.6 (6.8)	7.5 (2.7)	4.6 (0.74)	3.7 (0.30)
$TLR3^{-/-}$	42	54.2 (6.0)	5.7 (1.4)	6.8 (0.65)	5.3 (0.86)
C57BL/6	90	52.7 (3.5)	4.1 (0.83)	4.0 (1.6)	0.59 (0.23)
$MyD88^{-/-}$	90	55.9 (4.2)	3.4 (1.0)	3.2 (0.48)	0.56 (0.22)
$TLR3^{-/-}$	90	58.9 (5.7)	4.0 (1.3)	3.7 (0.84)	0.62 (0.16)

[a] C57BL/6, $MyD88^{-/-}$ and $TLR3^{-/-}$ mice infected with 1,000 PFU intranasal.

[b] Cell subsets were derived from initial FACS gating on live lymphocyte populations of cell preparations from spleens. The data shown are the mean percentage values for each subset determined from FACS analysis of four to six individual mice in triplicate. Numbers in parenthesis represent +/- standard deviation.

[c] Cell subsets were derived from initial gating on $CD19^{+}$ cells and then further fractionated into the indicated subsets using a additional surface markers as indicated.

Table 2. Analysis of B-cell Phenotype by Surface Antigen Markers in Infected $MyD88^{+/+}MyD88^{-/-}$ Chimera Mice

Cell population [a]	dpi	$CD19^{+}CD69^{hi}$ [b,c]	$CD19^{+}$ $GL7^{+}CD95^{+}$	$CD19^{+}$ Isotype $Switch^{+}$
$MyD88^{+/+}$	16	18.9 (4.8)	12.5 (1.5)	12.1 (3.6)
$MyD88^{-/-}$	16	4.8 (3.2)	3.2 (1.3)	3.6 (1.6)
$MyD88^{+/+}$_	42	4.7 (1.4)	5.2 (1.3)	3.7 (1.5)
$MyD88^{-/-}$	42	3.3 (1.9)	3.7 (1.2)	2.9 (1.5)

[a] Mice were infected with 1000 PFU of wild type virus by intranasal inoculation.

[b] Cell subsets were derived from initial FACS gating on live lymphocyte populations of cell preparations from spleens. The data shown are the mean percentage values for each subset determined from FACS analysis of four to ten individual mice in triplicate. Numbers in parenthesis represent +/- standard deviation.

[c] Cell subsets were derived from initial gating on $CD19^{+}$ cells and then further fractionated into the indicated subsets using a additional surface markers as indicated.

FIGURE LEGENDS

Figure 1. Development and phenotype of the B cell response is attenuated in MyD88$^{-/-}$ mice. Cells were prepared from spleens harvested at indicated time points from 4-6 mice per group that were infected with 1,000 PFU i.n, as well as 2-3 naïve uninfected mice. Representative flow cytometry plots from stained cells. Values shown in the upper right quadrant are the percentages of CD19^{+} cells that express CD69 activation marker (panel A), germinal center markers CD95 (Fas) and GL7 (panel C) and class switch by a cocktail of antibodies (IgA, IgE, IgG1, IgG2a/b and IgG3 (panel E) Bar graph of absolute cell numbers of CD19^{+}CD69^{+} (panel B), CD19^{+}GL7^{+}Fas+ (panel D), and CD19^{+} Isotype switch (panel F), from all mice for that time point. Error bars represent standard deviation between individual mice. The percentage of activation, germinal center and class switched B cells from infected MyD88$^{-/-}$ mice were significantly different from infected C57Bl/6 at 16 dpi, $p = 0.0324$, $p = 0.0005$ and $p = 0.0359$, respectively.

Figure 2. MyD88$^{-/-}$ mice have a decrease in MHV68 specific antibody response. Blood was taken from 3-5 mice for each of the indicated groups (panel A). Serum from infected C57Bl/6 and MyD88$^{-/-}$ mice was analyzed using a MHV68-specific ELISA to detect the presence of anti-viral antibodies. The difference in antibody titers determined at 30 and 42 dpi were statistically significant p=0.0012 and p=0.0002, respectively. Naïve serum from both strains of mice had undetectable levels of MHV68 specific IgG (data not shown). Pooled serum from 42-45 dpi (panel B) or 180 dpi (panel C) was used to

immunize C57Bl/6 mice, followed by MHV68 infection and analysis of virus reactivation from latency at day 16 post-infection.

Figure 3. Acute replication in the lungs is unaffected in MyD88$^{-/-}$ and TLR3$^{-/-}$ mice. C57Bl.6, MyD88$^{-/-}$ and TLR3$^{-/-}$ mice were inoculated with 1,000 PFU intranasally with wild-type MHV68. Lungs were harvested at 4 and 9 dpi. . The results were compiled from two independent experiments with 4-6 mice per group. Virus titers in lungs were determined by plaque assay on NIH 3T12 monolayers. Each point represents the virus titer from an individual mouse. The solid bar represents the geometric mean and the dashed line represents the limit of detection of the assay (50 PFU).

Figure 4. Splenic latency is markedly reduced in MyD88$^{-/-}$ mice. Spleens were harvested from mice that were infected with 1,000 PFU wild-type MHV68 intranasally. Frequency of bulk splenocytes harboring viral genomes at 16 dpi (panel A), 42 dpi (panel C) and 3 months dpi (panel D) as determined by limiting dilution PCR. Frequency of cells that have reactivating virus at 16 dpi (panel B).

Figure 5. MHV68 reactivation from latently infected PEC is enhanced by loss of MyD88. PECs and spleens were harvested from mice that were infected with 1,000 PFU wild-type MHV68 via intraperitoneal inoculation. Frequency of bulk PECs (panel A) and splenocytes (panel C) harboring viral genomes at 18 dpi were determined by limiting dilution PCR (Materials and Methods). The frequency of PECs (panel B) and splenocytes

(panel D) reactivating virus upon explant at day 18 post-infection was determined using a limiting dilution ex vivo reactivation assay (Materials and Methods).

Figure 6. Decrease in B cell response and MHV68 latency observed in MyD88$^{-/-}$ mice cannot be solely attributed to a lack of NF-κB signaling. MyD88$^{-/-}$ mice were infected with 1,000 PFU of MHV68-IκBαM.1 or MHV68-IκBαM.MR via intranasal inoculation. At day 7 post-infection, lungs were harvested and titered on NIH 3T12 fibroblasts. The data shown represent two independent experiments of 3 mice per group (panel A). Frequency of splenocytes harboring viral genome, compiled from 3 independent experiments each containing 4-6 mice per group (panel B).

Figure 7. MyD88$^{+/+}$/MyD88$^{-/-}$ mixed bone marrow chimeric mice were successfully reconstituted and able to control acute lytic replication in the lungs. Mice were bled by submandibular bleed and PBMC were examined for reconstitution Wild-type are Ly5.1 MyD88$^{-/-}$ are Ly5.2 and chimeras express both markers, Ly5.1 and Ly5.2 (panel A). Shown is a representational plot. After reconstitution was confirmed, mice were infected with 1,000 PFU wild type MHV68 intranasally and lungs were harvested. Viral titers were determined by plaque assay on NIH 3T12 monolayer as previously described (panel B).

Figure 8. In MyD88$^{+/+}$MyD88$^{-/-}$ chimeric mice there is a selective failure of MyD88$^{-/-}$ B cells to differentiate. Cells were prepared from spleens harvested at indicated time points from 5-10 mice per group that were infected with 1,000 PFU i.n, as well as 5 naïve

uninfected mice. Bar graph of absolute cell numbers of $CD19^{+}CD69^{+}$ (panel A), $CD19^{+}GL7^{+}CD95+$ (panel B), and $CD19^{+}$ Isotype switch (panel C), from $MyD88^{+/+}$ (Ly5.1) and $MyD88^{-/-}$ (Ly5.2) cells from $MyD88^{+/+}$ /$MyD88^{-/-}$ chimeric mice for that time point. Error bars represent standard deviation between individual mice. Asterisks indicate difference between $MyD88^{-/-}$ and $MyD88^{+/+}$ B cells are statistically significant, for CD69 expression ($p < 0.0001$), germinal center ($p < 0.0001$), and class switch ($p < 0.0001$) for 16 dpi.

Figure 9. In $MyD88^{+/+}MyD88^{-/-}$ chimeria mice, MHV68 latency is preferentially established in $MyD88^{+/+}$ B cells. At the indicated times intranasal infection of $MyD88^{+/+}MyD88^{-/-}$ chimeric mice, 4-10 spleens were pooled and sorted for Ly5.1 $MyD88^{+/+}$ B cells or Ly5.2 $MyD88^{-/-}$ B cells. Frequency of MHV68 latency is determined by limiting-dilution PCR at 16 (panel A) and 42 (panel C) dpi. MHV68 reactivation from latency (panel B) as determined by scoring for CPE on MEF monolayers (Materials and Methods).

Chapter III

Signaling Through Toll-like Receptors Induces Murine Gammaherpesvirus 68 Reactivation In Vivo

Gargano, LM, Forrest, JC, Speck, SH. *J. Virol.* 2008;83(3):1474-82.

ABSTRACT

Murine gammaherpesvirus 68 (MHV68) establishes a life-long infection in mice and is used as a model pathogen to study the role of viral and host factors in chronic infection. Maintenance of chronic MHV68 infection, at least in some latency reservoirs, appears to be dependent on the capacity of the virus to reactivate from latency *in vivo*. However, the signals that lead to MHV68 reactivation *in vivo* are not well characterized. Toll-like receptors (TLRs), by recognizing specific patterns of microbial components, play an essential role in the activation of innate immunity. In the present study we investigated the capacity of TLR ligands to induce MHV68 reactivation – both *in vitro* and *in vivo*. Stimulation of latently infected B cell lines with ligands for TLRs 3, 4, 5, and 9 enhanced MHV68 reactivation; e*x vivo* stimulation of latently infected primary splenocytes, recovered from infected mice, with poly I:C, LPS, flagellin, or CpG DNA led to early B cell activation, B cell proliferation, and a significant increase in the frequency of latently infected cells reactivating virus. *In vivo* TLR stimulation also induced B cell activation and MHV68 reactivation, resulting in heightened levels of virus replication in the lungs which correlated with an increase in MHV68-specific $CD8^+$ T cell responses. Importantly, TLR stimulation also led to an increase in MHV68 latency, as

evidenced by an increase in viral genome positive cells 2 weeks post-*in vivo* stimulation by specific TLR ligands. Thus, these data demonstrate that TLR stimulation can drive MHV68 reactivation from latency and suggests that periodic pathogen exposure may contribute to homeostatic maintenance of chronic gammaherpesvirus infection through stimulating virus reactivation and reseeding latency reservoirs.

INTRODUCTION

Gammaherpesviruses are characterized by their capacity to establish life-long latent infection within host lymphocytes. Virus reactivation is thought to be necessary for transmission of the virus to new hosts and may also be required to maintain reservoirs of latently infected cells in the chronically infected host (21, 51, 72, 126, 127). The switch between latency and the lytic cycle for the human gammaherpesviruses, Epstein-Barr virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV), has been extensively characterized in established latently infected cell lines *in vitro* (50, 108, 140). Initiation of the EBV lytic cycle can be stimulated by several different reagents, including anti-immunoglobulin (anti-Ig), calcium ionophore, sodium butyrate, and tetradecanoyl phorbol acetate (TPA) (108). KSHV reactivation can also be induced by stimulation with phorbol esters and sodium butyrate (22, 85).

