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Andrea Michelle Siegel Date

M2 and Blimp-1:
Studies in Murine Gammaherpesvirus 68 Pathogenesis
and B cell Biology

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By

Andrea Michelle Siegel
B.S., Brandeis University, 2001

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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

Graduate Division of Biological and Biomedical Sciences

2009

ABSTRACT

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By Andrea Michelle Siegel

Gammaherpesviruses establish life-long infections in lymphcytes and are associated with a variety of lymphomas and carcinomas. The murine gammaherpesvirus68 (MHV68), which naturally infects wild murid rodents, has emerged as a model pathogen to study latency and reactivation *in vivo*. MHV68 latently infects B cells, dendritic cells, and macrophages; the long-term latency reservoir is the memory B cell. This thesis explores the role of B cells in MHV68 latency from both the pathogen and host viewpoint. Firstly, I examined how M2, a latency-associated MHV68 gene product, manipulates B cell biology to enable MHV68 to establish and reactivate from latency. I demonstrate that M2 expression in primary murine B cells leads to B cell proliferation, survival, and differentiation into an activated, pre-plasma memory B cell phenotype. M2-driven proliferation is dependent on IL-10, and M2 expression during MHV68 infection is associated with a significant increase in serum IL-10 levels at the onset of latency. Secondly, I studied the role of B-lymphocyte induced maturation protein-1 (Blimp-1), the master transcriptional regulator of plasma cell differentiation, in MHV68 pathogenesis *in vivo*. I observed that Blimp-1 expression in infected cells plays a significant role in the establishment of latency and reactivation from latency. Additionally, Blimp-1 expression in splenocytes is needed for the maintenance of MHV68 long-term latency. Together, these studies of M2 and Blimp-1 demonstrate how the biology of the B cell is intertwined with MHV68 pathogenesis.

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AGKNOWLEDGEMENTS

I would like to thank my mentor, Sam Speck, for guiding me through this process. There were many ups and downs, and his encouragement and patience, especially during the rocky beginning, enabled me to persevere and reach the end of the marathon that is a doctorate. Sam allowed me to forge my own experimental path, developing techniques and protocols that were novel to the lab. I will carry my sense of independence and fortitude in the lab throughout my future scientific pursuits.

I owe a dept of gratitude to all of the members of the Speck lab who helped me grow not only as a student and a scientist, but as a teacher as well. I would particularly like to thank Laurie Krug for her mentoring and Jeremy Herskowitz for his longtime collaboration. Finally, I would like to thank my rotation students, Udaya Rangaswamy and Ruth Napier, for their patience as I learned to teach.

I would like to thank the members of my thesis committee, Drs. Max Cooper, Joshy Jacob, Jacqueline Katz, and Aron Lukacher. All of my committee members have been valuable sources of guidance and discussion of my science and career. A great thank you to Eli Kraus from the Tainsky and Haber labs who helped start me on this journey.

To my parents, I owe tremendous thanks for their support, love, and patience. A special thanks to Isa Bielsky for her experimental karma.

Thank you, my husband (and one-time physics lab partner), Nisan, for all of your love and encouragement and for our most important collaboration, Clara Alice. I hope we will continue to grow together as a family. This is for you both.

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CHAPTER I

INTRODUCTION

Gammaherpesviruses: lymphotropic viruses

The viruses in the family *Herpesviridae* are enveloped, DNA viruses with large, transcriptionally complex genomes. Viruses are classified as Herpesviruses based on their morphology: a spherical virion with a DNA core packaged into an icosahedral capsid, surrounded by tegument proteins, enclosed by a lipid envelope studded with viral membrane glycoproteins (7). Herpesviruses share the ability to establish latent infections that persist in the absence of infectious virion production. During latency, the viral genome circularizes into an extra-chromasomal episome that is duplicated along with the cellular genome during replication. These periods of latency can be interrupted by re-expression of viral genes and production of infectious, lytic virions in a process known as reactivation.

Herpesviruses are divided into three families based upon genetic homology as well as latency-associated tropism. The alphaherpesvirues such as Herpes Simplex Virus (HSV) and Varicella-zoster Virus (VZV) establish latency in neurons. The betaherpesviruses establish latency in monocytes. For example, Cytomegalovirus (CMV), a human betaherpesvirus, establishes latency in T cells and macrophages. The gammaherpesviruses establish life-long, latent infections in lymphocytes and are common within the human population.

Gammaherpesviruses display exquisite species-specific tropism, and all are associated with oncogenesis in lymphocytes. The gammaherpesvirus family consists of the two genera *Lymphocryptovirus* (or γ1-herpesviruses) and *Rhadinovirus* (also referred to as γ2-herpesviruses). Infection with Epstein-Barr Virus (EBV), a human lymphocryptovirus, is so ubiquitous that more than 95% of people are believed to be latently infected (162). EBV infection is associated with a variety of lymphomas and carcinomas including Burkitt's lymphoma, nasopharyngeal carcinoma, and Hodgkin's disease. Kaposi's Sarcoma Associated Herpesvirus (KSHV) is an important human rhadinovirus and evidence for KSHV-associated tumorigenesis is found in Kaposi's sarcoma tumors as well as in primary effusion lymphomas (PELs) and multicentric Castleman's disease (MCD). Both EBV and KSHV establish latency in B cells, with memory B cells being the primary long-term reservoir for EBV (8, 14, 145). EBV and KSHV transform B cells; Burkitt's lymphoma, PELs, and lymphoblastic variants of MCD are all tumors of B cell origin. T cells, monocytes, and endothelial cells have also been shown to be positive for KSHV DNA (7).

All gammaherpesvirues share regions of extensive genetic homology and share phenotypic similarities (see Figure 1). Due to the restricted host range of the gammaherpesviruses, careful study of the pathogenesis of EBV and KSHV is hampered by an inability to perform primary infections, and two animal models are commonly used to study gammaherpesvirus pathogenesis. Herpesvirus saimiri (HVS) infects new world primates, establishes latency in T cells, and is responsible for T cell lymphomagenesis. HVS long served as the only model gammaherpesvirus pathogen. However, studies were hindered by the expense

and difficulty in handling primate infections. Recently, the field has turned to the murine gammaherpesvirus 68 (MHV68 or γHV68), a natural pathogen of wild murid rodents that causes lymphomas in certain strains of immunocompromised mice (16, 103, 159). MHV68 is also referred to as Murid herpesvirus 4 (MuHV-4) and has been classified as a member of the genus *Rhadinovirus*. MHV68 displays extensive genetic and phenotypic similarities with both KSHV and EBV. Examination of MHV68 pathogenesis in inbred and outbred strains of mice has emerged as a model for gammaherpesvirus biology (146, 176).

Murine Gammaherpesvirus68 as a model pathogen

The study of MHV68 as a model pathogen for gammaherpesvirus biology has many advantages. Firstly, the natural hosts of MHV68 are wild murid rodents, including the wood mouse (*Apodemus sylvaticus*), and, as such, the study of MHV68 in the ubiquitous lab mouse is a faithful recapitulation of a natural host/pathogen relationship (15, 16). This allows investigators to take advantage of the numerous knockout and transgenic mouse models to explore the contribution of the host's biology to pathogenesis (for example, those described in (55, 114, 182)). Importantly, the MHV68 genome was cloned into a bacterial artificial chromosome (BAC), facilitating the generation of recombinant viruses in *E.coli* by homologous recombination (1, 2, 106). The ease and speed at which recombinant strains of MHV68 are generated has lead to an explosion of knowledge concerning viral genes as well as their regulation (for example (34, 39, 62, 63, 106)). Additionally, our lab identified a phenotypically neutral locus

within MHV68 that allows for efficient expression of transgenic constructs within the virus without discernable disruption of latency (29, 82, 108).

Murine Gammaherpesvirus68 and B cells

Given that MHV68 infection of mice presents a unique opportunity to study gammaherpesvirus pathogenesis, many studies have focused on *in vivo* dissection of MHV68 biology. During an acute infection of the lung epithelium, MHV68 induces viremia that is resolved into a latent infection of B cells, dendritic cells, and macrophages (46, 157). There is also evidence of latent infection of lung epithelium, and a recent study points to the lung endothelium as an additional source of lytic replication (107, 152, 154). $CD8^+$ T cells are required for the control of MHV68 lytic infection, and they regulate the latent viral load in the spleen (37). B cells are responsible for carrying latent MHV68 from the lungs to the spleen (152, 183). MHV68 infection leads to a $CD4^+$ T cell-dependent splenomegaly (168). Additionally, both latent genomes as well as cells capable of efficient reactivation have been associated with proliferating B cells *in vivo* (108). Viral replication in B cells, but not macrophages, is necessary for the maintenance of latency in the peritoneal cavity (89). As the infection progresses, there is an increase in both virus-specific and nonspecific hypergammaglobulanemia, and, in certain strains of inbred mice, an expansion of a distinct population of $CD8^+$ T cells carrying the Vβ4 T cell receptor (45, 128, 164). Similar to EBV pathogenesis, memory B cells are the long-term reservoir for latent MHV68 (8, 76, 185). Increased frequency of lymphomagenesis is

associated with immunodeficiency; MHV68 infection of β2-microglobulin-deficient Balb/C mice leads to a high incidence of B cell lymphomas (159).

MHV68, unlike KSHV, grows to high titers in tissue culture, enabling efficient *in vitro* studies as well. Initial studies focused on the characterization of the S11 cell line, a IgM^+ B cell line derived from a tumor from a MHV68-infected Balb/C mouse harboring latent MHV68 (170). When stimulated with phorbol 12-myristate 13-acetate (TPA), S11 cells reactivate from latency, but studies were limited by lack of an uninfected, negative control (170). The field was furthered by the recent generation of latently infected cell lines derived from the A20 $IgG2a^+$ B cell lymphoma line (50). These cell lines, designated A20-HE1 and 2, harbor a recombinant strain of MHV68 wherein a hygromycin-EGFP cassette was inserted to allow for drug selection and detection of latently infected lines (50). A20-HE1 and 2 allow for comparison of MHV68 latently infected, reactivation competent cell lines to a true, uninfected negative control A20 cell line, permitting careful *in vitro* study of MHV68 biology (50).

B lymphocytes were named for their site of maturation in birds, the bursa of Fabricius. The name B cell was fitting, for the major site of maturation in both mouse and man is the bone marrow. The key effector function of B cells is their ability to secrete immunoglobulin (Ig) or antibody molecules. In the bone marrow, developing B cells undergo rearrangement of their V, D, and J loci to create unique surface immunoglobulin (sIg or B cell receptor (BCR)) molecules. Once sIg is successfully displayed on the cell surface, the mature B cell exits the bone marrow and homes to the lymphoid tissues.

When a B cell encounters the antigen that binds to its specific BCR in the context of T cell help and cytokines, the B cell can enter the germinal center reaction wherein somatic hypermutation, class-switching, and affinity maturation take place through a process of proliferation and selection to create an antibody with high specificity and unique effector function for a given antigen. During the germinal center reaction, a B cell has two fates: death or differentiation. B cells that mutate their BCR specificity away from the antigenic stimulus or fail to produce a functional BCR altogether die by apoptosis. B cells with high affinity BCRs specific for the antigen can differentiate either into a plasma cell (otherwise known as an antibody-secreting cell or ASC), migrate to the bone marrow and secrete antibodies, or they can become a long-lived memory B cell, remain in the spleen and retain surface immunoglobulin expression (Figure 2). Upon re-encounter with its cognate antigen, a memory B cell can differentiate into a plasma cell, with or without entering a germinal center reaction, or remain a memory B cell, providing long-term humoral immunity. Plasma cells, too, can be long-lived, with a certain subset residing in the bone marrow and providing a steady stream of secreted antibody.

Manipulation of B cells by Gammaherpesviruses

Gammaherpesvirues actively manipulate their host lymphocytes to establish latent infections and enable reactivation, leading to shedding, transmission, and maintenance of infection by infection of new lymphocytes. Both EBV and KSHV are B cell tropic and associated with development of lymphoproliferative diseases, and each virus has its own unique arsenal of viral

proteins capable of altering B cell biology. The unique open reading frames found at the left end of the herpesvirus genomes often are transformative and capable of manipulating B cell signaling (Figure 1).

EBV is hypothesized to actively drive infected, naïve B cells into the germinal center reaction through the expression of the "default program" of latency-associated genes (162). In the default program, EBV expresses LMP-1, LMP-2A, and EBNA1 (162). LMP-1 expression in B cells can functionally substitute for CD40 ligation; LMP-2A can serve as a BCR and drive B cells to the periphery in $Rag1^{-/-}$ mice (22, 166). EBNA1 tethers the EBV genome to the host chromosome, allowing for maintenance of latency (187). LMP1 is a potent activator of the NF-κB, p38, and JNK pathways, and expression LMP1 in transgenic mice is capable of transforming B cells *in vivo* (17, 84). Additionally, LMP1 is critical for EBV-mediated transformation of primary B cells *in vitro* (32). LMP2A binds to Lyn, Fyn, Syk, and Csk kinases, and dampens BCR signaling in EBV-transformed lymphoblastoid cell lines (LCLs), maintaining EBV latency (17). Together, LMP1 and LMP2A manipulate B cell signaling in order to establish EBV latency while EBNA1 expression facilitates genome maintenance.

KSHV K1 protein, like LMP1 and LMP2A, is a transmembrane glycoprotein and is capable of mimicking BCR signaling by inducing constitutive NFAT and NFκB signaling through interactions with Vav, p85, and Syk (32). K1 expression in rodent fibroblasts is transformative; K1 transgenic mice develop tumors similar to a spindle cell sarcomatiod tumor and maliganant plasmablastic lymphomas (88, 118). Additionally, KSHV encodes an ortholog of microRNA -155 (miR-155), miR-K12-11 (57, 143). MiroRNA-155 (miR-155) is necessary for

generation of an optimal germinal center response in part by controlling expression of cytokine genes, and miR-155$^{-/-}$ mice have attenuated immune function of B, T, and dendritic cells (126, 161). When miR-155 is deleted only in the B cell compartment, mice are unable to elicit strong extrafollicular or germinal center responses and produce antigen-specific, class-switched plasma cells, indicating a direct role for miR-155 in plasmablast differentiation and survival (175). Overexpression of miR-155 is observed B cell tumors, and miR-155 transgenic mice develop B cell lymphomas (23). The KSHV miR-K12-11 silences a similar set of mRNAs as miR-155, and only the viral miR-K12-11 is expressed in KSHV-positive peritoneal effusion lymphomas (57, 143). Skalsky et al suggest that perhaps miR-K12-11 sustains the germinal center response after miR-155 has been silenced, deregulating B cell proliferation and leading to lymphomagenesis (143).

M2: a latency-associated Murine Gammaherpesvirus68 protein

Multiple viral factors are involved in MHV68 latency and reactivation, some of which are unique to MHV68. M1 is necessary for the suppression of reactivation in the peritoneal cavity (28). ORF73, the MHV68 LANA homolog, is required for the establishment of latency following intranasal inoculation (106). Additionally, MHV68 encodes a viral cyclin, ORF72, that is critical for reactivation from latency following intraperitoneal inoculation (173). ORF72's role in lytic replication was only appreciated when an ORF72-null strain of MHV68 was administered intranasally and found to be defective in replication in the lung (167). Thus, the complexity of MHV68 gene function during various

stages of infection has been demonstrated by varying the route of administration of virus.

The M2 open reading frame was hypothesized to encode a protein involved in latency and reactivation due to M2's position at the left end of the viral genome, homologous to many of the other latency-associated genes in the gammaherpesvirus family. The M2 ORF was also predicted to encode for a unique protein with no known homologs in virus, mouse, or man. Indeed, M2 is transcribed *in vivo* in latently infected B-cell deficient mice, but no transcripts are detected during a lytic infection of fibroblasts *in vitro* (178). S11 B cells which harbor latent MHV68 also express M2 transcripts (66). In confirmation of M2's proposed role in gammaherpesvirus pathogenesis, M2 plays critical roles in the establishment and reactivation from latency *in vivo* while being dispensable for lytic replication (62, 68, 97).

Characterization of the M2 transcript in S11 B cells revealed a spliced transcript with exon 1 encoded from 5924-5815 and exon 2 from 4609-3375 (all nomenclature WUMS MHV68 sequence, ATCC VR-1465) (66). Recently, *in vivo* 5' RACE performed on RNA from latently infected splenocytes revealed multiple transcriptional initiation sites for M2 clustered close together with the majority at 5861 (34). *In vivo* analysis also confirmed the 3' end of the M2 transcript as well as the conserved splicing from 4609 (splice acceptor) to 5815 (splice donor) (34). Interestingly, mapping of the M2 promoter in S11 and WEHI231 B cells revealed both a proximal and distal promoter, and deletion of either promoter in MHV68 impaired the establishment and reactivation from latency following intranasal

inoculation (34). These studies hint at a potentially complex, tightly controlled regulation of M2 expression during MHV68 infection.