Toll-like receptors (TLRs) are important pattern recognition receptors (PRR) in innate immunity. Following TLR engagement by ligands of microbial origin, dendritic cells undergo maturation, which in turn, activates the adaptive immune response. Pathogen-associated molecular patterns (PAMPs) recognized by TLRs can come from bacteria, fungi, protozoans, insects or viruses. TLR1/2 and 2/6 recognize bacterial components (e.g., lipoproteins) and components from yeast (e.g., zymosan) (92, 122-125), TLR4 recognizes LPS (98) and flagellin is recognized by TLR5 (54). TLRs that sense viral PAMPs include TLR3 recognition of double-stranded RNA (5), TLR7 and TLR8 (33, 56, 57, 65, 78) recognition of single-stranded RNA and TLR9 sensing of unmethylated CpG DNA (26, 58, 70, 77).

Engagement of TLR ligands, as well as heterologous viral infections, can trigger reactivation of latent infections. Signaling through TLRs 2, 4, or 9 enhance viral replication from HIV-1 latently infected mast cells (115). LPS can also induce KSHV reactivation from BCBL-1 cells (87) and reactivation of latent murine cytomegalovirus (MCMV) (27). HIV-1 infection of primary effusion lymphoma cell lines, BC-3 and BCBL-1, triggers KSHV reactivation (84, 135). Cytomegalovirus (CMV) superinfection of cell lines latently infected with EBV (BJAB-B1 and P3HR-1) induce reactivation of EBV (7).

Although EBV and KSHV reactivation have been extensively studied in tissue culture, little is known regarding triggers of gammaherpesvirus reactivation *in vivo*. Murine gamma-herpesvirus 68 (MHV68) is closely related to EBV and KSHV. Infection of mice with MHV68 provides a tractable small-animal model in which to study the pathogenesis of gamma-herpesviruses *in vivo*. Ectopic expression of the MHV68 *orf*50 gene product, RTA (the lytic transactivator), in an MHV68 latently infected B lymphoma cell line, S11, can drive expression of both early and late viral genes and production of lytic virus (133, 143). We have previously demonstrated that, like EBV and KSHV, TPA and anti-Ig can stimulate MHV68 reactivation from MHV68 latently infected B cell lines. In addition, we have shown that *ex vivo* stimulation of latently infected splenocytes with anti-Ig/anti-CD40. as well as LPS stimulation, enhances MHV68 reactivation (89).

In the present study we investigated the role of TLR stimulation in inducing MHV68 reactivation. We demonstrate that stimulation of MHV68 latently infected B cell lines with ligands for TLRs 3, 4, 5, and 9 increases output viral titer. *Ex vivo* stimulation of primary splenocytes latently infected with MHV68 with these same TLR ligands led to

B cell activation, proliferation, and an increase in the frequency of cells reactivating virus. Lastly, administration of LPS or CpG DNA *in vivo* at 42 days post-infection (dpi) triggered an increase in MHV68 reactivation, early B cell activation and $CD8^+$ T cell responses. MHV68 reactivation in the spleen was accompanied by an increase virus replication in the lungs. Importantly, the increase in MHV68 reactivation/replication lead to an increase in the frequency of MHV68 latently infected splenocytes 14 days post-stimulation.

MATERIALS AND METHODS

Cells, virus and virus culture. MHV68 strain WUMS (ATCC VR1465) was used for all virus infections unless indicated. Virus passage, maintenance, and titers were performed as previously described (25). NIH 3T12 cells and mouse embryonic fibroblast (MEF) cells were maintained in Dulbecco's modified Eagle's medium supplemented with 100 U penicillin/ml, 100 mg streptomycin/ml, 10% fetal calf serum, and 2mM L-glutamate (cMEM). Two independently derived hygromycin-EGFP MHV68 latently infected cell lines, A20-HE1 and A20-HE2 cells (44) and A20 were maintained in RPMI supplemented with 100 U penicillin/ml, 100 mg streptomycin/ml, 10% fetal calf serum and 2mM L-glutamate, for the A20-HE cells were maintained with 20ng/ml hygromycin and 300μg/ml B. sulfate in the media. Cells were maintained at 37^{o} C in a 5% CO_2 environment. MEF cells were prepared from C57Bl/6 as previously described (97).

Mice, infections and organ harvests. 6-8 week old C57Bl/6 mice (Jackson Laboratory, Maryland) were housed and bred in the Whitehead Building vivarium at Emory University School of Medicine in accordance with all federal, university, and facility regulations. Mice were placed under isofluorane anesthesia prior to intranasal inoculation with 1,000 PFU of virus in 20 μl of cMEM. Mice were anesthetized with isofluorane prior to sacrifice by cervical dislocation. Spleens were homogenized in cMEM and filtered through a 100-μm-pore-size nylon cell strainer (Becton Dickinson). Erythrocytes were removed with red blood cell lysis buffer (Sigma, St. Louis, MO).

Splenocytes from 3 to 5 mice were used in all experiments. Lungs were harvested intact into cMEM and stored at -80^{o}C.

A20-HE cell stimulation. A20-HE cells were resuspended at 1x10^{6}/ml of RPMI complete and stimulated for 24 hours with either 20ng/ml PMA, 0.5μg/ml Pam3CSK4, 65μl for 6x10^{7} cells, 10μg/ml poly I:C, 1μg/ml LPS, 600μg/ml flagellin, 0.5μg/ml FLS-1, 0.5μg/ml ssRNA or 1μM CpG (Invivogen). After 24 hours supernatants were harvested and freeze/thawed 3 times prior to plaque assay.

Plaque assays. Plaque assays were performed as previously described (25), with minor modifications. NIH 3T12 cells were plated onto six-well plates 1 day prior to infection at 2.5 x 10^{5} cells/well. Serial 10-fold dilutions of supernatants were plated into NIH 3T12 monolayers in a 200 μl volume and allowed to absorb for 1 h at 37^{o} C with rocking every 15 min. Immediately after infection, plates were overlaid with 2% methylcellulose in cMEM. After 7 d, plates were stained with a neutral red overlay, and plaques were scored the next day. The limit of detection for this assay is 50 PFU/ml.

Flow cytometry. For flow cytometry analysis, cells were resuspended at 1 x 10^{6} cells/ml in PBS containing 2% FCS and prior to staining they were Fc blocked with rat anti-mouse CD16/CD32 for 10 min. Then stained with a combination of the following antibodies fluorescein isothiocyanate (FITC) conjugated antibodies to CD69; phycoerythrin (PE) conjugated antibodies to CD19, CD44 and CD62L; Peridinin chlorophyll protein (PerCP) conjugated antibodies to CD8 and CD4; PE-Cy7 conjugated

antibodies to CD69 and CD11a; PacificBlue conjugated antibodies to CD8, all purchased from BD Pharmingen, and allophycocyanin (APC) conjugated antibody to CD8 purchased from eBioscience. Tetramer to $ORF6_{487-495}$ ($H\text{-}2D^b$) were synthesized at the NIH Tetramer Core Facility at Emory University and conjugated to streptavidin-APC (Molecular Probes) according to core protocol. Antibodies were used at a 1:100 dilution of all antibodies except PE- and PE-Cy7-conjugated antibodies which were used at 1:200, for 20 min on ice in the dark. Cells were then washed with PBS/2% FCS and resuspened in 200 μl. Data were collected on a LSR II (BD Biosciences) and analyzed using FloJo software (TreeStar).

Limiting-dilution ex vivo reactivation analyses. Limiting-dilution analyses to determine the frequency of cells spontaneously reactivating from latency upon explant, was performed as described previously (136, 139). Briefly, bulk splenocytes were resuspended in cMEM and plated in serial 2-fold dilutions (starting with 10^6 cells) onto MEF monolayers in 96-well tissue culture plates. Twelve dilutions were plated per sample, and 24 wells were plated per dilution. Wells were scored for cytopathic effect at day 21 post-plating. To detect preformed infectious virus, parallel samples of mechanically disrupted cells were plated onto MEF monolayers. This process kills more than 99% of live cells, which allows preformed infectious virus to be discerned from virus reactivating from latency infected cells (136, 138, 139). The level of sensitivity of this assay is 0.2 PFU (138). Unless otherwise indicated, significant levels of preformed virus were not detected in these assays.

Limiting-dilution nested-PCR detection of MHV68 genome-positive cells. The frequency of cells harboring the MHV68 genome was determined using a limiting dilution analysis coupled with a single-copy sensitivity nested PCR assay to detect the MHV68 *orf*50 gene sequence, as previously described (138, 139). Briefly, bulk splenocytes were thawed, counted, and resuspended in isotonic buffer. A series of six threefold serial dilutions, starting with 10^4 cells/well, were plated in a background of 10^4 uninfected NIH 3T12 cells in 96-well PCR plates. Cells were lysed prior to nested PCR by 6 h treatment at 56^o C in the presence of detergent and proteinase K. Then, 10 μl of round 1 PCR mix was added to each well. Following first round PCR, 10 μl of round 2 PCR buffer was added to each well and samples were subjected to second round of PCR. All cell lysis and PCR were performed on a PrimusHT thermal cycler (MWG Biotech). Products were resolved by ethidium bromide staining on 2% agarose gels. Twelve PCRs were performed for each sample dilution, and a total of six dilutions were performed for each sample. Every PCR plate contained control reactions (uninfected cells and ten copies, one copy and 0.1 copy of plasmid DNA in a background of 10^4 cells). All of the assays demonstrated approximately single-copy sensitivity with no false positives.

Ex vivo stimulation of latently infected splenocytes. Splenocytes were harvested from day 42 infected mice. 10^6 cells were stimulated with media alone, 20ng/ml TPA, 0.5μg/ml Pam3CSK4, 65μl for $6x10^7$ cells, 10μg/ml poly I:C, 1μg/ml LPS, 600μg/ml flagellin, 0.5μg/ml FLS-1, 0.5μg/ml ssRNA or 1μM CpG DNA. The stimulated splenocytes were labeled with 1μM carboxyfluorescein diacetate succinimidyl ester (CFSE) and harvested at different times to assay cellular proliferation, or were plated into

the limiting-dilution *ex vivo* reactivation assay, in the presence or absence of TLR ligands.

In vivo administration of TLR ligands. C57Bl/6 mice were infected with 1,000 PFU intranasally and at 42 days post-infection were injected intraperitoneally with either 200μl PBS, 15μg LPS or 20μg CpG DNA in 200 μl PBS. Splenocytes and lungs were then harvested at days 1, 3, 7 and 14 post-stimulation and processed as described above.

Measurement of viral persistence in the lung. Persistent MHV68 replication in the lungs was measured using a modified form of the limiting-dilution *ex vivo* reactivation assay described above. Briefly, the left lung from each animal was mechanically disrupted as described previously (134). This disrupts >99% of the cells present, but has a less than a two-fold effect on the titer of the preformed infectious virus (136). The homogenate was plated in a two-fold dilution series onto MEFs in 96-well tissue culture plates - 12 dilutions of 16 replicate wells were plated. The appearance of CPE was monitored microscopically and read 21 days post-plating. Individual lungs from 3 to 5 mice were used in all experiments.

Statistical analyses. All data were analyzed by using GraphPad Prism software (GraphPad Prism). Viral titer data was statistically analyzed using the Mann-Whitney nonparametric two-tailed *t*-test. Based on Poisson distribution, the frequency of reactivation and viral genome-positive cells were obtained from nonlinear regression fit of the data where the regression line intersects 63.2%. The frequencies of reactivation

and viral genome-positive cells were statistically analyzed by unpaired two-tailed *t*-test of the log 63.2% effective concentration.

RESULTS

B cell stimulation by TLR ligands drives MHV68 reactivation from latently infected B cell lines. To determine whether TLR ligands can trigger MHV68 reactivation, we used murine A20 B cells latently infected with MHV68 (A20-HE cell lines) which we have previously shown are responsive to a number of reactivation stimuli (44). Two independently derived A20-HE cells lines were treated with different TLR ligands or PMA as a positive control. The supernatants from untreated and treated A20-HE cells were recovered at 24 hours post-induction and assayed for changes in virus production by plaque assay on murine NIH 3T12 fibroblasts (Fig. 1A). To control for the possible toxic effects of these treatments on NIH 3T12 fibroblasts, a parallel series of treatments of the MHV68-negative parental murine A20 B cell line was carried out. Importantly, supernatants from treated A20 cells were also plated onto monolayers of NIH 3T12 cells and no cytopathic effect was detected under any of the treatment conditions (data not shown). Stimulation of A20-HE cells with PMA resulted in strong induction of virus reactivation (Fig. 1A). In addition, stimulation with the TLR ligands poly I:C (TLR3), LPS (TLR4), flagellin (TLR5) and CpG DNA (TLR9) also elicited substantial increases in virus reactivation/replication (Fig. 1A). It should be noted that we performed RT-PCR using intron-spanning, gene-specific primers for mouse TLRs 1-9 and found that the A20-HE cells express all of these TLRs (data not shown). While a failure to respond could be a true lack of response it may also be due to experimental setup. Thus, we conclude that these data demonstrate that stimulation through a subset of TLRs can promote MHV68 reactivation from latently infected B cell lines.

HIV infection can induce KSHV reactivation (84, 135). It has also been shown that TLR7 can recognize HIV ssRNA (9, 56, 83). Although both occurring on the same cell has not been demonstrated, we wanted to further investigate the role of TLR7 signaling and MHV68 reactivation. To be sure that the concentration of ssRNA we used to stimulate the A20-HE cells would be sufficient to induce MHV68 reactivation we performed a dose-response with ssRNA (Fig. 1B). We found that varying the concentrations from .25μg to 10μg made no difference in the inability of TLR7 stimulation to induce MHV68 reactivation (Fig. 1B).

TLR stimulation leads to early B cell activation and proliferation, which correlates with an increase in virus reactivation from primary splenocytes. B cell receptor-dependent stimulation of MHV68 latently infected splenocytes leads to an increase in B cell activation and proliferation that correlates with viral reactivation (89). To investigate the impact of TLR stimulation, we harvested splenocytes from mice 42 dpi after intranasal infection. Notably, by 42 dpi there is barely detectable spontaneous virus reactivation from splenocytes detected in our limiting dilution *ex vivo* reactivation assay (128). We focused our analysis on those TLR ligands that generated the largest stimulation of virus reactivation from the A20-HE cell lines (Fig. 1). The TLR ligands poly I:C, LPS, flagellin and CpG DNA were added to bulk splenocytes and B cell activation and proliferation examined over several days post-stimulation. Within 24 hours post-stimulation a significant number of the B cells were activated, as evidenced by an up-regulation of CD69 compared to unstimulated control cells (Fig. 2A). We also assessed proliferation by CFSE labeling cells. CFSE is a dye that labels cells by

incorporation into the cell and with each cell division leads to dilution of the dye in daughter cells. The CFSE labeled cells were stimulated with TLR ligands and analyzed by flow cytometry for loss of CFSE staining at several times points post-stimulation. At 24 hours post-stimulation, there was very little evidence of B cell proliferation (Fig. 2B, open histogram). However, by 120 hours post-stimulation, all the B cells present in the cultures had proliferated to varying extents with those cultures treated with TLR ligands exhibiting greater proliferation than the unstimulated culture (Fig. 2B, shaded histogram). These data indicate that TLR stimulation is effective in activating B cells and driving them to proliferate.

To asses whether TLR-dependent B cell activation and proliferation had any impact on MHV68 reactivation from splenocytes, we recovered splenocytes 42 dpi and plated them in a limiting-dilution reactivation assay either with media alone, or with the addition of either PMA, poly I:C, LPS, flagellin or CpG DNA. Notably, e*x vivo* stimulation with PMA and or the TLR ligands resulted in a substantial increase in the frequency of splenocytes reactivating from latency (Fig. 3). Since the readout of the *ex vivo* reactivation assay is the presence of CPE on monolayers of mouse embryo fibroblasts (MEFs), we also assessed whether any of these treatments alone caused the appearance of CPE on monolayers of MEFs. Notably, none of the treatment conditions used led to detectable CPE on the MEF monolayers (data not shown). Furthermore, qPCR analysis of MHV68 genomes demonstrated an increase in viral genomes at 48 and 72 hours following LPS or CpG DNA stimulation (data not shown). Taken together, these findings demonstrate that *ex vivo* TLR stimulation induces B cell activation, proliferation and enhanced MHV68 reactivation from latency.

TLR4 and TLR9 stimulation in vivo drives B cell activation and increased virus reactivation/replication. We next tested the capacity of TLR agonists to induce B cell activation *in vivo*. For these analyses we used LPS and CpG DNA, the two most potent ligands for driving reactivation *ex vivo*. Also, LPS is a known B cell mitogen and CpG DNA directly triggers B cell activation (69). Mice were intranasally infected with 1,000 PFU intranasally and at 42 dpi the mice received intraperitoneal (i.p) administration of either PBS, 15μg LPS or 20μg CpG DNA. Splenocytes were harvested at different times post-stimulation and analyzed by flow cytometry for B cell activation by an up-regulation of CD69 expression. We observed that similar to *ex vivo* stimulation; the peak of B cell activation occurred one day after both LPS and CpG DNA were administered (Fig. 4). We observed an ca. 3-fold increase in B cell activation in LPS and CpG DNA treated mice when compared to PBS treated control mice (Fig. 4B). Importantly, these data demonstrate that TLR ligand-induced B cell activation occurs in mice latently infected with MHV68.

We next assessed whether LPS or CpG DNA treatment of mice *in vivo* impacted MHV68 reactivation. After i.p administration of LPS or CpG DNA, splenocytes were harvested at different times post-stimulation and plated for reactivation. Notably, no further stimulation was given upon explant of splenocytes. At day 1 post-stimulation, we did not detect any stimulation-induced change in MHV68 reactivation (Fig. 5A) or any increase in the presence of preformed infectious virus in the spleen (the latter was assessed by mechanically disrupting splenocytes) (Fig. 5B). In contrast, by 3 days post-stimulation we were able to detect a >2-fold increase in MHV68 reactivating from

latency in mice treated with LPS or CpG DNA (Fig. 5A). This modest increase in virus reactivation correlated with a substantial increase in the presence of preformed infectious virus in the spleens of mice that received LPS or CPG DNA treatment (Fig. 5B). The induction of virus reactivation and the presence of preformed infection virus was apparent at both days 3 and 7 post-stimulation, and appeared to largely subside by 2 weeks post-treatment (Fig. 5). These data provide compelling evidence that *in vivo* TLR stimulation can trigger virus reactivation *in vivo*.

To extend these analyses, we assessed whether TLR stimulation led to recrudescence of virus in the lungs, we performed a limiting-dilution assay on lung homogenate. Following an intranasal infection, the lungs are the primary site of acute lytic replication, which is cleared by ca. 12 days post-infection (128). At day 42 post-infection the PBS treated mice had very low levels of replicating virus in the lungs (Fig. 6). As observed in the spleen, there was a significant increase in virus replication in the lungs of mice that received either LPS or CpG DNA - which could be detected at both days 3 and 7 post-treatment and was largely cleared by 2 weeks post-treatment (Fig 6).

MHV68 reactivation in vivo correlates with an increase in virus-specific, activated and effector CD8$^+$ T cells. If LPS and CpG DNA induce MHV68 reactivation and subsequent virus replication *in vivo*, we reasoned that a CD8$^+$ T cell response to the reactivating virus would ensue. To test this hypothesis we assessed the frequency of CD8$^+$ T cells by following viral specific CD8$^+$ T cells, activated and effector CD8$^+$ T cells. One day post-stimulation there was little difference in the MHV68-specific CD8$^+$ T cell response between PBS treated and LPS or CpG treated mice, as examined by

tetramer staining of MHV68 ORF6$_{487\text{-}495}$, (data not shown). However, by day 7 post-stimulation there was a statistically significant increase in the MHV68-specific CD8$^+$ T cell response (Fig. 7A). This increase in the MHV68-specific response corresponded with a global increase in CD8$^+$ T cell activation in LPS and CpG treated mice, as seen by a down-regulation of CD62L (Fig. 7B). Finally, we observed an increase in effector CD44^{hi} CD62L^{lo} CD8$^+$ T cells in mice that received LPS or CpG DNA (Fig. 7C). These data indicate that an increase in MHV68 reactivation/replication corresponds to an increase the CD8$^+$ T cell response, suggesting re-engagement of an adaptive immune response to MHV68 reactivation/replication.

TLR-mediated induction of MHV68 reactivation/replication leads to an increase in MHV68 latency in vivo. Homeostatic maintenance of latency during chronic infection might involve periodic reactivation and reseeding of latency reservoirs. To test whether TLR induced reactivation leads to changes in the levels of virus latency in the spleen, we analyzed changes in the frequency of splenocytes harboring MHV68 between 1 and 14 days post-stimulation. We found the frequencies of viral genome positive cells were similar at day 1 following PBS (1/3600), LPS (1/4000) and CpG (1/4500) treatment (Fig. 8). Remarkably, while PBS treated controls exhibited the same frequency of viral genome positive splenocytes at day 14 (1/4000), mice treated with LPS (1/550) or CpG (1/650) had a substantial increase in the frequency of viral genome positive cells at day 14 post-stimulation (Fig. 8). Therefore, we conclude that TLR stimulated induction of MHV68 reactivation appears to seed latency *in vivo*. These data suggest that periodic

heterologous infections may contribute to the homeostatic maintenance of latent gammaherpesvirus infection through TLR-mediated virus reactivation/replication.

DISCUSSION

The stimuli for herpesvirus reactivation *in vivo* during natural infections are largely unknown. Here we report that signaling through specific TLRs can induce MHV68 reactivation, both *in vitro* and *in vivo*, raising the question of whether heterologous infections play a significant role in episodic gammaherpesvirus reactivation/replication in the chronically infected host. Notably, we observed that 14 days post-TLR stimulation there was an increase in the frequency of MHV68 latently infected splenocytes, suggesting that TLR-driven virus reactivation/replication may be one mechanism by which chronic MHV68 infection is maintained/renewed. Virus reactivation is clearly important for horizontal transmission of herpesviruses, and may for some herpesviruses play an important role in maintaining reservoirs of "functional" latently infected cells in the chronically infected host (21, 51, 72, 126, 127). The latter issue may be particularly important for gammaherpesviruses, whose genomes are known to be heavily methylated in the long-term latency reservoirs (24, 95). Methylated cytosines are prone to oxidative deamination leading to the production of thymine which, if not repaired prior to cellular proliferation, can become fixed mutations in the viral genome (11, 12). Thus, episodic virus reactivation may ensure the persistence and propagation of "functional" viral genomes (and perhaps reflects an ongoing mechanism by which viral fitness is maintained).