Biochemically, M2 most resembles an adapter protein. Early studies showed that M2 localized to the cytoplasm and plasma membrane of B cells, leading to the hypothesis that M2 plays a role in the manipulation of cell signaling to facilitate the establishment and reactivation from latency (97). The M2 protein contains nine PXXP motifs through which it could potentially interact with SH3 domain containing proteins (66). Additionally, M2 has two potential tyrosine phosphorylation motifs, which, if phosphorylated *in vivo*, could facilitate binding of proteins with a SH2 domain (66). Biochemical studies have shown that M2 interacts with Vav1 and Fyn, leading to phosphorylation of M2 at tyrosine$_{120}$ and Vav1 as well as downstream activation of the Rac1 pathway (98, 116, 125). It is unclear, however, from these studies as to the role of the M2/Vav1 interaction *in vivo*. Mutation of proline$_{160}$ and proline$_{163}$ by our group (as discussed further), proposed to be critical for this interaction, revealed a very mild defect *in vivo*, leading to the possibility that this is one of many pathways potentially manipulated by M2 in the context of an infection (63). Mice deficient in Vav have impaired B cell compartments, confounding studies using infection of knockout animals (35, 125, 160).

M2 inhibits the STAT1/2 pathway leading to inhibition of the interferon response in A20 B cells (91). Additionally, M2 dampens the DNA damage response by interacting with the DDB1/COP9/Cullin repair complex and the ATM DNA damage signal transducer in fibroblasts (90). Neither of these interactions has been confirmed in the context of a MHV68 infection.

Nevertheless, these studies support the hypothesis that M2 is a potent modulator of cell signaling – potentially influencing multiple cellular pathways by interactions with many proteins.

M2 is dispensable for lytic replication of MHV68 both *in vitro* and in the lungs following intranasal inoculation, regardless of the dose of virus (62, 68, 97). However, when an M2-deficient strain of MHV68 (MHV68/M2.Stop) was administered intraperitoneally, no defect was observed at day 4 post-infection, but there was a profound defect in lytic replication at day 9 post-infection as compared to wild-type MHV68 (68). This observed phenotype may be linked to the role of M2 in reactivation *in vivo*. M2 is required for reactivation from latency following intraperitoneal inoculation at day 16 post-infection in a dose-dependent manner (62, 68). Perhaps reactivation at day 9 post-infection following intraperitoneal infection fuels a burst of lytic replication that is detected by plaque assay, and without M2, reactivation is compromised, leading to lower levels of lytic virus in the spleen. M2 is also required for the establishment of latency at day 16 post-infection following intranasal inoculation in a largely dose-independent manner (62, 68, 97). By varying the dose and route of administration of MHV68, M2 was found to play a role both in the establishment of as well as reactivation from latency. However, by day 42 to 3 months post-infection, depending on the dose of virus administered, the frequency of latently infected splenocytes was similar in animals infected intranasally with M2-deficient and wild-type viruses (62, 68).

Careful examination of recombinant strains of MHV68 wherein only specific PXXP motifs in M2 were altered demonstrated a role for three specific

motifs, designated P1, P8, and P9 in MHV68 pathogenesis (63). Mice were infected intranasaly with a low-dose (100 pfu) of virus, and the frequency of latently infected cells and cells capable of reactivation was examined at day 16 post-infection. Mutation of the P1 PXXP motif in M2 resulted in a decrease in the ability of the virus to reactivate from latency (63). In comparison, mutation of two PXXP motifs in the C-terminus of M2, P8 and P9, including the putative Vav1 binding site, resulted in a decrease in the establishment of splenic latency, but failed to recapitulate the M2-null phenotype (63). These studies also demonstrated that both $tyrosine_{120}$ and $tyrosine_{129}$ of the M2 protein play significant roles in the reactivation from MHV68 latency, supporting the importance of these motifs in the M2-Vav1 interaction (63). Interestingly, although phenotypically similar *in vivo*, *in vitro* studies of P8 and P9 mutant forms of M2 in primary murine B cells indicate separate roles for these motifs in the manipulation of B cell biology (63). As a whole, *in vivo* mutational analysis confirmed the role of M2 as a multifactorial protein, manipulating multiple pathways within the context of a MHV68 infection.

There is a K^d restricted $CD8^+$ T cell epitope in M2 from amino acids 91-99 (66). Kinetic studies in infected Balb/C mice have demonstrated that the M2-specific $CD8^+$ T cell response peaks around day 16-18 post-infection, at the onset of splenic latency, and contracts by day 20 post-infection (169). Transcriptional analysis has shown M2 to be transcribed in activated, PNA^{high} B cells at the onset of splenic latency (day 14 post-infection) as well as in germinal center and memory B cells at three months post-infection (142). Mutation of the K^d anchor

residues in M2 leads to an increase in MHV68 latency, suggesting that periodic expression of M2 elicits a potent $CD8^+$ T cell response that limits viral loads (99).

Analysis of the frequency of latently infected cells in different B cell populations indicates a potential role for M2 in the manipulation of B cell biology during MHV68 infection. Fractionation of B cells into naïve (IgD^+) and mature (IgD^-) populations 42 days post-intraperitoneal inoculation revealed a role for M2 in the efficient transition of latently infected B cells to the long-term latency reservoir, the memory B cell (62). A 50-fold higher frequency of latently infected naïve B cells was observed in animals infected with a M2-deficient strain of MHV68 versus the wild-type virus, and this trend continued as far out as six months post-infection (62). Intriguingly, a similar frequency of latently infected mature B cells was observed in mice infected with either virus throughout the time-course (62). These results indicate a M2-independent route of direct infection of mature B cells with MHV68. Complementing these studies, careful examination of B cell subsets following intranasal inoculation reveals an inability to efficiently transition MHV68 latency from the germinal center to memory B cell populations without M2 at 150-180 days post-infection (142). Histological examinations of spleen sections from infected animals at the onset of latency revealed that seeding of latency in the splenic follicles was impaired in the absence of M2 (142). In other words, without M2, seeding of latency in the splenic follicles but not expansion of the latent load in the germinal center reaction was compromised, and the latent MHV68 genomes were not efficiently exiting the germinal center pool into the long-lived memory B cell population. Together, the data from these studies demonstrate a role for M2 in the

manipulation of B cell biology to facilitate both the establishment of and reactivation from latency.

One major goal of this body of work was to carefully examine the role of M2 in MHV68 manipulation of B cell biology in order to better understand the mechanisms of gammaherpesvirus latency and reactivation. Previous studies expressed M2 in transformed cell lines; we wished to explore the effect of M2 expression in primary murine B cells, a system capable of differentiation in order to more faithfully mimic the biology of M2 observed *in vivo*. The second question addressed in this dissertation is what effect the differentiation state of the host B cell has on latency and reactivation. Specifically, this study sought to address the role of B lymphocyte-induced maturation protein-1 (Blimp-1), the master transcriptional regulator of plasma cell differentiation, in the establishment of and reactivation from MHV68 latency.

Plasma B cell Differentiation

Plasma B cells arise through similar differentiation pathways depending on the antigenic stimulation received by their B cell precursors (reviewed in (21, 41, 93)) (Figure 2). B-1 and marginal zone B cells are considered "innate-like" B cells and respond first to pathogens. Marginal zone B cells reside adjacent to the marginal sinuses in the spleen where they are bathed with blood-borne antigens. The BCR specificity of marginal zone B cells is biased towards T-independent antigens (such as NP-Ficoll), and, upon BCR and toll-like receptor (TLR) stimulation, they can rapidly differentiate into short-lived plasmablasts in the periarterial lymphatic sheath (PALS) without entering a germinal center reaction.

Marginal zone B cells are thought to contribute the early burst of low affinity IgM in an immune reaction, but they can enter the germinal center reaction if stimulated with a T-dependent antigen. B-1 B cells reside in the peritoneal cavity, can self-renew, and are responsible for the natural antibody found on mucosal surfaces. Like marginal zone B cells, B-1 B cells respond to microbial T-independent antigens, although they secrete IgA when they become plasma cells.

Conventional B-2 B cells, or follicular B cells, are the lymphocytes most commonly thought of as "B cells". Following stimulation of the B-2 BCR in the context of T cell help by CD40 ligation in the T-cell zone of lymphoid tissues, naïve B cells have two fates: 1) differentiate into short-lived plasmablasts and secrete low affinity antibodies in extrafollicular sites or 2) become activated and migrate to the primary follicles where they encounter follicular dendritic cells (reviewed in (79)). B-2 B cells rapidly proliferate within the follicle forming a germinal center reaction characterized by a dark zone made of densely packed centroblasts and a light zone of centrocytes surrounded by a network of follicular dendritic cells, T cells, and macrophages. These organizations are hardly static – there is recent evidence using intravital microscopy that B cells move between the light and dark zones during an active germinal center reaction and that cells proliferate in both areas (4, 60, 134). In the germinal center, centroblasts undergo somatic hypermutation and class-switch recombination mediated by the activity of activation-induced cytosine deaminase (AID) (67, 95, 110, 122). BCRs with the highest affinity for antigen are selected and cells further differentiate into memory B cells, residing in the periphery, or long-lived plasma cells which home to the bone marrow and secrete high-affinity antibody.

While it is clear that B cells must undergo a germinal center reaction to generate high affinity, class-switched immunoglobulin, there is a disagreement in the literature as to how exiting B cells reach a terminally differentiated state. One view is that there is a bifurcation in differentiation during the germinal center reaction towards either plasma or memory B cell (72). The second model proposes a linear progression of differentiation from a centrocyte to a memory B cell to a pre-plasma memory B cell to a plasmablast, and, finally, into a plasma cell (139). In order to exit the germinal center reaction and differentiate into a plasma cell, a highly orchestrated series of events must occur in order to ensure that somatic hypermutation, affinity maturation, and selection proceed. Likewise, tight control is needed to ensure that differentiation from a memory B cell to a plasma cell happens only upon re-stimulation with cognate antigen. Three transcriptional regulators have been shown to be key in directing differentiation towards a plasma cell fate *in vivo*: B lymphocyte-induced maturation protein-1 (Blimp-1), IFN-regulatory factor-4 (IRF-4), and X box-binding protein-1 (XBP-1) (78, 120, 139). This study will focus on the role of Blimp-1 in MHV68 pathogenesis.

Blimp-1: master transcriptional regulator of plasma cell development

B lymphocyte-induced maturation protein-1 (Blimp-1) is necessary for plasma cell differentiation *in vitro* and *in vivo* (139, 165). Blimp-1 was isolated by a subtractive hybridization screen of BCL1 B cells stimulated by IL-2 and IL-5 to secrete IgM versus unstimulated cells (165). Ectopic expression of Blimp-1 in

BCL1 cells leads to induction of J chain synthesis, upregulation of Syndecan-1 (CD138), as well as increased size and granularity (165). It was quickly confirmed that primary splenic B cells, too, upregulate Blimp-1 and IgM in response to stimulation with IL-2, IL-5, LPS, in conjunction with crosslinking the BCR (133). Transcriptional analysis showed that Blimp-1 is transcribed during plasma cell differentiation and highest Blimp-1 expression appears to be in the long-lived plasma cell population (73, 165). Blimp-1 contains five tandem Krüppel zinc finger motifs and localizes to the nucleus, leading to the early hypothesis that Blimp-1 mediates its effects by binding DNA (165).

The Blimp-1 protein was earlier isolated as a repressor of interferon-β, designated PRDI-BF1, (75) and it is now appreciated that Blimp-1 is a potent transcriptional repressor of many key genes in B cell biology (recently reviewed in (41, 100)). Blimp-1 expression leads to exit from the germinal center reaction and extinguishes the mature B cell gene expression pattern by repression of *c-myc*, *CIITA*, *PAX5*, *Spi-B*, *Bcl-6*, and *Id3*, silencing key pathways involved in cell proliferation, mature B cell lineage commitment, DNA synthesis and repair, BCR signaling, and isotype switching (94, 117, 121, 135). Additionally, Blimp-1 orchestrates plasma cell differentiation by inducing XBP-1 expression, a transcriptional activator that is necessary for the expansion of the ER in the unfolded-protein response, allowing for increased protein synthesis, thus turning a B cell into an antibody secreting factory (120, 135, 136). Bcl-6, a germinal center transcriptional repressor, and Blimp-1 form a developmental feedback loop; each protein represses the other, allowing a B cell to be either activated,

proliferating in the germinal center or an antibody-secreting plasma cell respectively (120).

In vivo, Blimp-1 is detected by immunohistochemistry in plasma cells, both short and long lived, arising from either T-dependent or T-independent immunizations, during both primary and secondary responses (6). Intriguingly, a Blimp-1^{+} population of B cells is observed in the germinal center that is of intermediate phenotype between germinal center and plasma B cells (proliferating, yet PNA^{-}, cytoplasmic light chain positive, $CD138^{+,}$, $IRF4^{+}$, $Bcl\text{-}6^{-}$) (6). Importantly, memory B cells do not express Blimp-1 (6). Mice with homozygous disruptions of the Blimp-1 locus die late in gestation, thus, studies of Blimp-1 *in vivo* have taken advantage of two different systems to delete Blimp-1 in the lymphocyte compartment.

The Blimp-1 protein is encoded by the *prdm1* gene, and in one system exons 5-8 of the *prdm1* gene have been flanked by LoxP sited to generate $prdm1^{flox/flox}$ mice that can be crossed to mice expressing Cre recombinase under the control of various promoters (referred to as conditional Blimp-1 mice) (101, 132, 139, 140). A second group has inserted green-florescent protein (Gfp) cassette into the *prdm1* locus ($prdm1^{Gfp/Gfp}$), creating Blimp-1 reporter mice that have nonfunctional Blimp-1 protein (73). $Rag1^{-/-}$ mice reconstituted with fetal liver cells from $prdm1^{Gfp/Gfp}$ embryos have Blimp-1 disrupted in all lymphocytes (referred to as Blimp-1 reporter mice) (73, 139). Both strains of Blimp-1-deficient mice lack plasma cells and have low levels of serum immunoglobulin, yet the memory B cell compartment is intact (72, 139). In the absence of Blimp-1 expression, mice do not secrete high levels of antibody when immunized with T-

dependent or T-independent antigens (72, 139). Without Blimp-1 expression in B cells (in $prdm1^{flox/flox}CD19^{Cre/+}$ mice), there is a post germinal center block in B cell development; memory B cells are present, but mice lack pre-plasma memory B cells, plasmablasts, and plasma B cells (72, 139). Data from heterozygous Blimp-1 reporter mice confirmed that Blimp-1 expression increases from maturation from a plasmablast to a long-lived plasma cell (73). Pax5 is downregulated in Blimp-1 reporter mice before Blimp-1 is expressed, indicating an early, Blimp-1-independent phase of plasmacytic differentiation termed a preplasmablast (72). These preplasmablasts secrete low levels of Ig, and they could be responsible for the low serum Ig observed in the conditional Blimp-1 mice (72). Although Blimp-1 may be dispensable for the initiation of plasma cell differentiation, it is required to both complete and maintain plasma cell differentiation in the bone marrow and, thus, for the high levels of serum antibody produced by terminally differentiated plasma cells (140). Additionally, B-1 B cells, an innate-like B cell in the peritoneal cavity that responds to conserved antigens, requires Blimp-1 expression for secretion of Ig (132).

Intriguingly, recent research revealed a role for Blimp-1 in T cell differentiation and homeostasis (74, 101). Mice with Blimp-1-deficient T cells ($prdm1^{flox/flox}Lck\text{-}Cre$) develop colitis and have increased numbers of effector and memory T cells (74, 101). Although overall numbers of T_{reg}s remain the same in these animals, their $CD4^+CD25^+$ T_{reg}s secrete less IL-10, whereas, following TCR ligation, the Blimp-1-deficient $CD4^+$ T cells proliferate more and produce increased amounts of IFNγ and IL-2 (101). Similar to the studies of Blimp-1 in B cells, Nutt et al. examined the role of Blimp-1 in T cells using Blimp-1 reporter

mice and found a similar increase in effector and memory T cell populations (74). Blimp-1 reporter mice develop a lethal lymphoproliferative syndrome characterized by extensive lymphocyte infiltration, tissue inflammation, and destruction of the lung, liver, and spleen, and Blimp-1-deficient $CD4^+$ T cells have enhanced proliferation and secretion of IFNγ and decreased IL-10 this system (74). Together, these studies indicate a key role of Blimp-1 in the regulation of not only B cell terminal differentiation, but in T cell fate and proliferation as well.

Plasma cells and gammaherpesvirus reactivation

In EBV pathogenesis, differentiation to a plasma B cell phenotype is associated with reactivation (30, 86) (see Figure 3). Histological studies of EBV latently infected B cell lines showed an association of lytic viral proteins with B cell maturation (30). Staining of sections from tonsils of infectious mononucleosis patients also revealed a correlation between lytic transcription and plasma cell differentiation (5). Using FACS sorting, tonsillar plasma cells from EBV patients were shown to be positive for BZLF1 transcripts *in vivo* (86). BZLF1 encodes for the protein Zta which is the master lytic transactivator for EBV, and, therefore, presence of BZLF1 transcripts implies lytic activity in the plasma cells (59). X-box Binding Protein-1s (XBP-1s), a transcription factor required for plasma cell differentiation, can transactivate the BZLF1 promoter (13, 120). During plasma cell development, the activation of the unfolded protein response (UPR) due to ER stress of producing copious amounts of antibodies leads to induction of XBP-1s. Recently, XBP-1s has been shown to directly bind

and activate the BZLF1 promoter, functionally linking plasma cell differentiation and EBV reactivation (155).