Is there any evidence indicating a role for heterologous infections triggering herpesvirus reactivation? In support of this idea, latently infected EBV cell lines can be induced to reactivate upon superinfection with CMV (7). Similarly, HIV-1 infection of

KSHV infected PEL cells causes KSHV reactivation (84, 135). Many infections, both bacterial and viral, trigger an immune response by their PAMPs. TLRs are expressed on a wide variety of cells including antigen-presenting cells. Signaling through TLRs can trigger HIV-1 replication in latently infected mast cells (115). KSHV infected PEL cells can be induced to reactivate in the presence of LPS (87), while *in vivo* administration of LPS also stimulates MCMV reactivation (27). Finally, we previously demonstrated that following intraperitoneal inoculation MyD88$^{-/-}$ mice exhibited a defect in MHV68 reactivation, but not establishment of latency (48). The latter suggested a potential role for TLR signaling in MHV68 reactivation. As a surrogate for heterologous infections we assessed whether TLR ligands could induce virus reactivation *in vivo* at 42 dpi. Day 42 is a time point at which very little spontaneous MHV68 reactivation is observed (142). We focused out analyses on B cell latency since by day 42 the vast majority of MHV68 latently infected splenocytes are B cells (142). LPS and CpG DNA treatment increased activated splenic B cells compared to PBS treated mice 1 day after administration. This was consistent with the early kinetics of B cells activation upon TLR stimulation *ex vivo*. *In vivo* TLR stimulation significantly increased MHV68 reactivation from latency – with both reactivation and the presence of preformed infectious virus in the spleen peaking at 7 days post-stimulation (cleared by day 14). As discussed above, by day 14 we observed a significant increase in the frequency of viral genome positive splenocytes in mice that received LPS or CpG DNA treatment compared to PBS treated mice. This increase implies either reseeding of latency following virus reactivation and subsequent virus replication, and/or expansion of the pool of latently infected cells through TLR-driven

cell proliferation. Either mechanism may facilitate maintenance of chronic gammaherpesvirus infection.

We have previously demonstrated that anti-Ig/anti-CD40 stimulation *ex vivo* can induce both proliferation of B cells and MHV68 reactivation/replication (89). In the present study we show similar results using TLR agonists. The immediate downstream pathway activated following TLR stimulation of a B cell are distinct from that following anti-CD40/anti-Ig stimulation; however, both pathways eventually lead to the activation of similar downstream transcription factors - notably NF-κB and AP-1, as well as interferon regulatory factors (IRFs) (120). Since multiple stimulation pathways lead to the activation of similar downstream transcription factors, one cannot clearly decipher what cellular factors and their expression patterns are necessary to drive reactivation of MHV68. Transcription Element Search Software (TESS) analyses have revealed putative NF-κB and IRF binding sites in the promoter of *orf*50, whose gene product RTA is the master lytic activator of MHV68 replication - being required for both acute virus replication and reactivation from latency (76, 96). Activation of one or more of these transcription factors may not only induce B cell activation and proliferation, but also bind to the *orf*50 promoter and cause production of RTA, leading to the initiation of the lytic cycle. Interestingly, over-expression of the NF-κB subunit p65 has seen shown to inhibit MHV68 replication in tissue culture (19). One interpretation is that over-expression of a single NF-κB subunit may alter the dynamic complex formation needed for reactivation. Notably, however, we have previously shown that NF-κB is not essential for reactivation *in vivo* (71). The Raf/MEK/ERK pathway is known to trigger KSHV reactivation (43).

Also, growth factors or cell cycle regulatory proteins induced by NF-κB may also play a combinatorial role in MHV68 reactivation.

TLR signaling likely represents one of many pathways that lead to MHV68 reactivation. Signals that lead to terminal differentiation of latently infected memory B cells are also a likely trigger (74), as has been shown for EBV and KSHV (64, 74, 145). Apoptotic signaling pathways triggered by activation through TLR-mediated pathways may be another (6). TLR signaling leads to an array of pro-inflammatory cytokine production (IL-12, TNFα, IFNα/β and/or IL-2), all of which have autocrine and paracrine affects that may contribute to MHV68 reactivation. TLRs induce the expression of selectins, chemokine and chemokine receptor genes that regulate cell migration to the sites of inflammation (60). Chemokines that cause B cell migration into secondary lymphoid organs during an immune response, such as secondary lymphoid tissue chemokine (SLC), B lymphocyte chemokine (BLC), as well as MIP-3α and MIP-3β, may also play a role in triggering virus reactivation *in vivo*. Furthermore, the latter may be a mechanism that could bring the reactivating B cell in close proximity to proliferating B cells, facilitating *de novo* latency establishment.

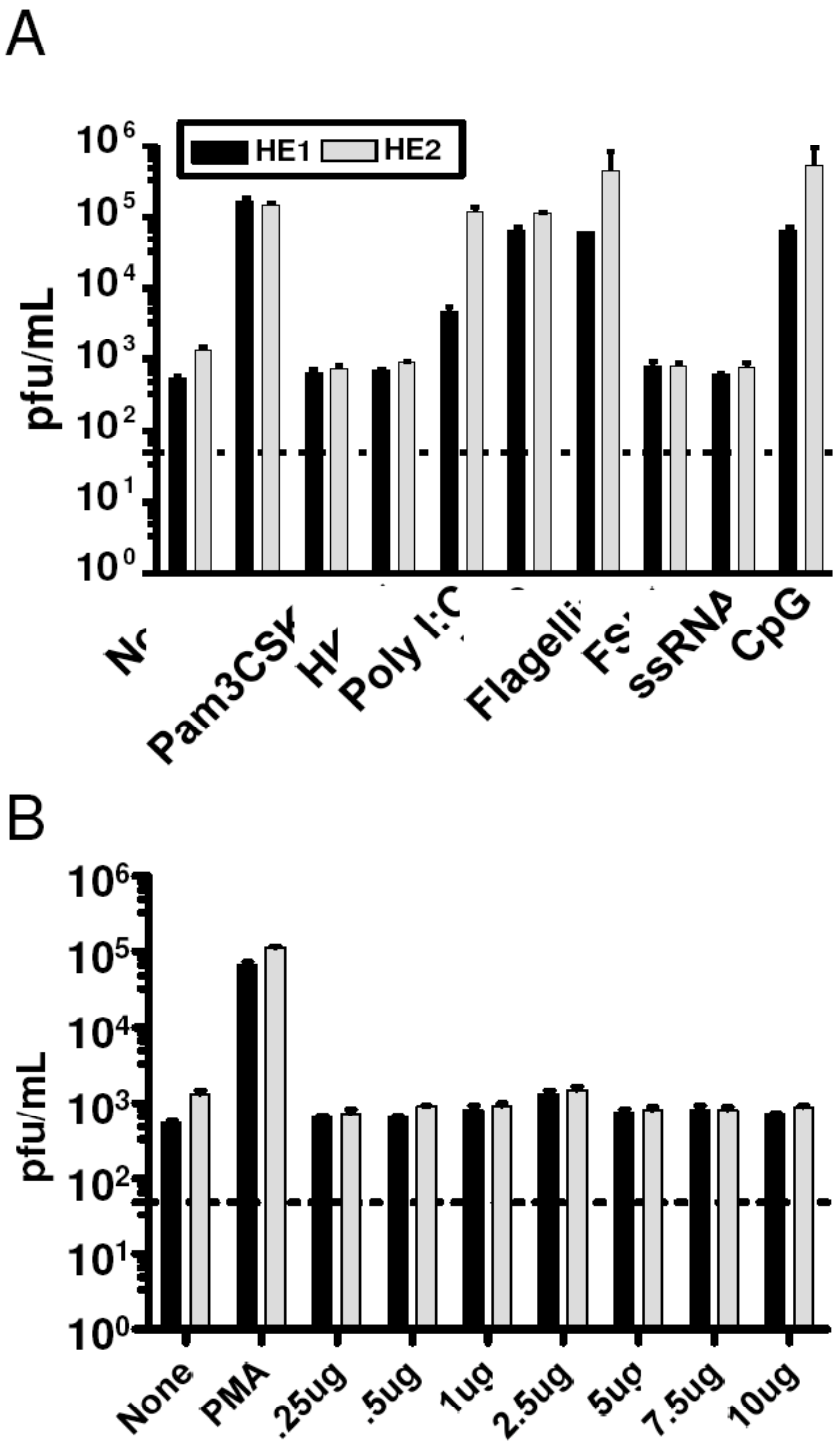
Coincident with the peak of *in vivo* reactivation following TLR stimulation, we also observed an increase in the $CD8^+$ T cell response. Both an increase in MHV68-specific CD8+ T cells, as well as a general increase in activated and effector CD8+ T cell responses were observed. This indicates that the host immune system responds to reactivating virus *in vivo*, likely in an attempt to control virus replication. Thus, periodic virus reactivation *in vivo* may, in turn, keep the MHV68 specific immune response primed to control chronic MHV68 infection.

Thus, in conclusion, we have demonstrated that TLR signaling can trigger MHV68 reactivation and subsequent virus replication *in vivo*. To our knowledge this is the first report demonstrating *in vivo* stimulation of gammaherpesvirus reactivation. We hypothesize that MHV68 may not only take advantage of this innate immune response to trigger reactivation, but also utilize the expansion of latently infected B cells and/or reactivation of MHV68 from B cells reseeding latency reservoirs, to facilitate homeostatic maintenance of MHV68 latency.

Acknowledgements

This work was supported by NIH R01 CA95318. SHS is also supported by NIH R01 grants CA52004, CA58524, CA87650, and AI58057. Thanks to members of the Speck lab for all of their helpful discussions,

Figure 1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10^5
10^4
10^3
10^2
10^1
10^0
Pam3CSK
Poly I:C
ssRNA
CpG
B
pfu/mL
10^6
10^5
10^4
10^3
10^2
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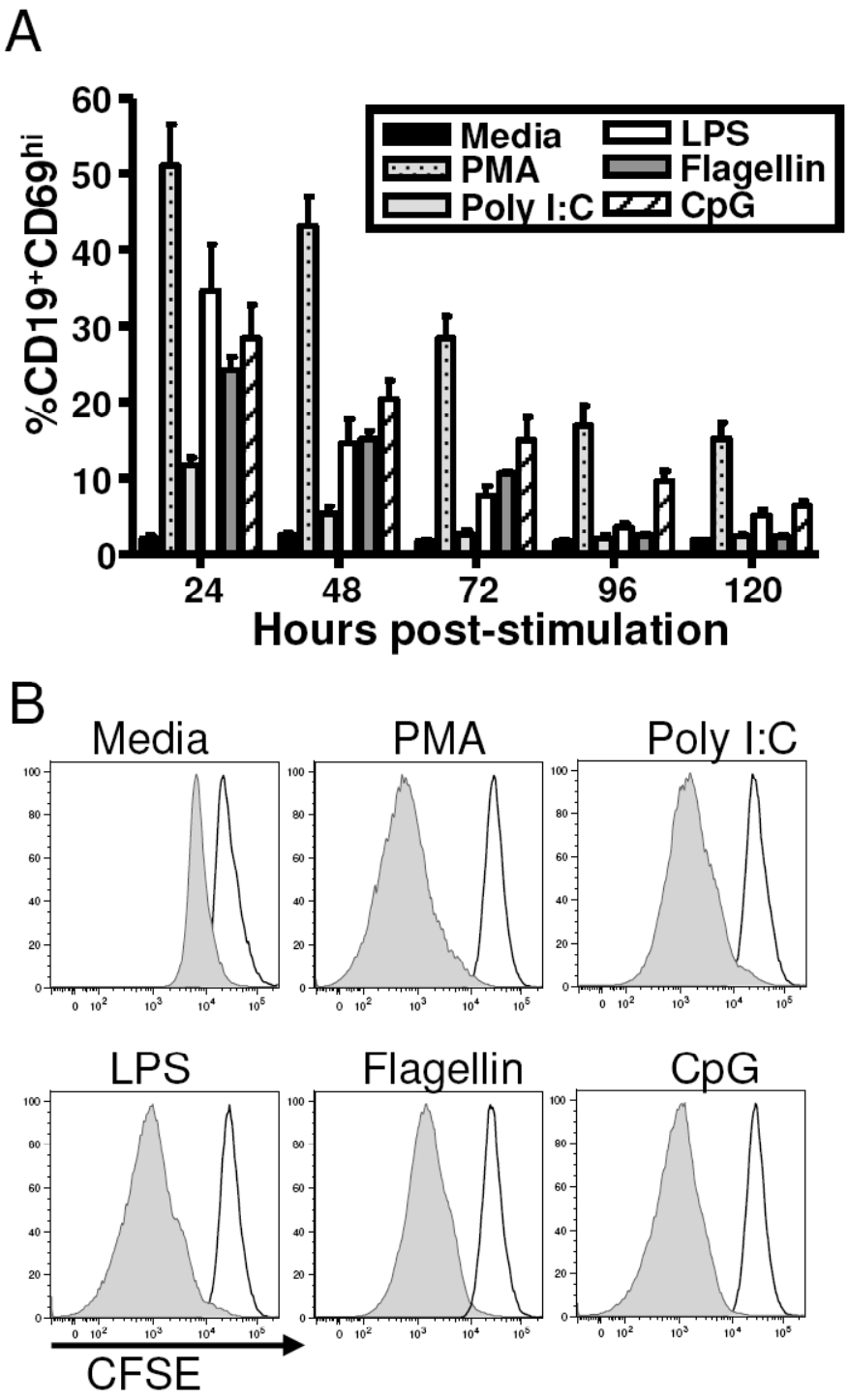
5ug
7.5ug
10ug

Figure 2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50
40
30
20
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0
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PMA
Poly I:C
LPS
Flagellin
CpG
24
48
72
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Hours post-stimulation
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Poly I:C
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Flagellin
CpG
CFSE

Figure 3

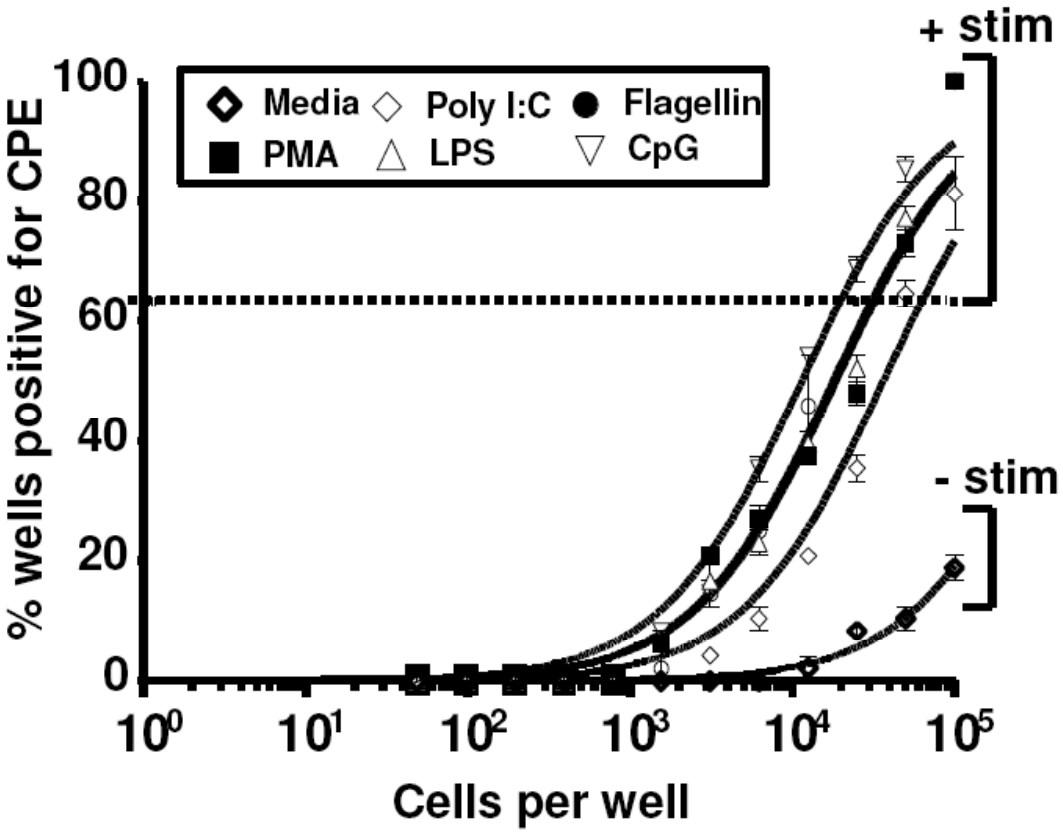
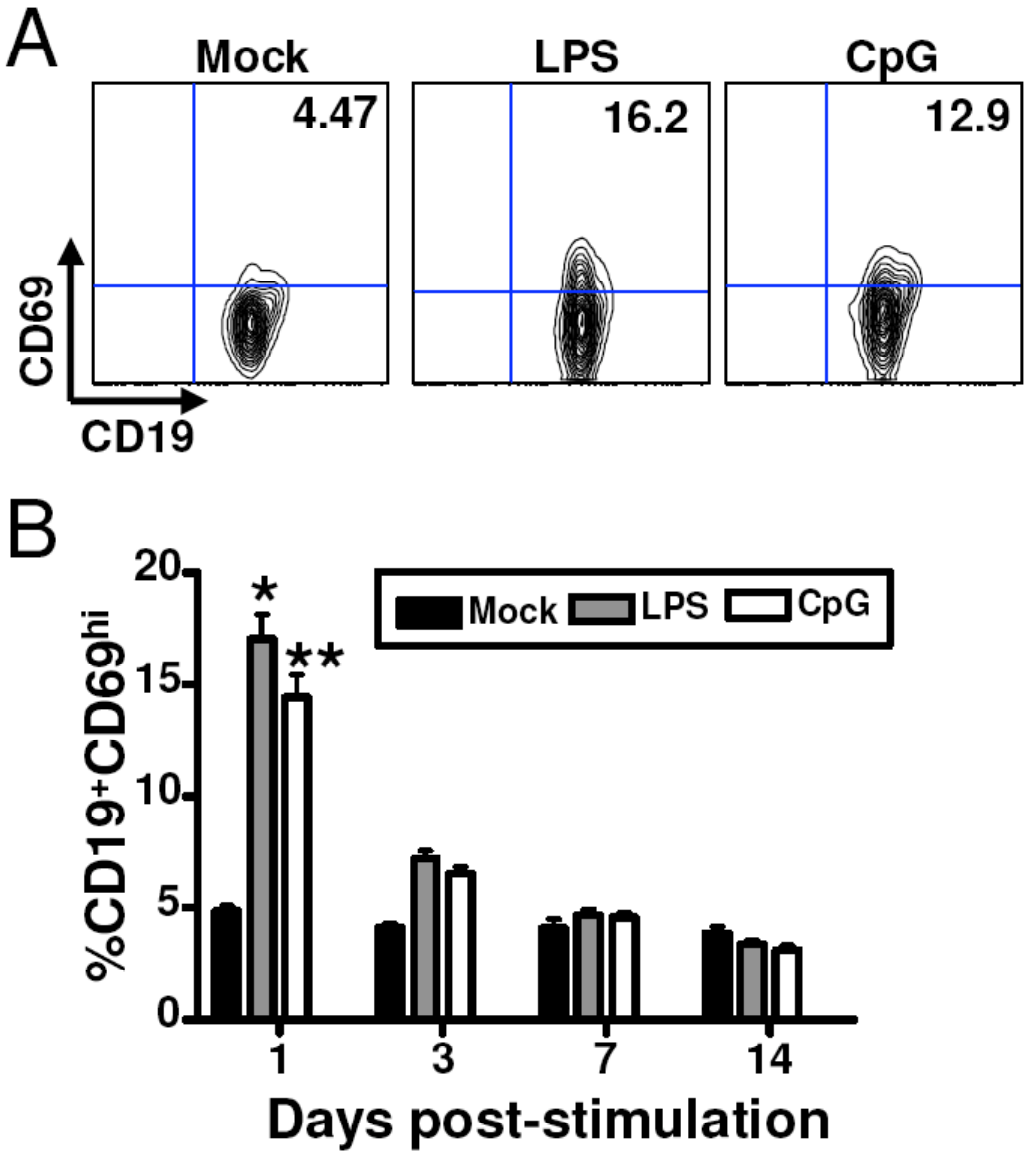

+ stim
- stim
Media
Poly I:C
Flagellin
PMA
LPS
CpG
% wells positive for CPE
100
80
60
40
20
0
10^0
10^1
10^2
10^3
10^4
10^5
Cells per well

Figure 4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Mock
LPS
CpG
4.47
16.2
12.9
CD69
CD19
B
Mock
LPS
CpG
%CD19+CD69hi
20
15
10
5
0
*
**
1
3
7
14
Days post-stimulation

Figure 5

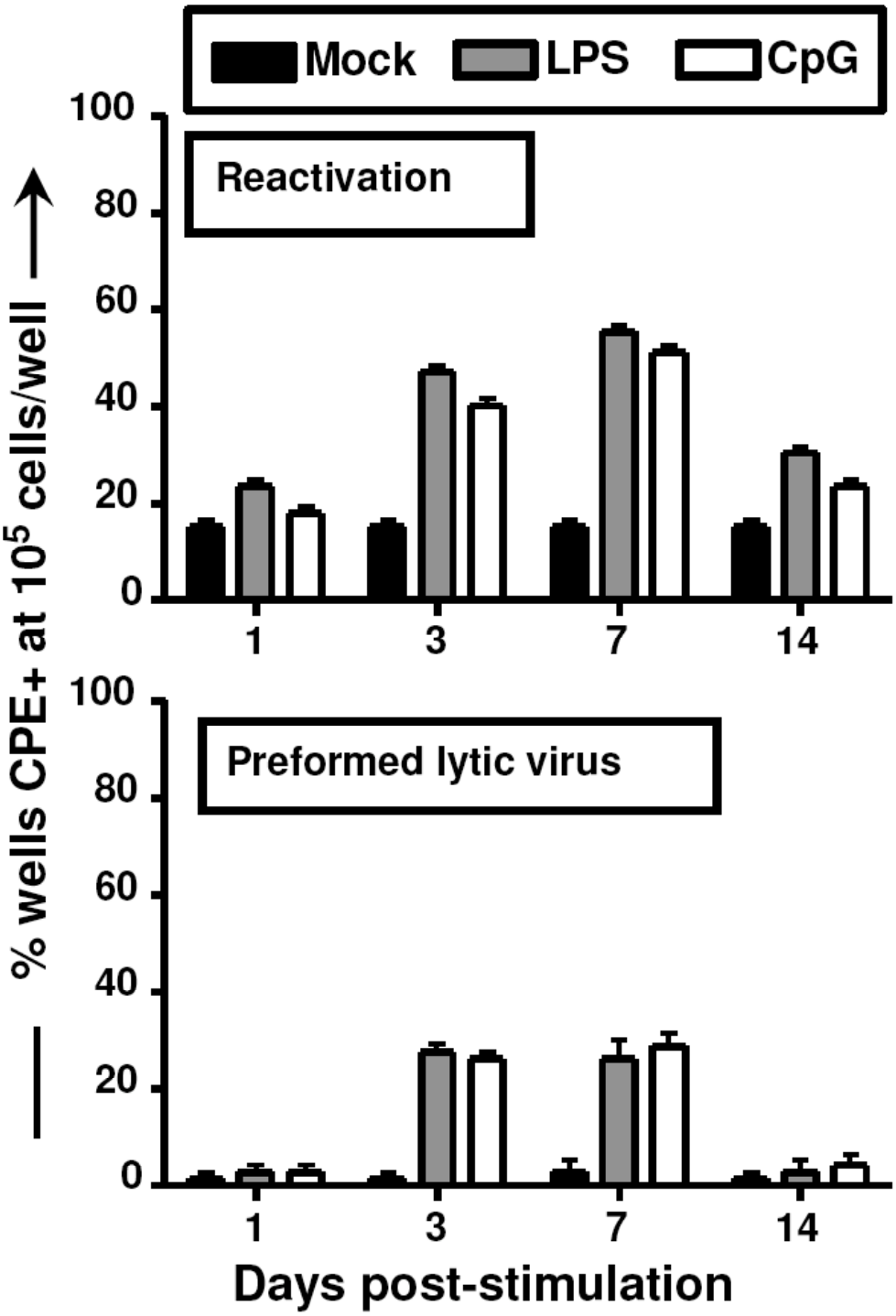
Mock
LPS
CpG
Reactivation
Preformed lytic virus
% wells CPE+ at 10^5 cells/well
100
80
60
40
20
0
1
3
7
14
Days post-stimulation