Plasma cell differentiation is also associated with KSHV reactivation. KSHV was initially isolated from Kaposi's sarcoma lesions from patients with AIDS, but the virus is also associated with primary effusion lymphoma (PEL), also termed body-cavity-based lymphoma (24, 25). PEL presumably originates from B cells for the lymphomas carry evidence of VDJ rearrangement, but many PELs lack surface or cytoplasmic immunoglobulin, have neither B nor T cell surface markers, and can express a variety of surface markers of activation (24). However, a high frequency of PELs do express surface CD138 (Syndecan-1, a surface marker of plasma cell differentiation), Blimp-1 transcripts, and genes involved in the unfolded protein response (52, 70, 80). Microarray experiments indicated that PELs display a plasmablastic gene expression profile, a post-germinal center intermediate between immuoblasts and fully differentiated plasma cells (70, 80). Ectopic expression of XBP-1s in KSHV latently-infected cell lines leads to activation of the RTA (replication and transcription activator) promoter in luciferase assays (188). RTA, encoded by ORF50, is the master transcriptional regulator of KSHV reactivation; RTA expression is both necessary and sufficient to induce the KSHV lytic cycle (96, 156). XPB-1s binds to the ORF50 promoter and induces KSHV reactivation from PELs and, therefore, plasma cell differentiation is associated with both lymphomagenesis and reactivation of KSHV (186).

Gammaherpesviruses famously manipulate B cell biology to establish and reactivate from latency, often leading to transformation of the host lymphocyte as

discussed above. We hypothesized that MHV68 pathogenesis, like EBV and KSHV, is intimately linked to the biology of the host B cell. Earlier studies of the M2 protein hinted at a possible role for M2 in the manipulation of B cell signaling, therefore, we examined the role of M2 in primary murine B cells. Due to the high degree of phenotypic similarity between the pathologies of EBV, KSHV, and MHV68, we sought to determine if there was a similar role of plasma cell differentiation in MHV68 reactivation and latency. Together, these studies of M2 in B cells and of plasma cell differentiation in reactivation reflect both sides of the host/pathogen relationship of MHV68.

FIGURES

Figure 1.

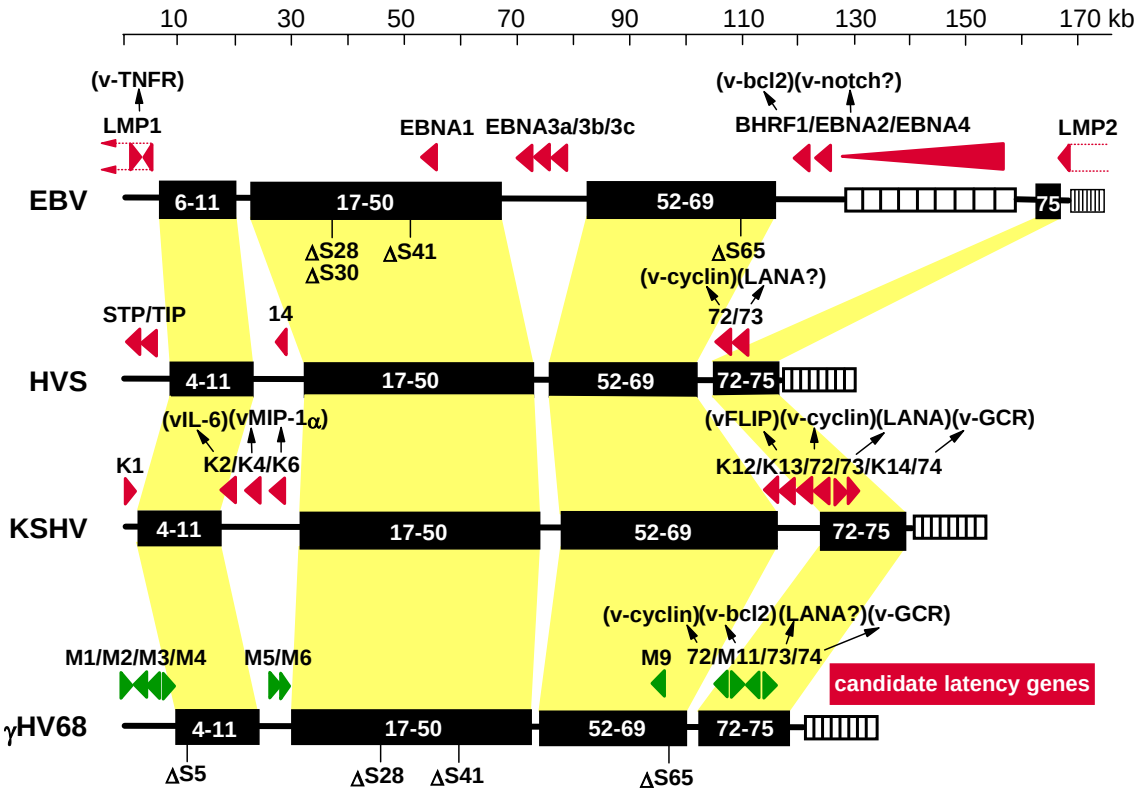

Figure 2.

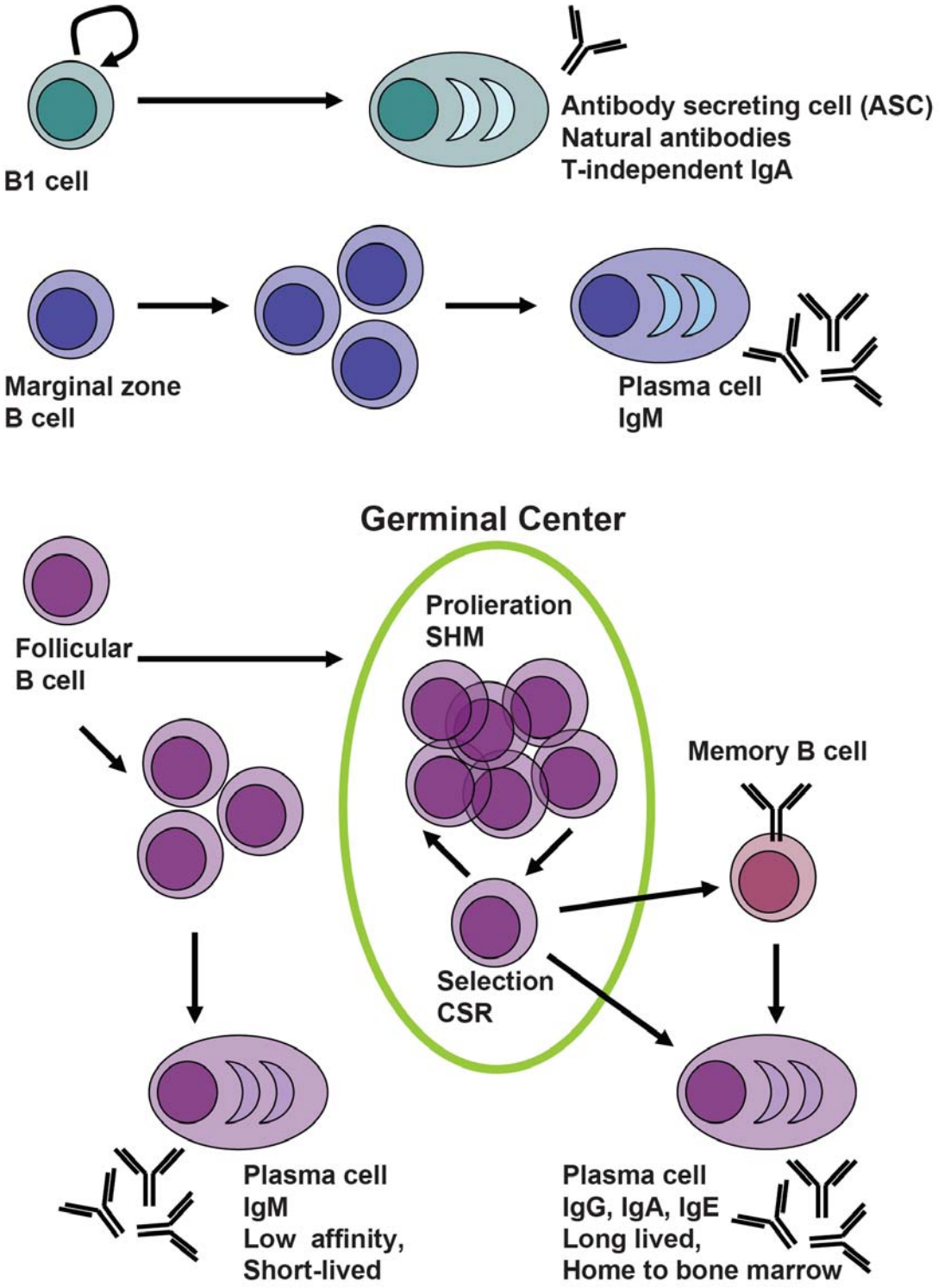
B1 cell
Antibody secreting cell (ASC)
Natural antibodies
T-independent IgA
Marginal zone
B cell
Plasma cell
IgM
Germinal Center
Follicular
B cell
Prolieration
SHM
Memory B cell
Selection
CSR
Plasma cell
IgM
Low affinity,
Short-lived
Plasma cell
IgG, IgA, IgE
Long lived,
Home to bone marrow

Figure 3.

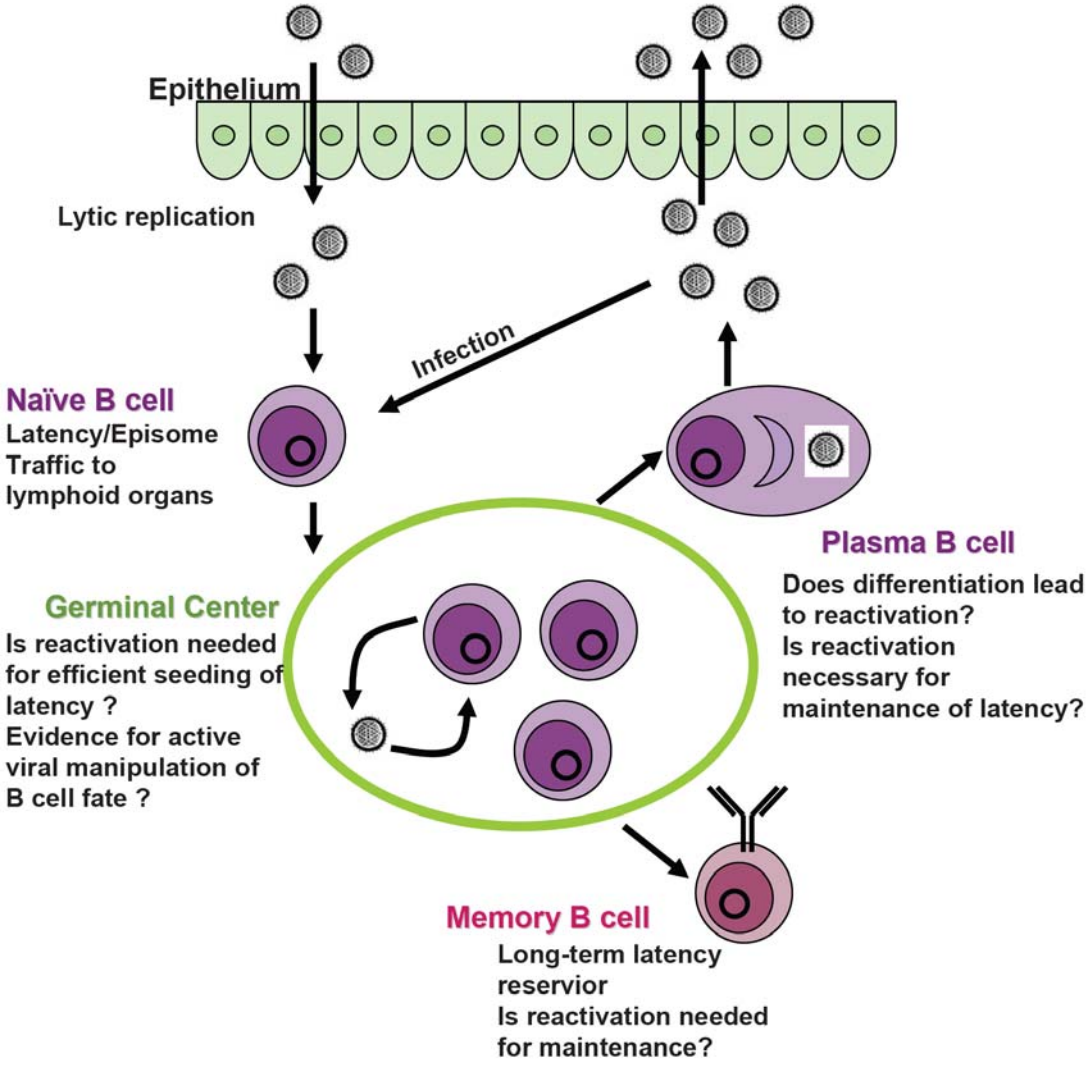
Epithelium
Lytic replication
Infection
Naïve B cell
Latency/Episome
Traffic to
lymphoid organs
Plasma B cell
Does differentiation lead
to reactivation?
Is reactivation
necessary for
maintenance of latency?
Germinal Center
Is reactivation needed
for efficient seeding of
latency ?
Evidence for active
viral manipulation of
B cell fate ?
Memory B cell
Long-term latency
reservior
Is reactivation needed
for maintenance?

FIGURE LEGENDS

Figure 1. Colinear depiction of the genomes of four members of the gammaherpesvirus family: EBV, KSHV, HVS, and MHV68. The human herpesviruses, EBV and KSHV, share regions of extensive genetic homology, shown in yellow, with the primate HVS and murine MHV68 viruses. These viruses display extensive phenotypic similarities as well. At the left and right ends of the viral genomes are regions unique to each virus with virus-specific genes shown in red and green. Some of the proteins encoded in this region, such as LMP1 and K1, manipulate B cell signaling. The M2 open reading frame, studied in this dissertation, is found in the left end of the MHV68 genome and plays a role in the establishment and reactivation from latency.

Figure 2. Plasma cells arise from multiple B cell subsets. B1 B cells have conserved BCRs and secrete natural antibodies and IgA. Marginal zone B cells are bathed in blood-borne antigens and are stimulated to secrete low-affinity IgM in response to T-independent stimuli. Follicular B cells can give rise to short-lived plasma cells or enter the germinal center reaction and differentiate into high-affinity, class-switched, long-lived plasma cells. Adapted from (138).

Figure 3. A model of MHV68 establishment and reactivation from latency. Following intranasal infection, lung epithelium are lytically infected with MHV68. Resident naïve B cells are infected and carry latent viral genomes to the spleen. MHV68-infected B cells then undergo the germinal center

reaction, and latency is expanded in the proliferating B cell subset. Reactivation of infected B cells is hypothesized to further disseminate virus and seed the latency pool. Upon exiting the germinal center reaction, MHV68 establishes long-term latency in memory B cells. We hypothesize that differentiation to a plasma cell phenotype will be necessary for MHV68 reactivation, as demonstrated for EBV and KSHV. Furthermore, we suggest that upon reencounter with its cognate antigen, memory B cells differentiate into plasma cells, stimulating MHV68 reactivation, infection of naïve B cells, and refilling of the latency reservoir. Adapted from (163).

CHAPTER II

Chapter II has been published in the Public Library of Science Pathogens (Siegel, A.M., Herskowitz, J.H., and Speck S.H. (2008) The MHV68 M2 Protein Drives IL-10 Dependent B Cell Proliferation and Differentiation. *PLoS Pathog*. 4(4): e1000039. doi:10.1371/journal.ppat.1000039).

INTRODUCTION

Herpesviruses establish life-long, latent infections characterized by episodic virus reactivation and subsequent virus shedding. Chronic infections with the lymphotropic gammaherpesviruses are associated with a variety of lymphomas and carcinomas which in humans includes Burkitt's lymphoma, nasopharyngeal carcinoma, Hodgkin's disease and Kaposi's sarcoma. The narrow host range of the gammaherpesviruses that infect humans, Epstein-Barr Virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV), has severely hindered detailed pathogenesis studies. Murine gammaherpesvirus 68 (MHV68; also known as γHV68 and murine herpesvirus 4) shares extensive genetic homology and biological similarity with both EBV and KSHV and is a natural pathogen of wild murid rodents. As such, MHV68 infection of inbred strains of mice has gained favor as a small animal model in which to evaluate viral and host determinants of gammaherpesvirus pathogenesis *in vivo*.

Upon intranasal infection, MHV68 infection results in acute viremia in the lung that is resolved into a latent infection of B cells, dendritic cells, and macrophages (46). B cells are necessary for trafficking of virally infected cells to the spleen, leading to the establishment of splenic latency (152, 183). The $CD8^+$ T cell response is critical for control of lytic infection in the lung as well as establishment of latent viral load in the spleen (37). MHV68 infection results in a $CD4^+$ T cell-dependent expansion of splenic B cells and both virus-specific and non-specific hypergammaglobulinemia (128, 168). Similar to EBV pathogenesis, memory B cells are the primary long-term reservoir of latent MHV68 in mice (8, 76, 185).