Figure 6

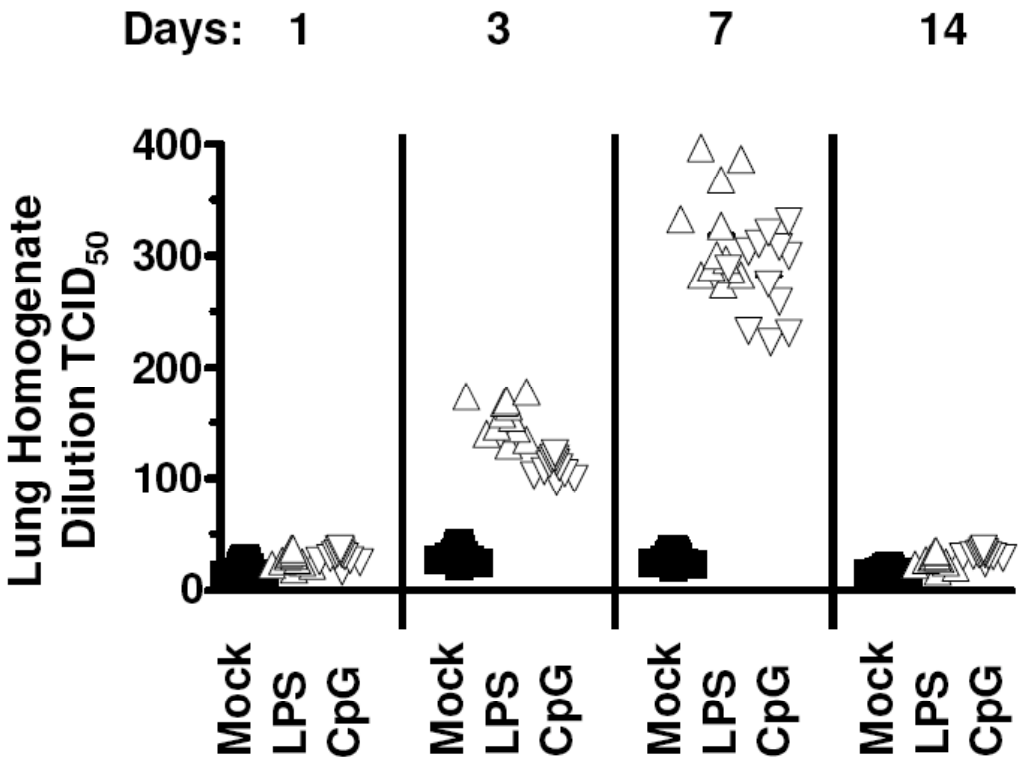
Days: 1
3
7
14
Lung Homogenate
Dilution TCID50
400
300
200
100
0
Mock
LPS
CpG
Mock
LPS
CpG
Mock
LPS
CpG
Mock
LPS
CpG

Figure 7

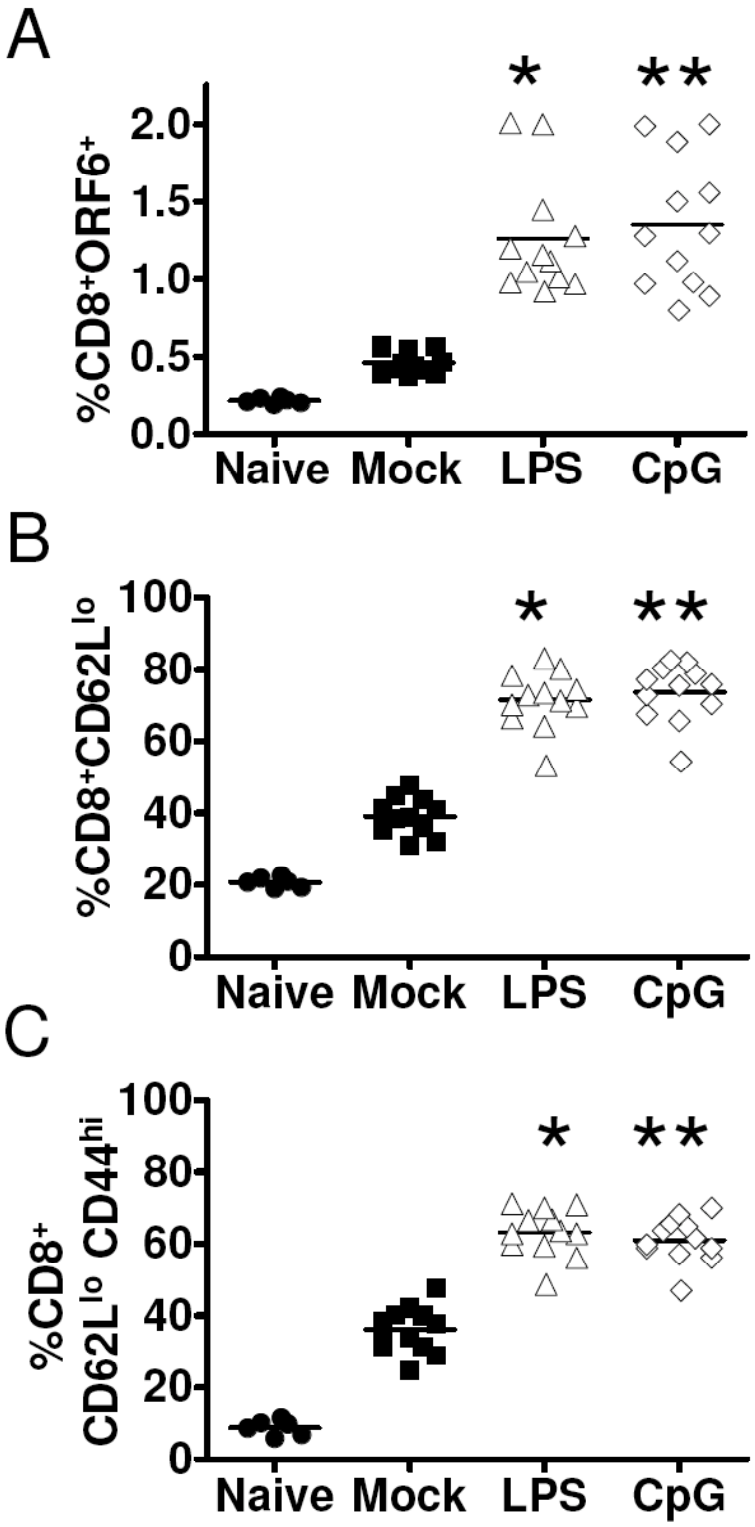
A
%CD8+ORF6+
2.0
1.5
1.0
0.5
0.0
Naive
Mock
LPS
CpG
*
**
B
%CD8+CD62Llo
100
80
60
40
20
0
Naive
Mock
LPS
CpG
*
**
C
%CD8+ CD62Llo CD44hi
100
80
60
40
20
0
Naive
Mock
LPS
CpG
*
**

Figure 8

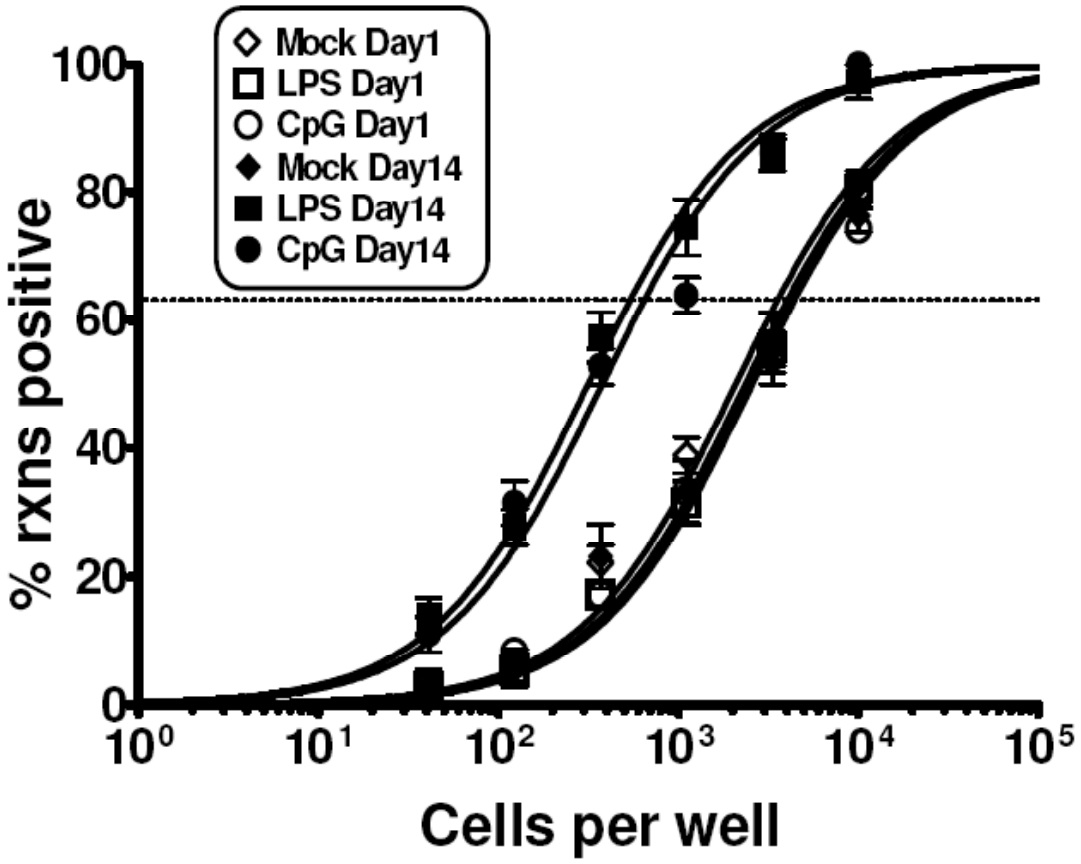
Mock Day1
LPS Day1
CpG Day1
Mock Day14
LPS Day14
CpG Day14
% rxns positive
100
80
60
40
20
0
10^0
10^1
10^2
10^3
10^4
10^5
Cells per well

FIGURE LEGENDS

Figure 1. Stimulation with TLR ligands enhances reactivation of MHV68 latently infected B cell lines *in vitro*. Two independently derived MHV68 latently infected cell lines (A20-HE cell lines) were treated with the indicated reagents. Tissue culture supernatants were harvested at 24 hour post-treatment and virus titers determine by plaque assay. Data shown are representative of results from three independent experiments. (A) The following ligands were used to stimulate specific TLRs as follows; Pam3CSK4 (TLR1/2), HKLM (TLR2), poly I:C (TLR3), LPS (TLR4), flagellin (TLR5), FSL1 (TLR6/2), ssRNA (TLR7) and CpG DNA (TLR9). (B) Dose response to ssRNA (TLR7).