All herpesviruses manipulate the host's immune system to establish and maintain a long-term, latent infection, and many of these immunomodulatory mechanisms are conserved among the members of the gammaherpesvirus family. Both KSHV and MHV68 encode proteins, K3 and mK3, respectively, that downregulate MHC I (151). MHV68 also encodes a viral bcl-2 homolog, a viral cyclin, and a chemokine-binding protein, M3 (53, 171, 172). The EBV proteins LMP1 and LMP2a mimic CD40 and tonic BCR signals, respectively, to manipulate B cell development, and they are believed to enable the virus to gain access to the memory B cell compartment independent of antigenic stimulation of the host B cell (22, 166). KSHV encodes both a viral IL-6 and a viral MIP-1α ortholog, while EBV encodes a viral IL-10 homolog, BCRF1 (or vIL-10) (111, 127).

Interleukin-10 (IL-10) was first noted as a cytokine synthesis inhibitory factor (CSIF) that is secreted by T_H2 cells and suppresses the activity of T_H1 cells (42). IL-10 enhances murine B cell viability and can activate human B cell

proliferation and class switching in culture (56, 127). In addition, IL-10 suppresses T_H1 responses through modulation of macrophage function by downregulation of MHC II and costimulatory molecules as well as inhibition of cytokine production and macrophage effector functions (43, 44). Dendritic cells exposed to IL-10 do not down-regulate costimulatory molecules but do secrete lower levels of IL-12, impairing their ability to induce a T_H1 response (33).

The M2 protein, unique to MHV68, has been shown to play a critical role in both the establishment of latency as well as reactivation from latency (62, 68, 97). A M2-null strain of MHV68 (MHV68/M2.Stop) replicates with wild-type efficiency in mice following intranasal inoculation but exhibits a dose-dependent defect in the establishment of latency at day 16 post-infection (62, 68). Under conditions in which the MHV68/M2.Stop mutant can efficiently establish a latent infection (high dose intranasal inoculation or low dose intraperitoneal inoculation), the M2-null virus exhibits a profound reactivation defect, revealing dual roles for the M2 protein in the viral life-cycle (62, 68). Additionally, efficient transition of latently-infected B cells from the germinal center reaction to the memory B cell reservoir appears to be stalled in the absence of M2, suggesting M2 may manipulate B cell signaling or differentiation to facilitate establishment of long-term latency in the memory B cell pool (62, 142). Numerous candidate SH3 binding motifs throughout M2 suggest the protein may function as a molecular scaffold that may modulate specific cellular signal transduction pathways. Consistent with this hypothesis, M2 has been shown to interact with a number of cellular proteins in vitro. M2 co-immunoprecipitates with Vav1 in S11 B cells, a MHV68 latently infected cell line, and M2 and Vav1

overexpression in A20 B cells leads to Vav1 phosphorylation, trimerization with Fyn, and downstream activation of Rac1 (125). In fibroblast cultures, M2 interacts with DDB1/COP9/cullin repair complex and ATM to suppress DNA-damage induced apoptosis (90). In addition, M2 can suppress STAT1/2 expression, leading to inhibition of the interferon response (91). However, to date the impact of M2 expression in primary murine B cells has not been reported.

Here we show that one function of the M2 protein is to induce expression of IL-10 in primary B cells, demonstrating a common immunomodulatory strategy utilized by those gammaherpesviruses encoding a viral IL-10 homolog and MHV68.

MATERIALS AND METHODS

Mice and infections. Female C57Bl/6 and IL-10$^{-/-}$ mice 6 to 8 weeks of age were purchased from the Jackson Laboratory. Mice were sterile housed and treated according to the guidelines at Emory University School of Medicine (Atlanta, GA). Following sedation, mice were infected intranasally with 1000 pfu of either MHV68/M2.Stop or MHV68/M2.MR in 20 μL of cMEM. Mice were infected with 100 pfu of MHV68/M2.Stop or MHV68/M2.MR in 500 μL of cMEM intraperitoneally. Mice were allowed to recover from anesthesia before being returned to their cages.

B cell isolation. Spleens were homogenized and erythrocytes removed by hypotonic lysis. B cells were enriched using negative selection by magnetic cell separation with the mouse B Cell Isolation Kit (Miltenyi Biotech). Purity was confirmed by staining for CD19, and B cells used in experiments were 93-97% pure. Cells were cultured in RPMI-1640 supplemented with 10% FCS, 100 U/mL penicillin, 100 mg/mL streptomycin, 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 10 mM non-essential amino acids, and 25 μg/mL of LPS (Sigma) overnight before retroviral transduction.

Cloning, retroviral production, and transduction. BglII sites were cloned flanking the M2 ORF with primers 5' CAG CTC AGA TCT ATG GCC CCA ACA CCC 3' and 5' CAG CTC AGA TCT TTA CTC CTC GCC CCA 3' and cloned into pCR-Blunt (Invitrogen). Positive clones were sequenced, digested with BglII, and

cloned into the pMSCV-IRES-Thy1.1 vector (a gift from Phillipa Marrack) to construct pMSCV-M2-IRES-Thy1.1. pMSCV-M2Stop-IRES-Thy1.1 was constructed in a similar manner. Retroviruses were produced using the BOSC23 producer cell line (ATCC). 2 x 10^6 BOSC23 cells were plated on 60 mM Collagen II coated plates. The following day, 10 ug of pMSCV vector was transfected into the BOSC23 cells using the LT-293T reagent from Mirus Biotech. Retroviral supernatants were harvested 48 to 72 hours post-transfection, centrifuged at 2000 rpm for 10 minutes to clear cell debris, and supplemented with 5 μg/mL of polybrene. B cells were transduced by removing 700 μL of media and replacing it with 1 mL of retroviral supernatant/polybrene. Cells were spun at 2500 rpm at 30°C for one hour. 750 uL of retroviral supernatant was removed and replaced with fresh, complete RPMI. Cells were rested for 48 hours before analysis.

In some analyses recombinant IL-10 was added back to transduced primary B cell cultures as follows. Primary murine B cells were harvested from C57Cl6 and IL-$10^{-/-}$ mice as previously described. Transduction effienceies were measured 48 hours post-transduction by flow cytometry. Following day 2 analysis, indicated cultures received 20 ng/mL of murine recombinant IL-10 (Peptrotech). B cell populations were analyzed on days 3-5 post-transduction by flow cytometry.

Immunoprecipitations and western blotting. Cells were lysed in a suitable volume of ELB buffer on ice for 20 minutes. Lysates were pre-cleared with pre-immune chicken IgY, and M2 precipitated with chicken anti-M2 IgY followed by capture by agarose anti-IgY beads (Aves Labs, Inc.). Precipitates

were run on a 15% acrylamide gel, transferred to nitrocellulose membranes, and blotted with rabbit anti-M2 antisera followed by donkey anti-rabbit HRP. Protein was detected using chemiluminescence on Kodak X-Omat Blue XB-1 film.

Flow cytometry. Rat anti-mouse CD16/32 (Fc block) was used prior to staining in most experiments. Cells were stained with the following antibodies: Thy1.1-FITC, -PE, or –APC (eBiosciences), CD44-FITC (Caltag), CD62L-PE (Caltag), GL7-FITC, $IgG_{1, 2a, 2b, 3}$-FITC, CD25-PE, CD138-PE, $I\text{-}A^b$-PE, CD4-PerCP, CD11a-PE-Cy7, CD19-APC, B220-APC, CD8-PacficBlue (BD Pharmigen except where noted). Tetramers were synthesized at the NIH Tetramer Core Facility at Emory University and conjugated to streptavidin-APC (Molecular Probes) according to core protocol. Intracellular bromodeoxyuradine incorporation was measured using BrdU-APC according to the manufacturer's protocol (BD Pharmigen). AnnexinV-PacificBlue and 7-AAD reagents were purchased in the Vybrant® Apoptosis Assay Kit #14 (V35124) from Molecular Probes and used per manufacturer's protocol. Cells were analyzed on FACScalibur or LSR II flow cytometer. Data was analyzed using FlowJo software (TreeStar, Inc., San Carols, CA).

Cytokine array and enzyme-linked immunosorbent assays (ELISAs). TranSignal™ Mouse Cytokine Antibody Arrays 1.0 (Panomics, Inc.) were used to screen for secreted cytokines as per manufacturer's instructions. Membranes were blocked in Blocking Buffer for two hours, washed, and then incubated for two hours at room temperature with day four supernatants from B cells

transduced with MSCV-M2 or MSCV-M2.Stop. Membranes were washed and incubated with Biotin Conjugated Anti-Cytokine Mix as per protocol. Membranes were washed and incubated with Streptavidin-HRP. After a final wash, bound cytokine was detected using chemiluminescence on Kodak X-Omat Blue XB-1 film. Cytokines were quantitated by ELISA. IL-6 and IL-10 were detected with reagents from BD Biosciences, and IL-2 and MIP-1α were detected with reagents from R&D Biosystems. IgM and IgG were detected with reagents from Bethyl Biosciences.

Transfection of murine A20 cell line. Triplicate cultures of 1 x 10^6 A20 B cells were nucleofected with 4 ug of pIRES2-EGFP (BD Biosciences Clontech), pM2-IRES-EGFP (63), or pBluescriptIISK (Stratagene) using Solution T with setting T-01 (Amaxa). Cells transduced with pBluescriptIISK were stimulated with 100 ng/mL of LPS following nucleofection as indicated. 48 hours post-nucleofection, supernatants were harvested and secreted IL-10 measured by ELISA (BD Biosciences).

MHV68 viral mutagenesis. A γHV68 genomic fragment containing the region from bp 2403 to bp 6262 (WUMS sequence) (177) was cloned into the Litmus-38 plasmid (Lit38-M2) as previously described (68). With Lit38-M2 as a template, a stop codon was introduced into the M2 ORF using the following oligonucleotides: Oligo1 (5' CCA CCA GGC CGA AGC TTA CGG ATT GGG AAT C) and Oligo2 (5' CCA ATC CGT AAG CTT CGG CCT GGT GGA TG) generating a translational stop codon at bp 4566 and introducing a *Hind* III restriction site.

The resultant product was ligated into the pCR Blunt plasmid (Invitrogen). In addition, an M2 marker rescue pCR Blunt plasmid was generated by PCR using Lit38-M2 as a template and designated as M2.MR. M2.Stop pCR Blunt plasmid and M2.MR were sequenced to verify the introduction of the site directed point mutations and the absence of unwanted mutations. Recombinant viruses were generated by allelic exchange in *E. coli*, as described by Smith and Enquist (106, 144). Briefly, the *Not* I and *Bam* HI restriction sites within pCR Blunt were used to liberate the γHV68 genomic region contained within the plasmid. This fragment was cloned into the suicide vector pGS284 which harbors an ampicillin gene and a levansucrase cassette for positive and negative selection, respectively. The resulting plasmid was transformed into S17λpir *E. coli* cells and mated to GS500 *E. coli* ($RecA^+$) containing wt γHV68 BAC. Cointegrants were selected on Luria-Bertani (LB) agar plates containing chloramphenicol (Cam) and ampicillin (Amp) and were resolved following overnight growth in LB medium with Cam. Next, bacteria were plated on LB agar plates containing Cam and 7% sucrose to select for loss of pGS284 vector sequence. Individual colonies harboring site specific point mutations within M2 were identified by colony PCR followed by restriction digest. Positive clones were grown in LB medium with Cam, and BAC DNA was purified with a Midi Prep Kit (Qiagen, Hilden, Germany) as described by the modified manufacturer's protocol. The presence of site specific point mutations and the absence of unwanted mutations within the region of homologous recombination were confirmed by sequencing and southern blot. Virus stocks were generated by Superfect (Qiagen, Hilden, Germany) transfection of recombinant γHV68 BAC DNA into Vero-Cre cells as previously described

(106). In wells showing cytopathic effect (CPE), virus was harvested, cleared of cell debris, and used to infect Vero-Cre cells in order to generate high-titer stocks. Following the presence of CPE in Vero-Cre cells, samples were harvested, homogenized, clarified, and aliquoted for storage at -80°C. Virus stock titers were determined by plaque assay as previously described (27, 68).

MHV68 viral assays. Limiting dilution assays for frequency of latent were performed as previously described (62, 68). To determine the frequency of cells harboring latent viral genomes, single-copy-sensitive nested PCR was performed. Frozen samples were thawed, washed in isotonic buffer, counted, and plated in three-fold serial dilutions in a background of 10^4 NIH 3T12 cells in 96 well plates. Cells were lysed by protease K digestion for six hours at 56°C. Two rounds of nested PCR were performed per sample with twelve samples per dilution, and the products were resolved on 2% agarose gels. In order to measure the frequency of reactivating splenocytes, bulk splenocytes were resuspended in cMEM and plated in serial two-fold dilutions on mouse embryonic fibroblast (MEF) monolayers in 96-well tissue culture plates. Parallel samples of mechanically disrupted cells were plated to detect preformed infectious virus. Wells were scored for cytopathic effect 14 to 21 days post-explant.

In vivo cytokine capture assay. The Mouse IL-10 *In Vivo* Capture Assay Set (BD Biosciences) was used to detect IL-10 *in vivo* during infections. On day 14-15 p.i., parallel groups of five mice were injected with 10 μg of biotinylated rat anti-mouse IL-10 antibody in 200 μl of sterile PBS. Mice were bled on day 15-16 p.i.

and serum collected. Samples were prepared and assayed by ELISA as per protocol. The limit of detection of this assay is 31.3 pg IL-10/mL of serum.

Statistical analysis. Data analysis was conducted using GraphPad Prism software. Error bars in all graphs depict standard error of the mean. For limiting-dilution analysis, data was subjected to nonlinear regression analysis with a sigmoidal dose-response algorithm for best-fit. Poisson distribution predicts that the frequency at which 63.2% of wells are positive for an event (PCR or reactivation) is the frequency at which there is at least one event present in the population. Statistical significance of the flow cytometry and ELISA data was determined by two-tailed, unpaired Student's T test with a confidence level of 95%.

RESULTS

MH68 M2 protein augments LPS-driven B cell proliferation.

The MHV68 M2 protein has been shown to be critical for both establishment and reactivation from B cell latency. M2 has no known homologous proteins, viral or cellular, and contains numerous SH3 binding motifs through which it can potentially manipulate B cell biology. Proliferating B cells harbor the majority of latent MHV68 genomes, and splenic B cell activation is associated with MHV68 infection at the onset of latency (82, 108). We asked whether expression of M2 in primary murine B cells in vitro altered proliferation or activation. B cells were purified by negative selection from mouse splenocytes, stimulated overnight with LPS, and transduced with either an M2 protein expressing recombinant murine stem cell virus (MSCV) retrovirus, MSCV-M2-IRES-Thy1.1, or a control retrovirus, MSCV-M2.Stop-IRES-Thy1.1, which harbors a translation termination codon near the 5' end of the M2 open reading frame at amino acid 13 (Fig. 1A). LPS stimulation is necessary for efficient retroviral transduction in this system because MSCV infection requires the cells to be in cycle (92). The presence of an IRES-Thy1.1 cassette readily allowed retroviral transduction efficiency to be monitored by flow cytometry for surface expression of Thy1.1. Notably, expression of the M2 protein from the retroviral construct could be detected as demonstrated by immunoprecipitation and immunoblotting of whole cell lysates harvested from primary B cells transduced with MSCV-M2-IRES-Thy1.1 at four days post-transduction (Fig. 1B). It should be noted that detection of M2 expression in the transduced primary murine B cells required

immunoprecipitation with a chicken anti-M2 antisera raised against two M2 peptides, followed by immunoblotting with a rabbit polyclonal antiserum raised against a bacterially expressed recombinant M2 protein. In contrast, M2 expression in the MHV68 latently infected B cell lymphoma line S11 can be detected by immunoblotting S11 lysates with the rabbit polyclonal anti-M2 antiserum (data not shown). Thus, it does not appear that M2 is "over-expressed" in transduced primary murine B cells.

Two days post-transduction, there were similar frequencies of transduced, Thy1.1$^+$ B cells in the control and M2-transduced B cell cultures. However, by 5-6 days post-transduction, nearly 100% of the B cell culture transduced with MSCV-M2-IRES-Thy1.1 was Thy1.1$^+$ as compared to ca. 20% of the culture transduced with the control vector, MSCV-M2.Stop-IRES-Thy1.1 (Fig. 1C). Notably, the dominance of M2-expressing, Thy1.1$^+$ B cells in the M2-transduced cultures was observed repeatedly. The increase in the percentage of Thy1.1$^+$ cells in the M2-transduced culture was gradual, and it did not correspond to an increase in overall cell number in the cultures or a decrease in cell death (Fig. 1D). The latter result suggests that in a mixed culture (M2 expressing and non-expressing cells), the non-transduced primary B cells are actively selected against. This could either be due to the secretion of a "toxic" factor by the M2 expressing cells or competition for a limiting factor necessary for cell survival.