Figure 2. *Ex vivo* TLR stimulation of splenic B cells induce B cell activation and proliferation. (A) Bulk splenocytes were harvested 42 dpi from mice and were either stimulated with the indicated TLR ligand, or left unstimulated in media. Cells were harvested at the indicated times post-stimulation and labeled with anti-CD69 and anti-CD19 antibodies, and analyzed by flow cytometry. Error bars represent standard deviation between individual mice. (B) Mice were intranasally infected with 1,000 PFU wild-type MHV68 and at 42 dpi bulk splenocytes were labeled with CFSE and stimulated with indicated treatments. At several time points post-stimulation, the cells were stained with anti-CD19 antibody and analyzed by flow cytometry for loss of CFSE. The flow cytometry histograms shown were gated on $CD19^+$ cells. The open histograms represent CFSE staining levels at 24 hours post-stimulation, while the shaded histograms represent

CFSE staining at 120-hours post- stimulation. The results shown are representative of results obtained from three independent experiments with 3-4 mice per group.

Figure 3. Stimulation of MHV68 reactivation from explanted splenocytes with TLR ligands. 42 days following 1,000 PFU wild-type MHV68 intranasal inoculation bulk splenocytes were obtained and were analyzed for the frequency of cells reactivating virus with and without TLR stimulation upon explant. The data shown represents results from three independent experiments, with cells pooled from 3-4 mice per experimental group. Based on these limiting dilution analyses the frequency of cells reactivating virus under each condition was as follows: no treatment (media), ~1 in 500,000; PMA, 1 in 30,000; poly I:C, 1 in 62,000; LPS, 1 in 30,000; flagellin, 1 in 30,000; and CpG DNA, 1 in 19,000.

Figure 4. LPS and CpG DNA treatment *in vivo* lead to B cell activation. 42 days after intranasal infection with 1,000 PFU of wild-type MHV68, mice were administered PBS, 15μg LPS or 20μg CpG DNA via intraperitoneal injection. Splenocytes were harvested at different times post-stimulation and stained with anti-CD19 and anti-CD69 antibodies, and analyzed by flow cytometry. (A) Representative flow cytometry plots of CD69 expression levels in purified B cell fractions (splenocytes were pooled from 4-5 mice per experimental group). Values shown in upper right quadrant are the percentages of $CD19^{+}$ cells that express CD69 activation marker. (B) Percentage of $CD19^{+}CD69^{hi}$ cells at the indicated times post-stimulation. Error bars represent standard deviation between individual mice. The percentage of $CD19^{+}CD69^{hi}$ cells in the spleens of mice

treated with LPS ($p<0.0001$), or CpG DNA ($p<0.0001$) at day 1 post-treatment were significantly different from PBS treated mice.

Figure 5. LPS and CpG DNA treatment of latently infected mice stimulate reactivation of MHV68. Mice were infected intransally with 1,000 PFU of wild-type MHV68. At 42 dpi mice were treated with PBS, LPS or CpG DNA via intraperitonal injection. Limiting dilution analyses assessed the impact of in vivo TLR stimulation on virus reactivation upon explant at the indicated days post-stimulation. Shown in the top panel is the percentage of wells in which viral CPE was detected when 10^5 cells were plated. Similarly, plating mechanically disrupted cells assessed the presence of preformed infection virus. The frequency of wells exhibiting CPE when 10^5 cell equivalents were plated is shown in the bottom panel. The data shown represents results compiled from three independent experiments, with cells pooled from 4-5 mice per experimental group.

Figure 6. Replication of MHV68 in the lungs following *in vivo* TLR stimulation. Mice infected with 1,000 PFU wild-type MHV68 intranasally were treated with PBS, LPS or CpG DNA at 42 dpi, followed by analysis of virus replication in the lungs at the indicated days post-treatment. Serial dilutions of lung homogenates were plated onto MEF indicator monolayers and scored for viral CPE 21 days post-planting with 16 replicates plated per dilution. Each point on the scatter plot represents the analysis of lung homogenate from an individual mouse, and represent analyses from three independent experiments containing 4-5 mice per condition per experiment. Statistically significant

differences were observed at both day 3 post-treatment (mock vs LPS treated, $p<0.0001$; mock vs CpG DNA treated, $p<0.0001$) and day 7 post-treatment (mock vs LPS treated, $p<0.0001$; mock vs CpG DNA treated, $p<0.0001$). Non-linear regression analyses were performed to determine the $TCID_{50}$.

Figure 7. Increase in CD8+ T cell responses following *in vivo* stimulation of MHV68 reactivation. Mice were infected intransally with 1,000 PFU of wild-type MHV68 and at 42 dpi were stimulated with PBS, LPS or CpG DNA and splenocytes were harvested at day 7 post-stimulation. (A) Cells were stained with anti-CD8 an MHC I tetramer to the an epitope in the MHV68 p56 antigen ($ORF6_{487-495}$) as an indicator of the MHV68 specific CD8+ T cell response. Each point on the scatter plot represents the percentage of $CD8^+$ $p56^+$ CD8+ T cells from the spleen of an individual mouse. The percentage of tetramer positive $CD8^+$ T cells in the spleens of mice treated with LPS ($p<0.0001$), or CpG DNA ($p<0.0001$) were significantly different from control (PBS treated) mice. (B) Splenocytes recovered from the control and treated mice were stained with anti-CD8 and anti-CD62L. Each data point on the scatter plot represents the percentage of $CD8^+CD62L^{lo}$ T cells, as a marker of activated $CD8^+$ T cells, from the spleen of a single mouse. The percentage of activated $CD8^+$ T cells in the spleens of mice treated with LPS ($p<0.0001$), or CpG DNA ($p<0.0001$) were significantly different from PBS treated mice. (C) To examine effector CD8+ T cells, splenocytes were stained with anti-CD8, anti-CD62L and anti-CD44. Each point on the scatter plot represents the percentage of $CD8^+CD62L^{lo}CD44^{hi}$ T cells in the spleen of a single mouse. The percentage of effector $CD8^+$ T cells in the spleens of mice treated with LPS ($p<0.0001$), or CpG DNA ($p<0.0001$) were significantly different

from PBS treated mice. The bar represents the mean percentage. Data shown represents three individual experiments with 4-5 mice per group, including naïve mice.

Figure 8. Stimulation of MHV68 reactivation *in vivo* leads to an increase in the frequency of latently infected splenocytes. Mice were infected with 1,000 PFU wild-type MHV68 intranasally and at 42 dpi were treated i.p with either PBS, LPS or CpG DNA. Bulk splenocytes from day 1 and day 14 post-treatment were assayed for the frequency of viral genome positive cells by limiting-dilution PCR. The data shown represent three independent experiments using 4-5 mice for each experimental group per experiment.

Chapter IV

Summary, Discussion and Future Directions

The MHV68 model system presents a unique opportunity to address the requirements for gammaherpesvirus pathogenesis in the context of a natural host infection (34, 35, 90, 105, 109). Toll-like receptors play an important role in the control of infections as well as the formation of a sufficient humoral response to infections (48, 52, 55, 93). This work is aimed to understand the role that TLRs play during an MHV68 infection. In chapter II, I found that TLR signaling on B cells is required not only for an optimal humoral response to MHV68 but also critical for the proper establishment of latency. These data support the hypothesis that B cell differentiation and the MHV68 life cycle are closely coupled (40, 41, 48, 71, 88). Reactivation from latency is also important to maintain MHV68 infection in the host (21, 51, 72, 126, 127). In the data collected in chapter III, I determined that TLR signaling induced MHV68 reactivation *in vitro*, *ex vivo* and importantly *in vivo*. This reactivation correlated with B cell activation, $CD8^+$ T cell response and increase in viral genomes. This provides a model that MHV68 utilized subsequent immune response to reseed latency reservoirs, as a way for homeostatic maintenance of latency in the host. Taken together these data shows that TLR signaling is a vital host determinant that is important throughout the life cycle of MHV68.

Analysis of TLR signaling in the establishment of MHV68 latency

MHV68 presents us with a murine model system in which we can utilize the vast array of transgenic mice to elucidate host determinants that influence gammaherpesvirus latency. In this study we used MyD88 deficient mice ($MyD88^{-/-}$) and TLR3 deficient mice ($TLR3^{-/-}$) to study both signaling pathways used by TLRs, MyD88 dependent and independent. We found an absence of a role for TLR signaling in controlling acute MHV68 replication. During MHV68 infection other pathways, most notably type I interferons (8, 36), may be the primary mechanisms involved in controlling acute replication. This does not rule out a double-stranded DNA sensor such as DAI (144), an as yet undiscovered PRR or other innate immune mechanisms being involved in controlling MHV68 replication.

As stated previously, the development of a B cell response seems to occur in conjunction with and the establishment of MHV68 latency. The decrease in B cell response we observed in the $MyD88^{-/-}$ mice was not absolute, indicating that other pathways that activate B cells, such as CD40-CD40L interaction, direct activation through the BCR, or a direct infection of memory B cells (a pathway that may be independent of B cell activation) may contribute to the pool of latently infected splenocytes. The reduction in splenic latency in $MyD88^{-/-}$ mice could reflect, in part, a role for this host cell factor in mediating inflammatory cytokine production or up-regulation of homing molecules to the spleen by infected cells that could lead to alterations in the recruitment, activation, and/or subsequent trafficking of infected cells to the spleen. MyD88 signaling may also be required for proliferation or survival signals upon entering the germinal center. Alternatively, MyD88 may be required for the

expansion, survival or migration of B cells during an immune response. Analysis of B cells for chemokine receptors, such as CXCR5, which recognizes chemokine CXCL13, and is necessary for homing of B cells to liver, spleen and lymph nodes could be done to investigate if there are differences in the ability of B cells to traffic in MyD88$^{-/-}$ mice during a MHV68 infection. It should also be noted that EBV infection can induces the up-regulation of a chemokine receptor, CCR7 on infected B cells (13). Study of differences in proliferation in MyD88$^{-/-}$ mice could be done with the use of BrdU or in *in vitro* studies using CFSE.

The extend to which MyD88 signaling plays a role in the formation of a B cell response appears to be system specific (48, 52, 55). This pathogen specific role might be due to direct recognition of the pathogen through the TLR, or, rather, as a tertiary signal following recognition of the antigen through the B cell receptor in conjunction with T cell help. The events involved in the activation of B cells, the transition of activated B cells into germinal center cells and succeeding formation of memory B cells are not completely understood. Nonspecific B cell activation might represent a mechanism by which the virus drives B cell participation in germinal center reactions to increase the likelihood of gaining access to the long-lived memory B cell reservoir. MHV68 may be a potent polyclonal B cell activator (35, 90). Germinal center B cells may represent the preferential reservoir for the establishment of latency because they represent a pool of highly activated B cells. The B cell activation signals required for the establishment of latency remain to be identified. This requirement of MyD88 in the establishment of MHV68 latency may define a unique differentiation step in the process of activation and transition of B cells into the germinal center and then to memory B cells. Ultimately,

MHV68 may exploit MyD88 signaling to gain access to memory B cells, its long-term latency reservoir (141). The discovery of MHV68 viral proteins that bind to MyD88 to induce its signaling independent of TLR ligation would uncover a mechanism by which MHV68 manipulates the infected B cell to drive it into a germinal center reaction then onto becoming a memory B cell. On the other-hand, discovery of a part of MHV68 may bind to a TLR to induce MyD88 signaling would also help uncover how MHV68 infection induces a humoral immune response.