Upon observing the M2-transduced cells dominating the culture, we asked whether M2 was influencing B cell survival, proliferation, or both. To directly assess B cell survival in M2-transduced and control retrovirus cultures (M2.Stop), cells were stained with anti-Thy1.1, Annexin V, and 7-AAD and

analyzed by flow cytometry. In contrast to the results obtained by trypan blue exclusion which measured the live/dead ratio in the entire culture (Fig. 1D), flow cytometry of the transduced and untransduced populations within the culture revealed a survival advantage of the M2-transduced B cells. At day 2 post-transduction, 20% more of the M2-transduced B cells were alive (AnnexinV$^-$ 7-AAD$^-$) than the untransduced cells in the same culture (Fig. 2 B). At day 3 post-transduction, there was a four-fold higher frequency of live cells in the M2-transduced population as compared to the untransduced cells in culture (Fig. 2, panels A & B). M2-transduced cells continued to survive better than the untransduced cells in the population, despite an equal frequency of cells entering apoptosis (data not shown).

At day 2 post-transduction, the M2-transduced cells have equal frequencies of live cells as the control M2.Stop retrovirus transduced cells (Fig. 2B). Analysis at day 3 post-transduction revealed a 20% increase in the frequency of live cells in the M2-transduced population as compared to the cells transduced with the control retrovirus (Fig. 2B). This trend continued until the end of the time-course, with a higher frequency of live cells found in the M2-transduced population versus the M2.Stop retrovirus control (Fig. 2B). The increased frequency of live cells in the M2-transduced population versus both the untransduced cells within the culture as well as the control retrovirus transduced population reveals a pro-survival effect of M2 protein expression in B cells.

We next addressed whether M2 protein expression altered proliferation in the B cell cultures thereby contributing to the expansion of transduced cells. To directly assess B cell proliferation in M2-transduced and control retrovirus

cultures, cells were pulsed with bromodeoxyuradine (BrdU) for 24 hours at different time points post-transduction. Cells were surface stained for Thy1.1 and proliferation was measured by intracellular staining for incorporation of BrdU. The time course analyses revealed that B cells transduced with either the M2 or control retrovirus exhibited equivalent frequencies (84-90%) of proliferating cells 2 days post-transduction (Fig. 2D). However, 80-90% of M2-transduced B cells continued to proliferate 3 and 4 days post-transduction as compared to 40-50% of the cells transduced with the control retrovirus (Figs. 2, panel C & D). By 5-6 days post-transduction there was a significant drop in the proliferation of M2-transduced B cells (Fig 2D). These results indicate that M2 protein expression is able to transiently augment murine B cell proliferation. Thus, these analyses indicated that the M2 protein contributes to both enhanced B cell survival as well as promoting continued B cell proliferation post-LPS stimulation – which together leads to dominance of M2-transduced B cells in the primary murine B cell cultures over the time-course analyzed.

M2 protein expression leads to B cell differentiation.

The transition from the germinal center B cell population to the long-lived memory B cell compartment is critical for establishment of MHV68 latency (76, 185). Latent genomes in mice infected with M2-deficent MHV68 accumulate in the germinal center compartment late in infection, leading to the hypothesis that M2 is capable of manipulating B cell differentiation (142). To determine whether M2-transduction leads to differentiation of B cells, surface expression of B cell differentiation markers was analyzed by flow cytometry. At four days post-

transduction, B cells expressing M2 were $CD19^{+}$, $CD25^{high}$, $GL7^{high}$, $B220^{low}$, $I\text{-}A^{b\ low}$, surface IgD^{-} (sIgD), $sIgG^{+}$, and $CD138^{low}$ when compared to untransduced cells within the culture (Fig. 3 A). Strikingly, M2-transduced cells expressed higher levels of CD25 as compared to cells transduced with the control retrovirus, although the MFI of CD25 was similar between the two populations (Fig. 3A). Both transduced populations became surface IgG positive, likely due to LPS stimulation coupled with retrovirus infection selecting for the LPS-driven proliferating B cell population. However, the M2 protein expressing B cells expressed more sIgG than the control retrovirus transduced cells. Similarly, the M2 and M2.Stop transduced populations both upregulated CD138, although the presence of M2 did not lead to high levels of CD138 indicative of plasma cell differentiation. Notably, the other changes observed in B cell differentiation were unique to the M2-transduced B cell population versus the cells transduced with the control retrovirus. In addition, the M2-transduced B cells secreted significantly higher levels of IgG on days 4-6 post-transduction than the cells transduced with the control retrovirus (Fig. 3B). Secreted IgM levels remained similar throughout the time-course for M2 and control retrovirus B cell cultures (Fig. 3C). Importantly, M2-transduced cells express surface IgG and remain $CD138^{low}$, indicating that they have not fully differentiated into plasma cells. Together, these data demonstrate that M2 expression leads to B cell activation and differentiation similar to a functional activated, pre-plasma memory B cell phenotype, namely $CD19^{+}$, $sIgG^{+}$, $sIgD^{-}$, $B220^{low}$, $CD138^{low}$ (36, 139). However, we can not formally rule out that M2 expression leads to differential survival and expansion of a population of pre-plasma memory B cells present in the

transduced culture – although this seems unlikely based on the very low frequency of this population in the purified naïve splenic B cells used for these studies.

M2 protein expression leads to secretion of IL-10.

To further investigate the proliferative effects of M2 protein expression in primary murine B cells, the supernatants of the transduced B cells were screened for a variety of cytokines using a mouse cytokine antibody array (see Materials and Methods). Supernatants of B cell cultures transduced with M2 and control retrovirus were compared at four days post-transduction (Fig. 4A). Cytokine arrays performed in duplicate time-course experiments revealed substantial increases in IL-10, IL-2, IL-6, and MIP-1α in the culture supernatants of B cells expressing M2 compared to the control retrovirus transduced B cell cultures (Fig. 4A). Cytokine levels throughout the time-course analyses were subsequently quantitated by ELISA. IL-2 levels in the M2-transduced cultures peaked at 50 pg/mL of supernatant at day 4 and waned by day 6 post-transduction, while only 1-2 pg/mL of IL-2 were detected in the control retroviral supernatants (Fig. 4B). From 3 days post-transduction until the end of the time-course, the supernatants from M2-expressing B cells contained levels of IL-6 twice as high as those of B cells transduced with the control retrovirus (780 pg/mL vs. 400 pg/mL) (Fig. 4C). There was also a 10-fold increase in the level of MIP-1α with M2-transduced cultures containing an average of 1845 pg/mL of MIP-1α versus 139 pg/mL in the control retrovirus supernatant (Fig. 4D). Notably, we observed a 20-fold increase in IL-10 levels in the B cell cultures transduced with M2 with 17.5 ng/mL of IL-10

in the M2-transduced cultures as compared to 0.9 ng/mL in the control retroviral supernatants (Fig. 4E). Notably, the number of cells in the M2 protein expressing and control B cell cultures were not significantly different, and thus the observed differences in cytokine levels cannot be explain by an increase in cell number. These data demonstrate that M2 expression in primary murine B cells leads to enhanced secretion of several cytokines, most notably IL-10. Finally, to further assess the ability of M2 expression to upregulate IL-10 secretion from B cells, we transfected the murine A20 B cell line with either a control expression vector (pIRES-EGFP) or an M2 expression vector (pM2-IEGFP) and assessed IL-10 secretion by ELISA (Figure 4F). Untreated A20 cells secrete significant levels of IL-10, which were only modestly enhanced by LPS treatment (Figure 4F). In addition, transfection of the control expression vector had no impact on the levels of IL-10 secreted by A20 cells (Figure 4F). However, transfection with the M2 expression vector lead to a substantial increase in the levels of IL-10 secretion (Figure 4F). The latter result provides further evidence that M2 is able to increase IL-10 secretion by B cells – independent of LPS stimulation.

IL-10 is required for the M2-driven B cell proliferation.

IL-10 has been demonstrated to be involved in the establishment of a latent MHV68 infection, and we asked whether IL-10 played a role in M2-driven B cell proliferation (47, 114). To address the role of IL-10 in M2-driven proliferation, B cells were isolated from wild-type and IL-10$^{-/-}$ mice, transduced with M2 or the control retrovirus, and surface Thy1.1^{+} expression was monitored over a six day time course. Although the percentage of M2-transduced C57Bl/6 B

cells increased from 40% to 85% of the culture, as previously observed (see Fig. 1C), there was only a modest expansion of the Thy 1.1^{+} population from 37% to 49% in the IL-10$^{-/-}$ cultures transduced with M2 expressing MSCV retrovirus (Fig. 5A). ELISAs of the supernatants from the transduced cultures confirmed that the IL-10$^{-/-}$ B cells do not secrete detectable levels of IL-10 (Fig. 5B). We noted an approximately 10% increase in the percentage of IL-10$^{-/-}$ M2 protein expressing B cells over the time course experiments, and we hypothesize that this small increase might be due to the ability of the M2 protein to manipulate proliferation and/or survival pathways independent of IL-10. Notably, IL-10$^{-/-}$ mice have been shown to have 20-fold higher levels of serum IL-6 than IL-10-sufficient mice (83), and indeed we observed a two-fold increase in IL-6 in the IL-10$^{-/-}$ B cell supernatants of the untransduced population at day 2 (Fig. 5C). Expression of the M2 protein led to a four-fold increase in the levels of IL-6 in the culture supernatants of IL-10$^{-/-}$ B cells (Fig. 5C). In addition, MIP-1α levels were four-fold higher in the IL-10$^{-/-}$ B cell cultures at day 2 post-transduction, and this increase was observed throughout the time-course (data not shown). However, the increased levels of IL-6 and MIP-1α observed in the M2-transduced IL-10$^{-/-}$ cultures could not compliment the loss of IL-10 in the cultures, leading us to hypothesize that IL-10 secretion is required for the expansion of the M2 protein-expressing B cells.

To more directly assess the role of IL-10 in M2 protein-mediated B cell proliferation and survival, we tested the ability of the cytokine enriched supernatants from transduced wild-type and IL-10$^{-/-}$ B cells to compliment loss of M2 and IL-10 expression in culture. After analysis of transduction efficiency at

two days post-transduction, one third of the supernatant from the WT MSCV-M2.Stop and IL-10$^{-/-}$ MSCV-M2 transduced cultures was replaced with an equal volume of supernatant from C57BL6 MSCV-M2 cultures from the respective days post-transduction. B cells were analyzed for Thy1.1 expression for the remainder of the time-course, and IL-10 levels were measured by ELISA (data not shown). Interestingly, addition of culture supernatants from C57BL6 M2 protein expressing B cells to C57BL6 B cells transduced with MSCV-M2.Stop failed to induce significant proliferation of the transduced B cells (Fig. 5D). In contrast, addition of IL-10 containing culture supernatants to IL-10$^{-/-}$ B cells expressing the M2 protein led to a steady proliferation nearly equivalent to that of C57BL6 B cell cultures expressing the M2 protein (Fig. 5D).

Finally, to formally demonstrate that IL-10 is required for the observed phenotype, we transduced IL-10$^{-/-}$ B cells with either the M2 or M2.Stop control recombinant MSCV viruses and assayed the frequency of Thy1.1 cells in the culture over time in the presence and absence of recombinant IL-10 (Figure 5E). As expected, the addition of recombinant IL-10 had no discernable effect on M2 expressing IL-10-sufficient B cells recovered from C57Bl/6 mice. However, addition of IL-10 to the M2 transduced IL-10$^{-/-}$ B cells (but not the M2.Stop transduced IL-10$^{-/-}$ B cells) rescued the dominance phenotype (Figure 5E). These results demonstrate that both intracellular M2 expression and IL-10 secretion are necessary for the observed proliferative expansion of the transduced B cell population, and that neither one alone is sufficient to induce this expansion. These data suggest that M2 manipulates intracellular signaling pathways which enhance the response to IL-10 signaling as well as induce IL-10 secretion.

Loss of M2 protein expression during MHV68 infection results in a significant reduction in serum IL-10 levels.

Previous studies have shown that in the absence of a functional M2 gene, establishment of MHV68 latency following intranasal inoculation is severely reduced (62). Similarly, inoculation of IL-10$^{-/-}$ mice with wild-type MHV68 leads to a decrease in the establishment of latency (47, 114). To determine whether M2 expression leads to IL-10 secretion *in vivo*, C57Bl/6 mice were infected (either 1,000 pfu via intranasal inoculation or 100 pfu via intraperitoneal inoculation) with either a recombinant MHV68 harboring the same translation termination codon near the 5' end of the M2 open reading frame as used in control retrovirus construction (MHV68/M2.Stop) or with a genetically repaired marker rescue isolate of the same locus (MHV68/M2.MR). Both intranasal and intraperitoneal inoculation of the M2-null mutant were assessed, since we have previously reported that route of inoculation impacts the latency phenotype observed (62). Serum IL-10 was measured by *in vivo* cytokine capture and ELISA (Fig. 6, panels A & B). Notably, mice infected with MHV68/M2.Stop had serum IL-10 levels that were only slightly elevated over the levels present in naïve mice and were 2- to 3-fold lower than the levels observed in mice infected with the marker rescue virus (MHV68/M2.MR). Notably, this phenotype was independent of the route of inoculation (Fig. 6, panels A & B). As we have previously reported (62), we observed defects in both establishment of latency (which was accentuated following intranasal inoculation), as well as reactivation from latency with the M2-null mutant MHV68 (Fig. 6, panels C & D). Notably, intraperitoneal

infection with MHV68/M2.Stop increased the establishment of latency eight-fold over intranasal inoculation, yet serum IL-10 levels were very similar to those observed following intranasal inoculation (Fig. 6). Importantly, the serum levels of IL-10 we observed in MHV68/M2.MR infected mice were similar to those previously observed (114). These results provide strong evidence that M2 induction of IL-10 secretion, either from latently infected B cells or some other latency reservoir (e.g., infected macrophages or dendritic cells), contributes significantly to the serum levels of IL-10 observed during MHV68 infection following either intranasal or intraperitoneal virus inoculation.

Loss of M2 expression during MHV68 infection correlates with an increase in virus-specific, activated CD8$^+$ T cells.

We next examined whether loss of M2 expression and the concomitant reduction in IL-10 expression might alter the CD8 T cell response to MHV68 since IL-10 is known to suppress T cell responses (42). Thus, we examined the MHV68-specific CD8$^+$ T cell response following infection of mice with either MHV68/M2.Stop or MHV68/M2.MR. Mice were infected intraperitoneally with 1000 pfu of MHV68/M2.Stop or MHV68/M2.MR and splenocytes were harvested at day 16 post-infection, a time at which lytic virus has been cleared and latency established. As previously reported, there was a ten-fold decrease in establishment of latency with a 20-fold decrease in reactivation (Fig 6D). Splenocytes from individual mice were stained for activated, tetramer positive CD8$^+$ T cells using tetramers specific to two MHV68 antigens encoded by ORF6 and ORF61 (Figs. 7, panels A - C). Both tetramers used in this analysis were

specific for viral antigens expressed during the virus lytic replication cycle. In two independent experiments, tetramer staining for two different lytic antigens revealed a statistically significant increase in the frequency of tetramer-specific, activated $CD8^+$ T cells in mice infected with MHV68/M2.Stop compared to MHV68/M2.MR (Fig. 7A-C). In contrast, there was no global change in overall CD8 activation as determined by the percentage of $CD8^+$ $CD11a^{high}$ T cells in the spleens of infected mice (Fig. 7D). $CD4^+$ T cell activation, as well as the percentage of $CD44^{high}$ $CD62L^{low}$ $CD4^+$ and $CD8^+$ T cells, was the same in the two groups of infected mice (data not shown). These data indicate that the loss of M2 during MHV68 infection specifically enhanced the MHV68-specific $CD8^+$ T cell response, despite a significant decrease in viral latency and reactivation (see Fig. 6D).

Overall, the immune response in the absence of M2 protein expression during infection is unique in that the MHV68-specific T cell response is increased correlating with a decrease in serum IL-10 levels. These data point to a potential role of M2 protein-mediated IL-10 secretion in the quiescence of the virus-specific T cell response *in vivo* which may facilitate both the efficient establishment of latency, as well as reactivation from latency.

DISCUSSION

The latency-associated M2 protein is critical for establishing splenic latency following low dose intranasal inoculation and for virus reactivation from latency following low dose intraperitoneal inoculation (62, 68). In the absence of M2, infected B cells are unable to efficiently transition from the germinal center to the follicles (142). Early in latency, there is an accumulation of latently infected naïve B cells in the absence of M2 protein, indicating a role for the M2 protein in manipulating B cell development during infection (62). Epstein-Barr virus is hypothesized to drive naïve B cells to enter the germinal center reaction in order to establish latency in the memory B cell pool (8, 71, 145). In long-term EBV carriers, lytic EBV gene transcripts are preferentially found in the plasma cell population, leading to a model whereby reactivation from latency is associated with differentiation from memory to plasma cell (86). B cell proliferation is necessary for the establishment of MHV68 latency, and, similar to EBV, memory B cells are the primary long-term latency reservoir (76, 108, 185). Reactivation is hypothesized to be needed for efficient seeding of the spleen during the establishment phase of MHV68 infection, and, as such, the M2-associated defects in establishment of latency and reactivation from latency may, in fact, be functionally linked. In this study we explored the impact of M2 protein expression in primary murine B cells - a system capable of differentiation.

M2 expression in primary B cells led to proliferation of transduced B cells, driving a rapid expansion within the culture, regardless of initial transduction efficiency. Although both the transduced ($Thy1.1^+$) and untransduced ($Thy1.1^-$) B

cell populations could be shown to be proliferating (by BrdU incorporation), the enhanced proliferation and survival of the M2-transduced B cells rapidly led to this population dominating the mixed culture. In primary murine B cells, M2-driven proliferation was dependent on the B cell's ability to secrete IL-10 and respond to the IL-10 signaling. Notably, transfer of culture supernatants from M2 expressing C57Bl/6 B cells or addition of recombinant murine IL-10 did not result in the dominance of the M2.Stop retrovirus transduced (i.e., Thy1.1^{+}) population in the absence of M2 protein expression, leading us to hypothesize that some other function(s) of the M2 protein augments IL-10 signaling. Culturing stimulated human memory B cells with IL-10 or IL-2 and IL-6 leads to plasma cell differentiation (3, 148). Also, an increase in MIP-1α transcription is associated with differentiation to a plasma cell phenotype (51). Human germinal center B cells can be induced to differentiate into plasma cells rather than memory B cells in the presence of IL-10 (26). In contrast to human B cells, IL-10 enhances murine B cell viability but does not drive proliferation (56). Our data suggest that the MHV68 M2 protein uniquely increases the murine B cell proliferative response to IL-10, mimicking the role of IL-10 signaling in human B cells.

M2-transduced B cells were B220^{low}, I-A$^{b\ low}$, sIgD^{-}, yet retained surface expression of CD19 and IgG, and remained CD138^{low}, indicating that they did not fully differentiated into plasma cells. Instead, the surface phenotype of the B cells expressing M2 most closely resembled that of a pre-plasma memory B cell, an intermediate stage in development between the memory and plasma cell phenotypes (102, 139). Together, this data supports a model wherein infection

of naïve B cells in the lung with MHV68 leads to M2 expression, B cell proliferation and activation, and differentiation to a pre-plasma memory B cell phenotype. Depending on other cytokines and signals in the area, M2-expressing B cells may further differentiate into memory B cells, establishing long-term latency, or plasma B cells, potentiating virus reactivation. Thus, in this model of MHV68 pathogenesis, M2 protein manipulation of B cell differentiation to an intermediate pre-plasma memory B cell phenotype could facilitate both virus reactivation as well as establishment of viral latency.

IL-10 has potent immunoregulatory activity, suppressing proinflammatory cytokine secretion and activation of antigen-presenting cells - functions which result in suppressed NK cell and T cell activity (105). IL-10 plays an important role in MHV68 pathogenesis, but prior to our analyses of M2 protein function no specific viral antigen had previously been shown to stimulate cellular IL-10 production. It has been shown that ex vivo stimulation of MHV68 latently infected splenocytes with MHV68-infected antigen presenting cells resulted in IL-10 secretion peaking at the onset of splenic latency, and B cells were shown to be responsible for a significant portion of the IL-10 secreted (130). Dendritic cells isolated from MHV68 infected mice express IL-10 transcripts, and dendritic cells infected ex vivo secrete IL-10 only when concurrently stimulated with LPS (47). Interestingly, these investigators showed that M2 is transcribed by infected dendritic cells, but they did not demonstrate that IL-10 secretion was mediated by M2 (47). In the absence of IL-10, establishment of MHV68 latency is decreased, concurrent with an increase in serum IL-12 p70 and splenomegaly,

demonstrating a role for IL-10 in both establishment of latency as well as immunosuppression (46, 114, 130).

We observed a significant decrease in serum IL-10 in mice infected with an M2-null MHV68 mutant. Notably, the decreased serum IL-10 levels correlated with an increase in the percentage of MHV68-specific $CD8^+$ T cells. Furthermore, it is important to note that this increased CD8+ T cell response was in the setting of an infection where virus reactivation was severely attenuated. Therefore, increased persistent virus replication cannot explain the increase in the tetramer-specific response. Thus, we hypothesize that during M2-mediated reactivation, there is concurrent IL-10 secretion, locally dampening the ability of the MHV68-specific $CD8^+$ T cells to clear the infected cells, leading to enhanced establishment and reactivation from latency.

Manipulation of the IL-10 signaling pathway appears to be a conserved mechanism used by a number of herpesviruses. EBV encodes a viral IL-10 homolog, BCRF1 (or vIL-10), that has been shown to increase human B cell proliferation following surface immunoglobulin crosslinkinking and induce B cells to secrete increased levels of IgM, IgG, and IgA in a similar manner to cellular IL-10 (127). In the absence of vIL-10, EBV can still efficiently establish latent, long-term lymphoblastoid lines (LCLs) (158). Exogenous vIL-10 added during the transformation of B cells by EBV enhanced both the rate and frequency of growth transformation, and antisense oligonucleotides to vIL-10 could negate this enhancement (104, 153). Human IL-10 could complement the loss of vIL-10 during EBV infection of B cells, demonstrating that it is IL-10 mediated signaling that augments B cell transformation following EBV infection

(104). LMP1, a functional CD40 ortholog encoded by EBV, is both IL-10 responsive and induces secretion of cellular IL-10 in stimulated Burkitt's lymphoma cells (77, 179). Patients with EBV-associated post-transplant lymphoproliferative disease also have elevated serum IL-10, but not IL-6 (9). However, *in vivo*, whether the primary role of vIL-10 is to suppress the immune response or trigger B cell proliferation and differentiation, or both, is unclear. Serum cellular IL-10 levels are elevated both during primary EBV infection as well as during EBV reactivation from latency, implying that IL-10 plays a role in both the establishment and reactivation from latency (64).

Human cytomegalovirus (HCMV), a beta herpesvirus, encodes a viral IL-10 (cmvIL-10) that has only 27% homology to cellular IL-10, but is nevertheless capable of binding the IL-10 receptor and mediating downstream STAT1/STAT3 signaling (81). Human cmvIL-10 is capable of downregulating MHC I and II, suppressing PBMC proliferation, and decreasing IFNγ, IL-1α, GM-CSF, IL-6, and TNF-α secretion in response to stimulation (147). Transcripts encoding cmvIL-10 have been detected in the bone marrow and mobilized peripheral blood during natural HCMV latency, indicating that cmvIL-10 may play a role in either establishment, maintenance, or reactivation from latency (69). Murine CMV (MCMV) does not encode an IL-10 homolog, although, parallel to the studies of Flano et al. on dendritic cells infected with MHV68 (47), *in vitro* MCMV infection of primary macrophages results in secretion of cellular IL-10 and downregulation of MHC II (119). Recently, IL-10 production by CD4 T cells has been shown to be of key importance in regulating MCMV persistence in the salivary glands. Blockade of the IL-10R resulted in a significant decrease in the titer of MCMV in

the salivary glands with a concurrent increase in the frequency of IFNγ-producing CD4 T cells, indicating a role for IL-10 in the maintenance of a MCMV infection, possibly through reactivation from latency (65). It seems reasonable to speculate that suppression of the host response may help these viruses both establish latency as well as reactivate from latency, reseeding the latency reservoir without clearance by memory T cell responses.

IL-10 has been implicated in the pathogenesis of both autoimmune as well as viral diseases, and the fact that many viruses carry IL-10 orthologs speaks to the potency of IL-10 in manipulating the host immune system (10, 174). The parapoxvirus, ORF virus, encodes a viral IL-10 homolog with 80% homology to ovine IL-10 and is capable of inhibiting T cell proliferation (48). Finally, two independent reports have demonstrated that blockade of the IL-10 receptor during chronic lymphocytic choriomeningitis virus infection led to clearance of the infection with enhanced IL-10 production by dendritic cells (18, 38).

We additionally observed a significant increase in IL-6 and MIP-1α in the B cell cultures transduced with M2. Interestingly, KSHV encodes homologs of both IL-6 and MIP-1α, suggesting that these cytokines have key roles in gammaherpesvirus immunomodulation that we have yet to appreciate (111). MIP-1α can be detected in the BAL and lung homogenate of MHV68-infected mice at the peak of lytic replication, but the contribution of this cytokine to latency and reactivation has not been studied directly (131, 184). During MHV68 infection, MIP-1α secretion in the lungs may attract B cells to the area of acute replication, facilitating viral infection and trafficking to the spleen. There is no significant difference in pathology or viral latency in IL-6$^{-/-}$ mice, despite the fact

that upon ex vivo stimulation, infected splenocytes secrete IL-6 (129). Further study is necessary to explore the links between M2 and these cytokines.

Does M2-driven IL-10 secretion play a critical role in MHV68 latency? The studies presented here provide an indication that the M2 protein has multiple functions – some of which are necessary for primary murine B cells to respond to IL-10 signaling in the retroviral transduction assays we have described. Our attempts to neutralize IL-10 in the B cell culture system have been unsatisfactory (data not shown) – perhaps owing to the difficulty of neutralizing the autocrine activity of IL-10 expressed from primary murine B cells. Thus, we anticipate that attempting to neutralize IL-10 in vivo during MHV68 infection will be difficult. As a distinct approach, we have recently published the analysis of a panel of point mutations in candidate functional motifs in the M2 protein (63). The latter studies have also provided evidence for the presence of multiple functionally important domains in M2 (63). Notably, two of these mutations (Y129F/P7 and P8) ablated the IL-10 dependent proliferative dominance phenotype in primary B cell cultures while the other mutation (P9) was similar to wild-type M2 (63). The latter result underscores that M2 is a multifunctional protein. In addition, these studies link M2 functional domains that play a critical role in MHV68 latency *in vivo* to M2-driven IL-10 secretion. Further studies will be required to assess the contribution of M2-driven IL-10 expression to chronic MHV68 infection.

In summary, the analysis of M2 protein function provides a unique insight into an immunomodulatory mechanism that is employed by many viruses, particularly the herpesvirus family. Our work demonstrates that the M2 protein,

a unique viral protein, manipulates B cell signaling to induce cellular IL-10 secretion and make cells more responsive to IL-10 signaling, leading to proliferation and enhanced survival of M2-expressing primary B cells in culture. M2 expression in primary murine B cells results in differentiation to a pre-plasma memory B cell phenotype, an intermediate in mature B cell development. In addition, M2 protein expression correlates with high serum IL-10 levels and an increased frequency of virus-specific $CD8^+$ T cells during MHV68 infection. We conclude that driving B cell proliferation, survival and differentiation, while simultaneously dampening the host immune response to the virus, is an elegant immunomodulatory mechanism used by MHV68 to both facilitate establishment of latency and subsequent episodic virus reactivation from latency.

FIGURES

Figure 1.

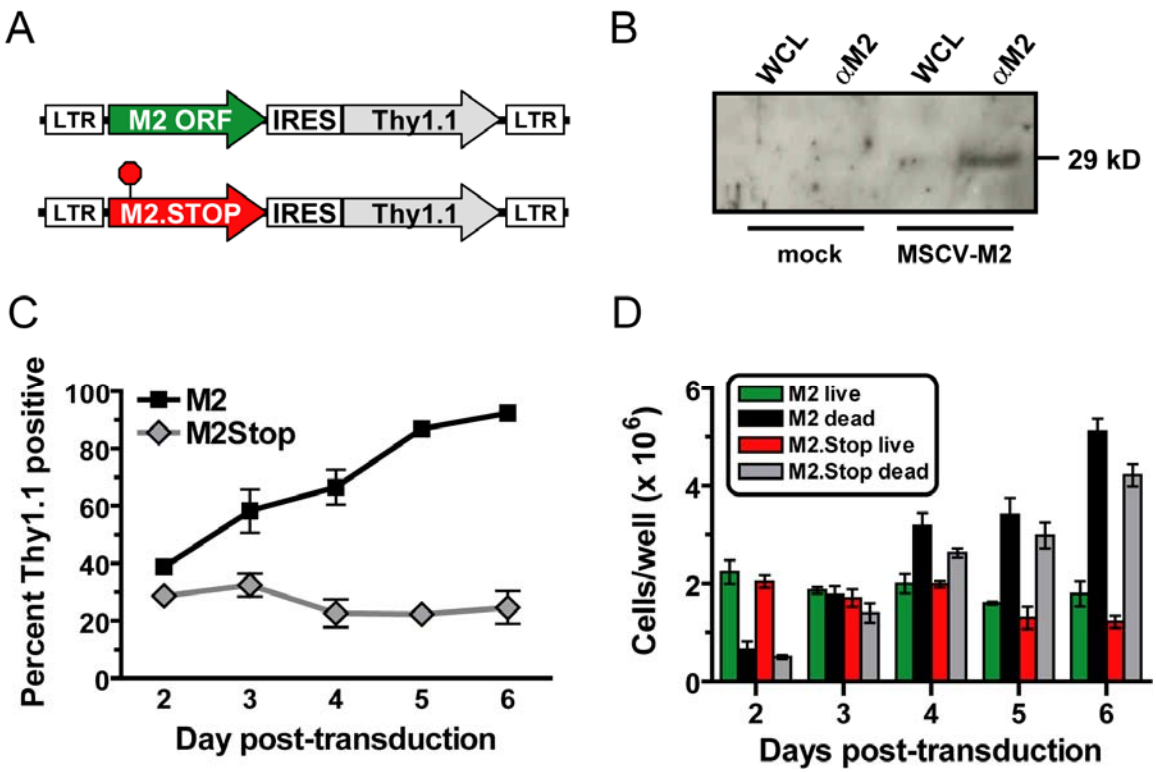

Figure 2.

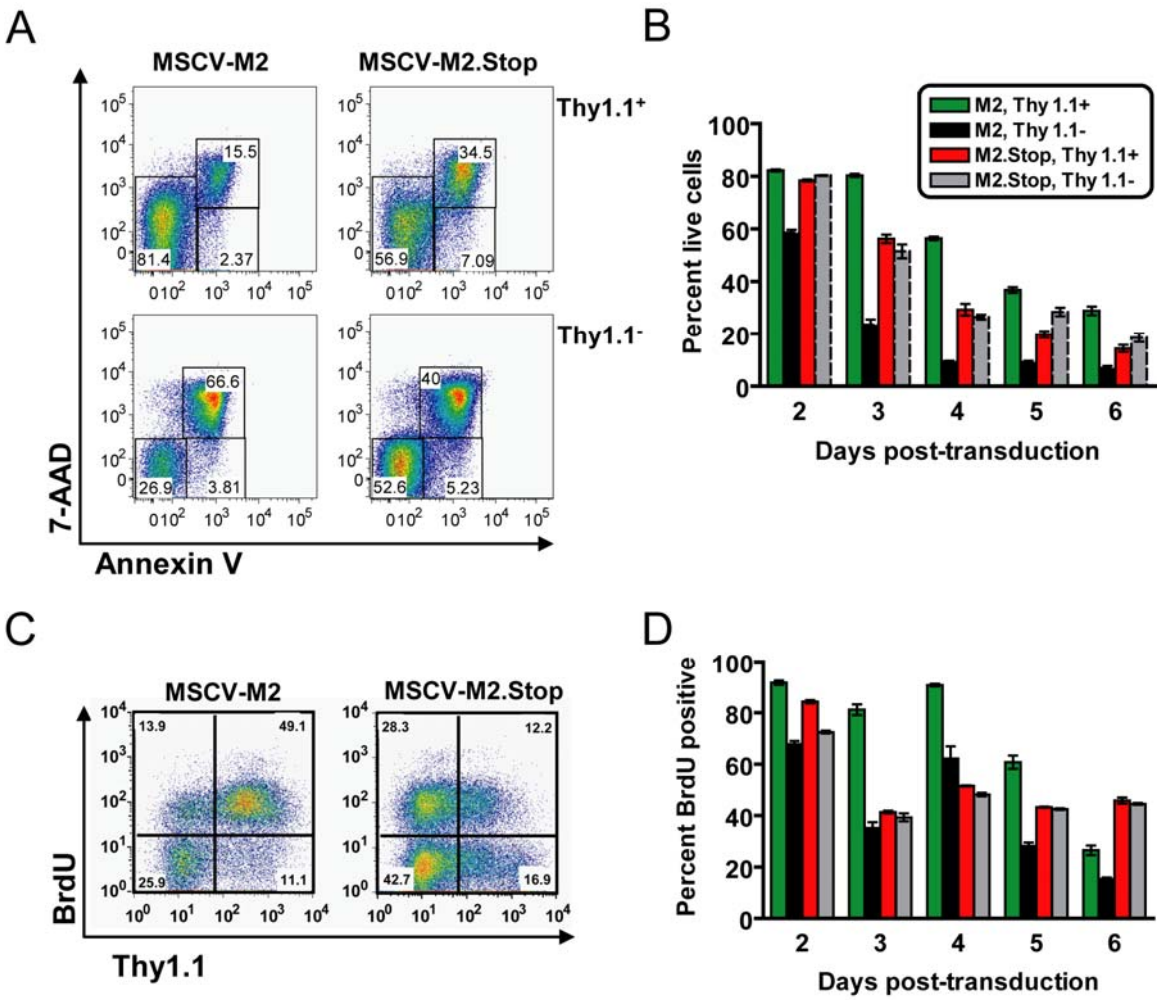
A
MSCV-M2
MSCV-M2.Stop
Thy1.1+
Thy1.1-
15.5
81.4
2.37
34.5
56.9
7.09
66.6
26.9
3.81
40
52.6
5.23
7-AAD
Annexin V
B
M2, Thy 1.1+
M2, Thy 1.1-
M2.Stop, Thy 1.1+
M2.Stop, Thy 1.1-
Percent live cells
Days post-transduction
C
MSCV-M2
MSCV-M2.Stop
13.9
49.1
25.9
11.1
28.3
12.2
42.7
16.9
BrdU
Thy1.1
D
Percent BrdU positive
Days post-transduction

Figure 3.