The recovery of an anti-MHV68 antibody response underscores the transient nature of the defective B cell response in MyD88$^{-/-}$ mice, indicating that ultimately other activation pathways over-come the loss MyD88 function. Further experiments should be done to identify if MyD88 signaling is required for a specific isotype pattern (for example, IgG1 vs IgG2a) during a MHV68 infection.

By switching from an intranasal route of inoculation to intraperitoneal route we uncovered that MyD88 signaling is not required for the establishment to MHV68 latency using this more permissive route of infection. However, unlike the intranasal route of inoculation, virus reactivation from splenocytes was significantly impaired in MyD88-deficient mice following intraperitoneal inoculation, suggesting route-specific differences in the latently infected cell populations and/or their activation state. A more efficient approach of testing for route-specific differences in the cells that become latently infected in MyD88$^{-/-}$ mice would depend on the ability to detect latently infected cells directly. Our lab as recently developed a recombinant MHV68 virus expressing a yellow fluorescence protein (YFP). (Collins et al., unpublished). This has allowed us to mark latently infected cells. This tool would also allow us to phenotype and track latently

infected cells and observe if there are difference between C57Bl/6 and MyD88$^{-/-}$ mice during a MHV68 infection.

NF-κB signaling is one of the major activation pathways downstream of TLR signaling. The compounding phenotype observed in MyD88$^{-/-}$ when we suppressed NF-κB signaling suggests that either the required MyD88 activation of NF-κB in B cells occurs prior to MHV68 infection and expression of the NF-κB super-repressor, or that an alternative activation pathway driven by MyD88 is involved in modulating MHV68 latency.

Experiments with mixed bone marrow chimeric mice provide a direct demonstration that the decrease in the response of MyD88$^{-/-}$ B cells is intrinsic to these B cells and not necessarily required to induce dendritic cell maturation and T-helper cell activation. In a mixed CD40$^{+/+}$/CD40$^{-/-}$ bone marrow chimeric mice both CD40-deficient and CD40-sufficient B cells were found to harbor viral genome at 14 dpi at similar frequencies, although ultimately latency in the CD40-deficient B cells waned at later times post-infection (68). In contrast, the MyD88$^{+/+}$/MyD88$^{-/-}$ mixed bone marrow chimera mice exhibit a defect in the establishment of latency at 16 dpi, suggesting that activation of MyD88 signaling plays an important role in addition to, and perhaps prior to, the engagement of infected cells with CD40L in germinal center reactions. When presented with both MyD88$^{-/-}$ and MyD88$^{+/+}$ B cells in the same mouse, MHV68 preferentially establishes latency in the MyD88$^{+/+}$ B cells, indicating a preference of MHV68 to establish latency in B cells that have undergone an optimal response. While the requirement for MyD88 signaling for the formation of a humoral immune response to MHV68 was intrinsic to B cells, we also reported that it was independent of IL-1R, a

cytokine receptor signaling that also signals through MyD88. However, it remains a formal possibility that several MyD88 dependent receptors are involved in the generation of the B cell response to MHV68, and as such a defect in only one may not impair formation of the humoral response.

Pasare *et al.* recently showed that TLR signaling must occur in B cells in order for the optimum antibody response to be generated (93). In these studies the mice were immunized with hen egg lysozyme or HSA-LPS but in follow-up studies by other groups, including ours, it was found that this requirement held true in the context of true pathogens, although the extent of the role of MyD88 in formation of the humoral response appears to be system specific (48, 52, 55). These data have implications for vaccine design in that they indicate that a robust and long lasting antibody response is achieved best in the presence of TLR stimulation. Most successful vaccines, such as attenuated pathogens, trigger TLR signaling, which is one of the reasons why they are successful. On the other hand, subunit vaccines generally do not work and the, few that do, do so because they either activate TLRs or some TLR equivalent component of the innate immune system. This is still an active area of investigation with some conflicting reports (49, 93). Interestingly, a recent report identified a pediatric patient who has had severe recurring infections since birth and is deficient in IL-1R-associated kinase 4 (IRAK-4), a downstream mediator of MyD88 signaling. In vaccination studies, this patient demonstrated defective B cell responses and was unable to generate long-term humoral immunity (30). This demonstrates that the MyD88 dependent pathways may be essential for generating a humoral immune response not only in mice but also in humans.

Investigation of the role of TLR signaling in MHV68 reactivation

The triggers for herpesvirus reactivation *in vivo* in a natural host are still not known. Reactivation studies are problematic in human hosts because of obvious ethical limitations. Fortunately, MHV68 infection has been well characterized and is similar to the human gammaherpesviruses. Our hypothesis during this study was that during infections of the host with heterologous pathogens may trigger MHV68 reactivation and that MHV68 may not only take advantage of this immune response to trigger reactivation, but also utilize the expansion of latently infected B cells and/or reactivation of MHV68 from B cells reseeding latency reservoirs, to facilitate homeostatic maintenance of MHV68 latency. Many infections, both bacterial and viral, trigger an immune response by their PAMPs. TLRs are expressed on a wide variety of cells including antigen-presenting cells, including B cells. As a surrogate for secondary infections we tested TLR ligands that would be presumably produced from a pathogen, could induce reactivation. We used a logical progression of experiments from cell culture to intact mice, to show that certain TLR ligands can reactivate virus.

Transcription Element Search Software (TESS) analyses have revealed putative NF-κB binding sites in the promoter of *orf*50, whose gene product RTA is the master lytic activator of MHV68 replication - being required for both acute virus replication and reactivation from latency (76, 96). One hypothesis might be that any mediator or signal capable of activating NF-κB might be capable of reactivating MHV68 from latency. Subsequent studies should ascertain if NF-κB binding in viral promoters could play a role in triggering MHV68 reactivation. Site-specific mutagenesis of NF-κB binding sites in MHV68 might be one method employed to test the importance of these sites for

reactivation. In this model of the way NF-κB signaling is triggered to initiate viral replication *in vivo* is not provided by the virus, but by activators of NF-κB, such as TLR, CD40 or Ig signaling. Another way NF-κB might play a role in triggering reactivation, which would allow for a more active role of MHV68 is one where a latency protein of MHV68, such as the M2 protein, actively binds to NF-κB and shuttles it to the viral genome so it can bind to MHV68 and induce reactivation.

In addition the activation of NF-κB, TLR signaling leads to an array of pro-inflammatory cytokine production (IL-12, TNFα, IFNα/β and/or IL-2), all of which have autocrine and paracrine affects that may contribute to MHV68 reactivation. TLRs induce the expression of selectins, chemokine and chemokine receptor genes that regulate cell migration to the sites of inflammation (60). Chemokines that cause B cell migration into secondary lymphoid organs during an immune response, such as secondary lymphoid tissue chemokine (SLC), B lymphocyte chemokine (BLC), as well as MIP-3α and MIP-3β, may also play a role in triggering virus reactivation *in vivo*. Furthermore, the latter may be a mechanism that could bring the reactivating B cell in close proximity to proliferating B cells, facilitating *de novo* latency establishment. One way to demonstrate is inflammatory mediators, such as IL-1 and TNFα, could induce MHV68 reactivation *in vivo* is to provide these cytokines to latently infected mice and assay for changes in reactivation, as was done with MCMV (27). Apoptotic signaling pathways triggered by activation through TLR-mediated pathways may be another (6).

TLR signaling likely represents one of many pathways that lead to MHV68 reactivation. Signals that lead to terminal differentiation of latently infected memory B cells are also a likely trigger (74), as has been shown for EBV and KSHV (64, 74, 145),

the role of plasma cell differentiation in MHV68 reactivation is currently being studied in our lab.

The clinical implications of these findings suggest that any patient latently infected with a gammaherpesvirus is at risk for reactivation. Immunosuppression, either by HIV infection or because of a transplant can increase the chance of reactivation disease by not only inability to control the latent herpesvirus infection but also failure to control subsequent infections and following uncontrolled reactivation. Understanding the molecular mechanisms of pathogen induced gammaherpesvirus reactivation that permit survival, propagation and subsequent involvement in disease development is critical for the development of more effective diagnostic, preventative, and treatment strategies to control these polymicrobial infection *in vivo*.

Concluding remarks

The goal of this dissertation was to better understand the complex interaction between the host and pathogen. In chapter II, I uncovered that as seen in other systems, MyD88 signaling influences the B cell response to MHV68. MyD88 signaling was required to form an optimal humoral response during MHV68 infection. I found that B cell activation, germinal center B cells and isotype switched B cells were all significantly decreased in MyD88$^{-/-}$ mice. This phenotype was not seen in TLR3$^{-/-}$ mice, demonstrating that this is a MyD88 dependent phenomenon. I also found that the loss of MyD88 signaling also lead to a decrease in the MHV68 specific antibody response. Analysis of splenocytes from MyD88$^{-/-}$ mice revealed that there was also a defect in the establishment of MHV68 latency that accompanied the substantial decrease in the B cell response. In

addition, in mixed bone marrow chimeric mice experiments, I found that MyD88$^{-/-}$ B cells had a selective failure to become activated, participate in germinal center reactions and class switch, indicating an intrinsic defect in the MyD88$^{-/-}$ B cell. It was also found that MyD88$^{-/-}$ B cells had a decrease ability to harbor MHV68 latent virus when compared to MyD88$^{+/+}$ B cells in the mixed bone marrow chimeras. This indicates that MyD88 is important for the establishment of MHV68 latency and is directly related to the role of MyD88 in the generation of a B cell response. This study further demonstrates how establishment of MHV68 latency is exquisitely tied to a B cell immune response. In chapter III, I sought to determine a mechanism by which MHV68 reactivates from latency. I found that TLR stimulation of MHV68 latently infected B cell lines induced reactivation. Also, when latently infected splenocytes were stimulated with poly I:C, LPS, flagellin or CpG DNA *ex vivo* they displayed an activated phenotype and underwent proliferation. *Ex vivo* treatment of the splenocytes also lead to MHV68 reactivation. Importantly, I showed that when TLR ligands LPS and CpG DNA induced B cell activation *in vivo* and this correlated with an increase in reactivation from splenocytes as well as increase in replicating virus in lungs. This increase correlated with an increase in the frequency viral specific CD8^{+} T cell response, as seen by tetramer staining as well as an increase in the global frequency of CD8^{+} T cell activation and effector CD8^{+} T cells. This increase in reactivation resulted in an increase in the frequency of splenocytes harboring viral genome at 14 days post-stimulation. These data demonstrate how TLR stimulation may be an important mechanism for gammaherpesvirus reactivation *in vivo* and suggests that periodic pathogen exposure may contribute to homeostatic maintenance of chronic gammaherpesvirus infection through stimulating virus reactivation and

reseeding latency reservoirs. Ultimately, studying the function of TLRs during MHV68 life cycle may provide insight to host determinants used to promote the establishment of latency and regulate virus reactivation during chronic infection. In addition, these analyses may lead to a greater understanding of the host's ability to shape gammaherpesvirus infections and on the role of the host immune responses in the formation of lymphoproliferative disease and tumorigenesis associated with gammaherpesviruses.

These studies are important in order to understand the complexity of not only gammaherpesvirus infection, but also all chronic infections. The words of Dr. James Lovelock "An inefficient virus kills its host. A clever virus stays with it", reminds us that these viruses have had millions of years to evolve with its host and develop ways to not only avoid being cleared by the immune system but how to use it to its advantage to survive. It will take work to understand the mechanisms by which gammaherpesviruses establish and maintain latency. In this work, I have illuminated some of the ways that gammaherpesviruses takes advantage of the host immune response to its own benefit. A better understanding of the relationship between gammaherpesvirus and the host will lead to better understanding of how disease is caused and development of preventative and treatment measures.

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