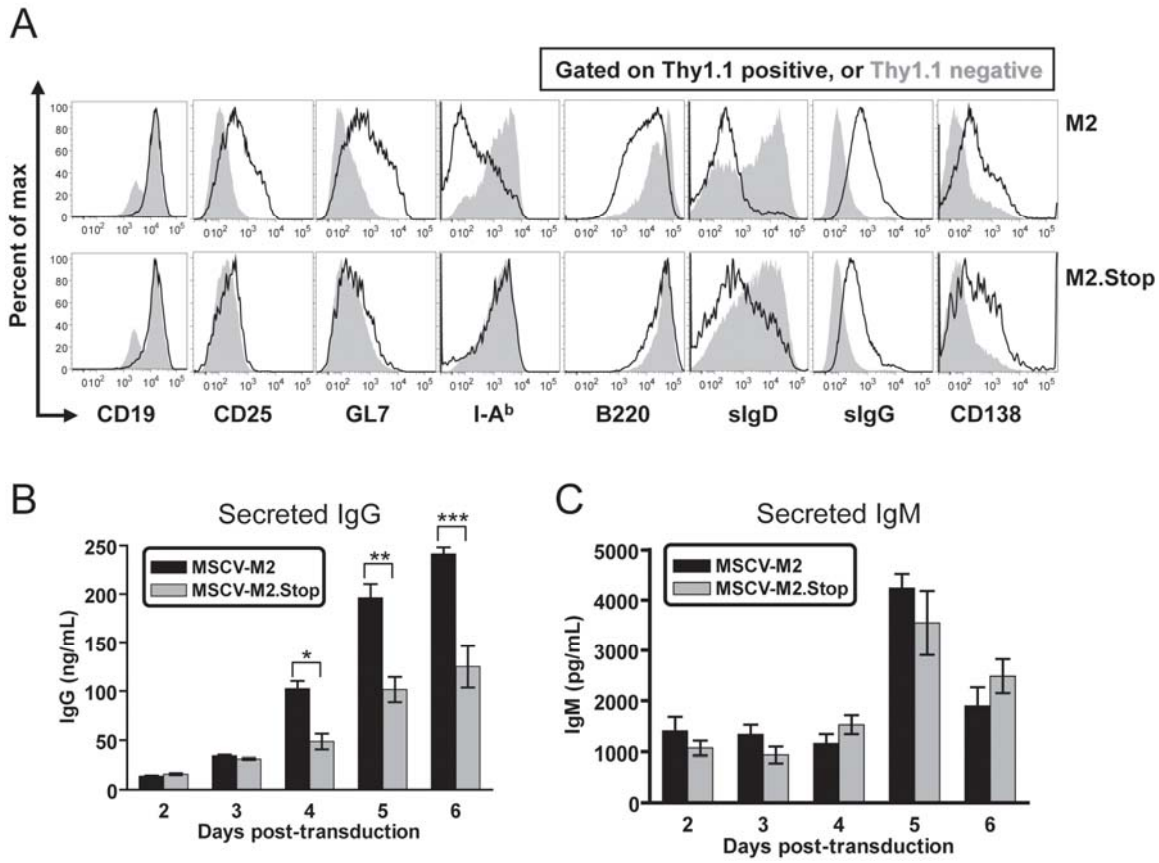
A
Gated on Thy1.1 positive, or Thy1.1 negative
Percent of max
M2
M2.Stop
CD19
CD25
GL7
I-Ab
B220
sIgD
sIgG
CD138
B
Secreted IgG
MSCV-M2
MSCV-M2.Stop
IgG (ng/mL)
Days post-transduction
C
Secreted IgM
MSCV-M2
MSCV-M2.Stop
IgM (pg/mL)
Days post-transduction

Figure 4.

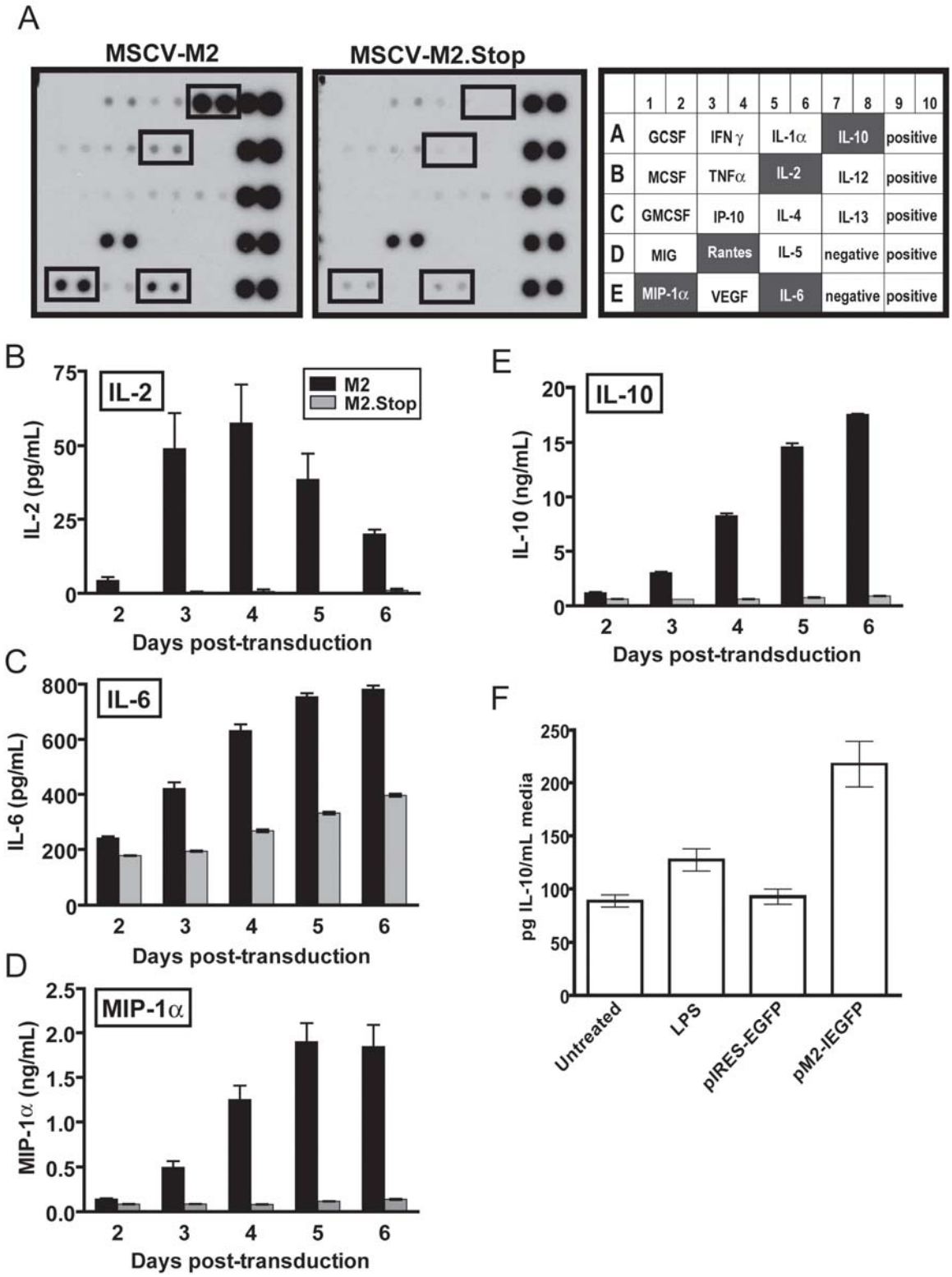
A
MSCV-M2
MSCV-M2.Stop
1 2 3 4 5 6 7 8 9 10
A GCSF IFNγ IL-1α IL-10 positive
B MCSF TNFα IL-2 IL-12 positive
C GMCSF IP-10 IL-4 IL-13 positive
D MIG Rantes IL-5 negative positive
E MIP-1α VEGF IL-6 negative positive
B
IL-2
M2
M2.Stop
IL-2 (pg/mL)
Days post-transduction
C
IL-6
IL-6 (pg/mL)
Days post-transduction
D
MIP-1α
MIP-1α (ng/mL)
Days post-transduction
E
IL-10
IL-10 (ng/mL)
Days post-trandsduction
F
pg IL-10/mL media
Untreated
LPS
pIRES-EGFP
pM2-IEGFP

Figure 5.

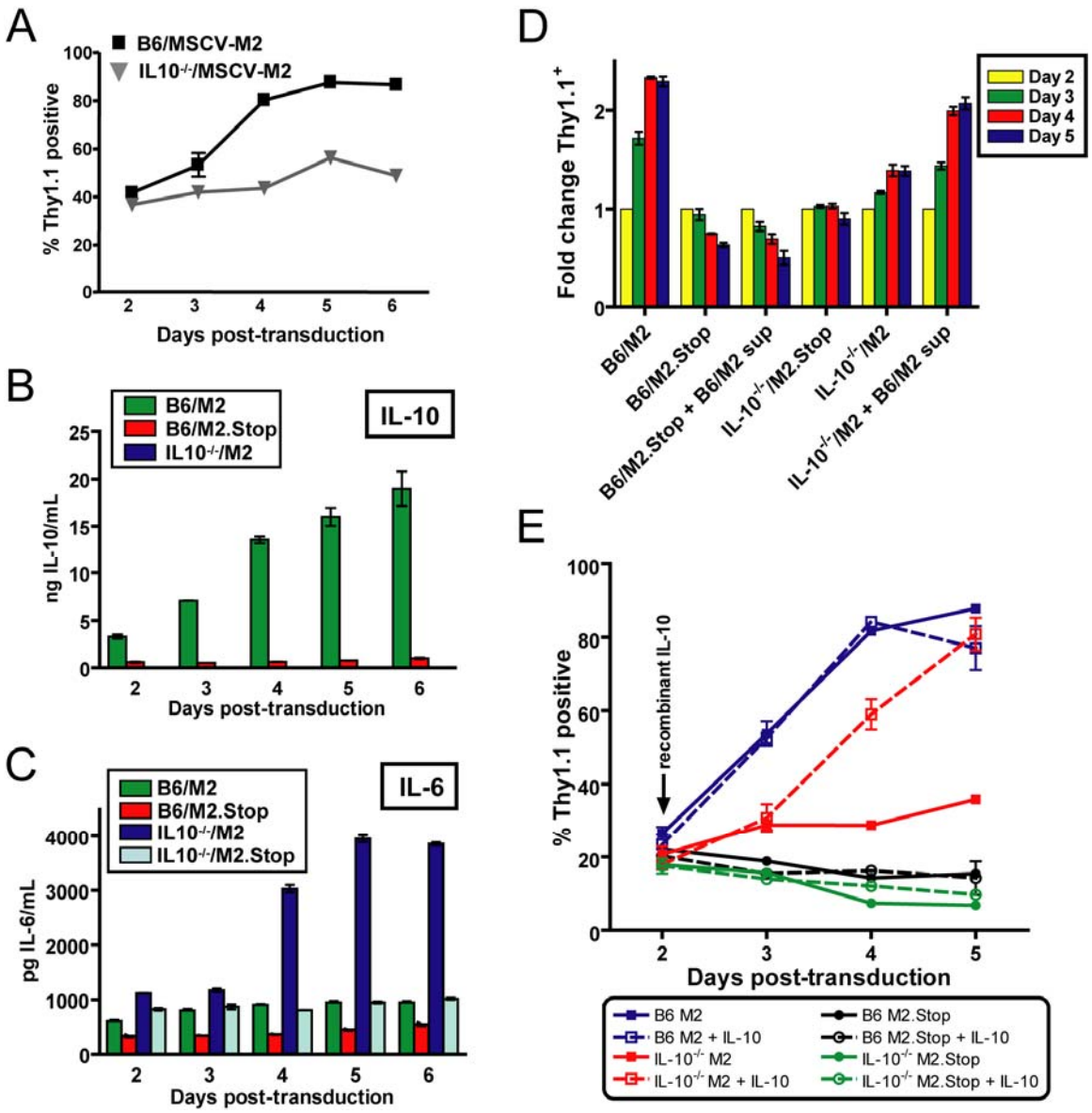
A
B6/MSCV-M2
IL10-/-/MSCV-M2
% Thy1.1 positive
Days post-transduction
B
B6/M2
B6/M2.Stop
IL10-/-/M2
IL-10
ng IL-10/mL
Days post-transduction
C
B6/M2
B6/M2.Stop
IL10-/-/M2
IL10-/-/M2.Stop
IL-6
pg IL-6/mL
Days post-transduction
D
Day 2
Day 3
Day 4
Day 5
Fold change Thy1.1+
B6/M2
B6/M2.Stop
B6/M2.Stop + B6/M2 sup
IL-10-/-/M2.Stop
IL-10-/-/M2
IL-10-/-/M2 + B6/M2 sup
E
% Thy1.1 positive
recombinant IL-10
Days post-transduction
B6 M2
B6 M2 + IL-10
IL-10-/- M2
IL-10-/- M2 + IL-10
B6 M2.Stop
B6 M2.Stop + IL-10
IL-10-/- M2.Stop
IL-10-/- M2.Stop + IL-10

Figure 6.

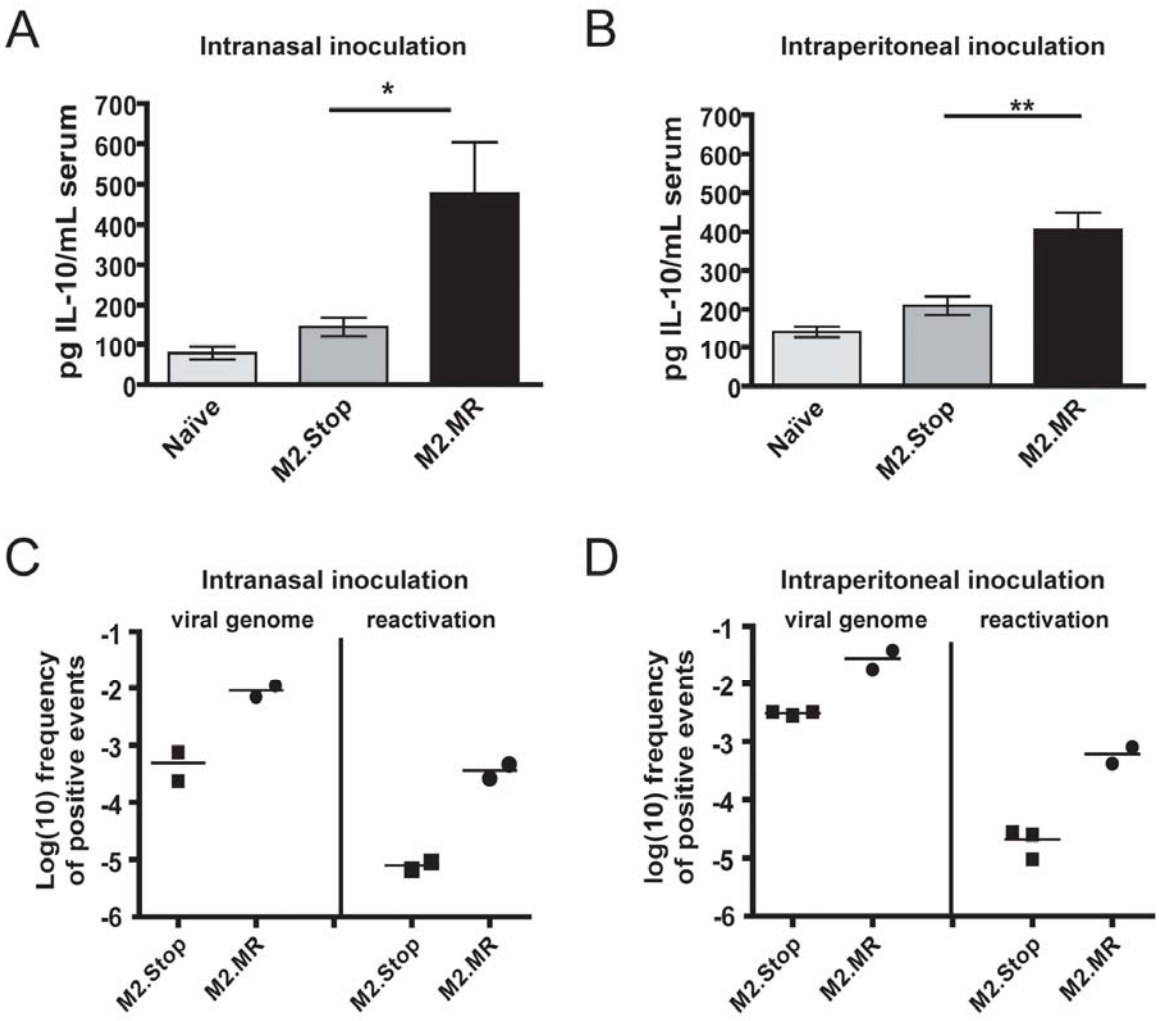
A
Intranasal inoculation
pg IL-10/mL serum
700
600
500
400
300
200
100
0
*
Naïve
M2.Stop
M2.MR
B
Intraperitoneal inoculation
pg IL-10/mL serum
700
600
500
400
300
200
100
0
**
Naïve
M2.Stop
M2.MR
C
Intranasal inoculation
viral genome
reactivation
Log(10) frequency of positive events
-1
-2
-3
-4
-5
-6
M2.Stop
M2.MR
M2.Stop
M2.MR
D
Intraperitoneal inoculation
viral genome
reactivation
log(10) frequency of positive events
-1
-2
-3
-4
-5
-6
M2.Stop
M2.MR
M2.Stop
M2.MR

Figure 7.

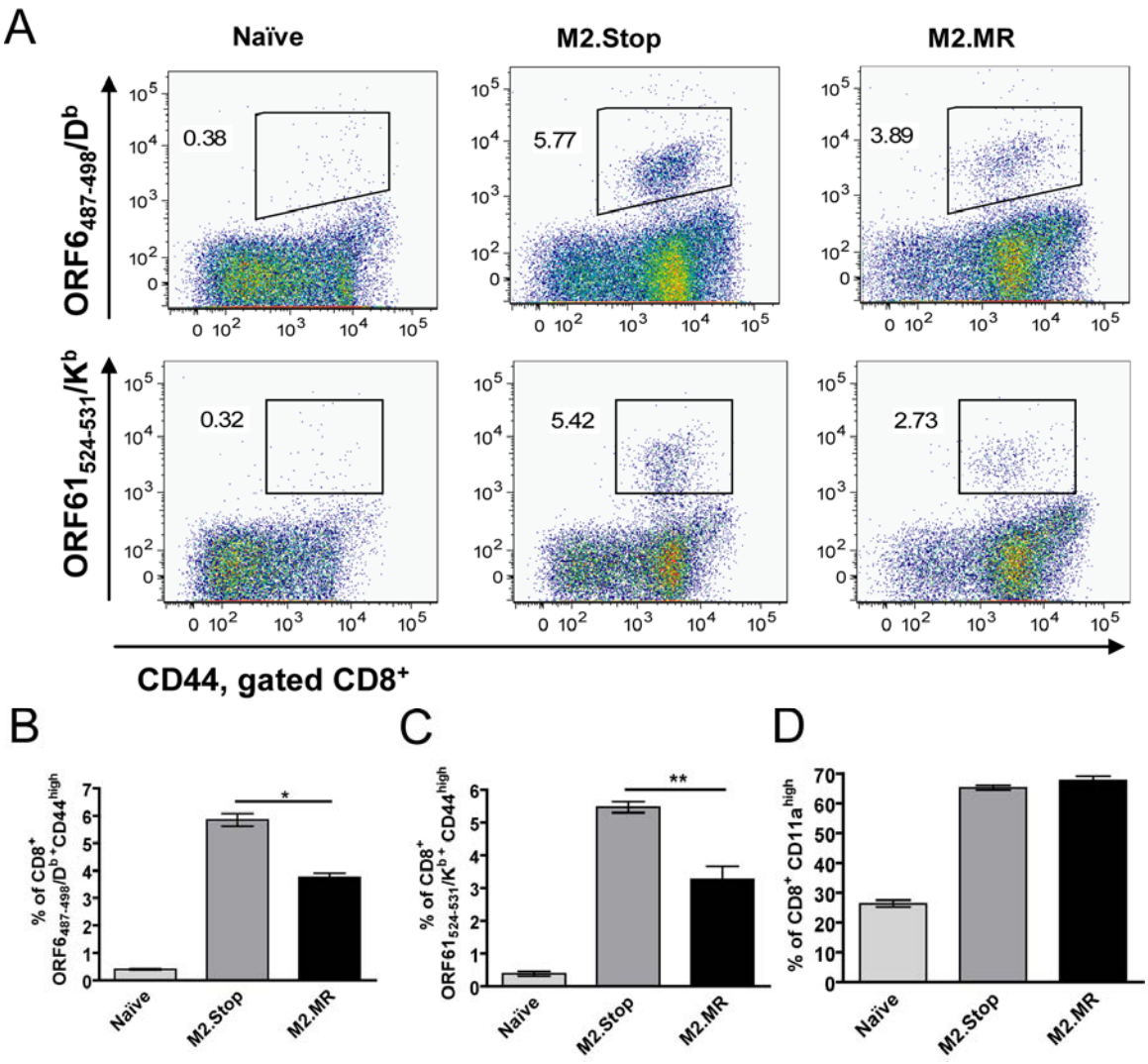
A
Naïve
M2.Stop
M2.MR
ORF6487-498/Db
ORF61524-531/Kb
0.38
5.77
3.89
0.32
5.42
2.73
CD44, gated CD8+
B
C
D
% of CD8+ ORF6487-498/Db+ CD44high
% of CD8+ ORF61524-531/Kb+ CD44high
% of CD8+ CD11ahigh
*
**
Naïve
M2.Stop
M2.MR

FIGURE LEGENDS

Figure 1. M2 expression in primary B cells leads to expansion of the transduced population. (A) M2 open reading frame and M2 ORF with a stop codon at amino acid number 13 (M2.Stop) were cloned into the MSCV-IRES-Thy1.1 vector. **(B)** Expression of M2 protein in primary murine B cells four days post-transduction. B cells were lysed in CHAPS-BOG and immunoprecipitated with chicken anti-M2 IgY and blotted with rabbit anti-M2 antisera. **(C)** M2-transduced B cells expand in culture over time. B cells were purified by negative selection MACS separation, and B cell cultures were between 94-97% pure (gated on $CD19^+$). Triplicate B cell cultures were transduced with M2 or M2.Stop retroviruses and allowed to rest for 48 hours before analysis. Cells were stained with anti-Thy1.1 and analyzed daily for expression. Data representative of more than three independent experiments. **(D)** Absolute numbers of cells per well are unchanged over time. Trypan exclusion was used to count absolute numbers of live and dead cells in triplicate wells per day. Data representative of at least two independent experiments.

Figure 2. M2 expression leads to enhanced survival and B cell proliferation. (A and B) M2-transduced cells have enhanced survival versus the untransduced cells in culture as well as M2.Stop-transduced cells. Triplicate cultures were stained with Thy1.1-PE, 7-AAD, and AnnexinV-PacificBlue and analyzed on a LSRII cytometer. **(A)** Representative data from 3 days post-transduction excluding obvious debris in the culture, gated on Thy1.1 expression.

(B) The percentage of live (AnnexinV^{-} 7-AAD^{-}) was determined from 2-6 days post-retroviral transduction with M2 or M2.Stop in transduced (Thy1.1^{+}) and untransduced (Thy1.1^{-}) populations. Data is representative of two independent time-course experiments. **(C and D)** M2-transduced B cells had enhanced proliferation early post-transduction. Triplicate B cell cultures were pulsed with 10 μM BrdU for 24 hours and stained with Thy1.1-PE and BrdU-APC and analyzed on a FACScalibur. **(C)** Representative data from day 3 post-transduction shows M2-enhanced proliferation of the transduced (Thy1.1^{+}) population, gated on viable cells. **(D)** Percent of transduced, proliferating cells over time demonstrates enhanced proliferation in B cells transduced with M2 at days 3 and 4 post-transduction. Proliferation of the untransduced populations were similar over the time-course. Statistical significance of the flow cytometry data was determined by two-tailed, unpaired Student's T test with a confidence level of 95%.

Figure 3. M2 expressing cells have an activated, pre-plasma memory phenotype. (A) M2-transduced cells were CD19^{+}, CD25^{high}, GL7^{high}, B220^{low}, I-A$^{b\ low}$, sIgD^{-}, sIgG^{+}, and CD138^{low} when compared to untransduced cells within the culture. Representative flow cytometry histograms at day 4 post-transduction. The black line open histograms reflect staining of the transduced (Thy1.1^{+}) B cell population, while the filled gray histograms reflect staining of the untransduced (Thy1.1^{-}) population. The top panels depict data from M2-transduced cultures; bottom panels depict data from M2.Stop-transduced cells. Data is representative of three samples per time-point with at least three

independent experiments per stain. **(B and C)** ELISA quantitation of the levels of IgM and IgG in supernatants of M2 and M2.Stop transduced B cell cultures. Three samples were analyzed per time point, and the data shown is representative of three independent experiments. Significance of differences in IgG secretion was determined by two-tailed, unpaired Student's T test with a confidence level of 95%. * p = 0.0182, ** p = 0.0352, *** p = 0.0118.

Figure 4. M2 expressing cells secrete more IL-2, IL-6, MIP-1α, and IL-10. (A) Cytokine antibody arrays were screened for the presence of cytokines in the supernatants of M2 and M2.Stop transduced B cell cultures at day 4 post-transduction. Data is representative of supernatants from two independent time-course experiments. **(B-E)** ELISAs of IL-2, IL-6, MIP-1α, and IL-10 levels in the culture supernatants M2 and M2.Stop transduced B cells. Data shown from triplicate wells analyzed from a single time-course analysis. The data shown are representative of three independent experiments. **(F)** IL-10 ELISAs of culture supernatants from the murine A20 cell line, either transfected with an empty plasmid (pBluescript II SK), and untreated, treated with LPS, or following transfection with a control expression plasmid (pIRES-EFGP) or an M2 expression plasmid (pM2-IEFGP). The difference between levels of IL-10 in the culture media of vector control and M2 expression vector transfected A20 cells was statistically significant (p = 0.0054).

Figure 5. M2 expressing B cells fail to expand in the absence of IL-10. (A) B cells from C57Bl6 and IL-10$^{-/-}$ mice were isolated from splenocytes

and transduced with either the M2 or M2.Stop retroviruses. Triplicate wells were analyzed per time-point. IL-10$^{-/-}$ B cells transduced with M2 failed to expand to the same extent as wild-type B cells. **(B)** Quantitative ELISA analyses confirm that IL-10$^{-/-}$ B cells fail to secrete IL-10. **(C)** IL-10$^{-/-}$ B cells transduced with M2 secrete significantly higher levels of IL-6 than C57Bl/6 B cells transduced with M2. However, IL-6 fails to compensate for the IL-10 deficiency. Data shown is representative of two independent experiments, each containing triplicate cultures. **(D)** Supernatants from C57Bl6 B cells transduced with M2 compliment for the IL-10$^{-/-}$ B cell proliferation defect. B cells from C57Bl6 and IL-10$^{-/-}$ mice were isolated and triplicate cultures transduced with M2 or M2.Stop retroviruses. On day 2 post-transduction, 500 μL of supernatant from the C57Bl6 B cells transduced with the M2.Stop retrovirus and IL-10$^{-/-}$ B cells transduced with the M2 retrovirus were replaced with 500 μL of tissue culture supernatant recovered from the C57Bl6/M2 B cell cultures from that respective day. Triplicate wells were analyzed per time-point. High IL-10 levels fail to complement the lack of M2 expression in the C57Bl/6 B cells, but did drive proliferation of the M2-transduced IL-10$^{-/-}$ B cells. Data are represented as the fold-change in percent Thy1.1 positive cells in the cultures over the percent Thy1.1 positive cells present at two days post-transduction. **(E)** Recombinant murine IL-10 rescues M2-mediated expansion of IL-10$^{-/-}$ B cells, but fails to expand either the C57Bl/6 or IL-10$^{-/-}$ M2.Stop transduced B cell populations. B cells from C57Bl/6 and IL-10$^{-/-}$ mice were isolated from splenocytes and transduced with either the M2 or M2.Stop retroviruses. IL-10 was added (final concentration of 20 ng/mL) to the

indicated samples starting on day 2 post-transduction (see text for description). Triplicate wells were analyzed per time-point.

Figure 6. Loss of M2 expression *in vivo* correlates with reduced serum IL-10 at the onset of latency. The reduction in serum IL-10 is independent of the route of infection and correlates with a significant reduction in reactivation from latency in the absence of M2. Groups of four to five mice were infected with 1000 pfu of M2 null mutant (MHV68/M2.Stop) or the M2 marker rescue virus (MHV68/M2.MR) intranasally or 100 pfu intraperitoneally (or naïve mice). On days 14 or 15 post-infection, mice were injected with biotin labeled anti-IL-10 antibody i.p. Twenty-four hours later, serum was collected for analysis of IL-10 levels **(A and B)**, and splenocytes were recovered for determinations of the frequency of viral latency and reactivation **(C and D)**. Data shown for intranasal inoculations is representative of two independent experiments, 4-5 mice per group, with IL-10 levels measured in individual mice and splenocytes pooled for viral latency assays (as described in Materials and Methods). Data shown for intraperitoneal inoculations is representative of three independent experiments, 4-5 mice per group, with IL-10 levels measured in individual mice and splenocytes pooled for viral latency assays. **(A and B)** Total serum IL-10 levels were significantly lower in mice infected with MHV68/M2.Stop compared to MHV68/M2.MR virus, independent of the route of infection. Intranasal infection data is from 10 individual mice from two independent infections; intraperitoneal data is from 15 individual mice from three independent infections. Two naïve animals were analyzed per infection * p

= 0.0117, ** p = 0.0005. Significance of IL-10 data was determined by two-tailed, unpaired Student's T test with a confidence level of 95%. **(C and D)** Frequencies of splenocytes from MHV68/M2.MR and MHV68/M2.Stop infected mice harboring latent viral genomes and reactivating from latency upon explant. The frequency of latency was determined by nested, limiting-dilution PCR (LD-PCR), as previously described. The frequency of cells reactivating from latency upon in vitro culture was determined by plating serial dilutions of live, intact splenocytes on mouse embryonic fibroblast monolayers and scoring cytopathic effect 14-21 days post-explant, as previously described. Data from both assays was subjected to nonlinear regression analysis with a sigmoidal dose-response algorithm for best fit.

Figure 7. Loss of M2 protein expression and reduction of serum IL-10 levels correlates with an enhancement in the MHV68-specific tetramer response. Groups of four to five mice were infected with 1000 pfu via intraperitoneal inoculation with either MHV68/M2.Stop or MHV68/M2.MR. Sixteen days post-infection, at the onset of latency, splenocytes were analyzed by flow cytometry. **(A)** Splenocytes were stained with tetramers for two MHV68 lytic antigens, $ORF6_{487-498}/D^b$-APC or $ORF61_{524-531}/K^b$-APC, anti-CD8-PacificBlue, and anti-CD44-FITC. The data shown is from mice with an intermediate phenotype representative of the median in each group. **(B and C)** Mean percentage of ORF6-specific (panel B) and ORF61-specific (panel C) T cells was significantly higher in the absence of the M2 protein (* $p < 0.0001$, ** $p = 0.0009$). Tetramer data was compiled from the analysis of individual mice and

represents two independent infections, with 5 mice per group per infection. **(D)** Mean percentage of activated $CD8^+$ T cells did not significantly change in the absence of the M2 protein. Data shown is from one representative set of infections of five mice from three independent experiments. Statistical significance of the flow cytometry data was determined by two-tailed, unpaired Student's T test with a confidence level of 95%.

CHAPTER III

Chapter III has been submitted to the Journal of Virology. (Blimp-1 dependent plasma cell differentiation is required for efficient maintenance of murine gammaherpesvirus latency and antiviral antibody responses. Andrea M. Siegel, Udaya Shankari Rangaswamy, Ruth J. Napier, and Samuel H. Speck.)

INTRODUCTION

Gammaherpesviruses establish life-long latent infections in lymphocytes and are associated with a variety of lymphomas and carcinomas. Over 95% of the human population is infected with Epstein-Barr Virus (EBV), which is the etiologic agent of infectious mononucleosis and is closely linked to the development of several cancers including the endemic form of Burkitt's lymphoma and nasopharyngeal carcinoma. Additionally, EBV is well known for its ability to immortalize primary human B cells *in vitro* (20). Evidence of Kaposi's sarcoma-associated herpesvirus (KHSV or HHV8) infection is found in Kaposi's sarcoma tumors and in primary effusion lymphomas (PELs), as well as an immunoblast variant of multicentric Castleman's disease (MCD). The murine gammaherpesvirus 68 (MHV68 or γHV68) is associated with B cell lymphoma development in β2-microglobulin-deficient Balb/C mice (51). EBV, KSHV, and MHV68 all establish latency in B cells and investigation of how B cell biology

shapes gammaherpesvirus pathogenesis is critical to understanding virus-mediated lymphomagenesis (9, 20, 50).

Herpesviruses are characterized by their ability to establish life-long latent infections with episodic productions of progeny virus. During latency the viral genome is almost completely transcriptionally silent, except for the expression of viral genes necessary for maintenance of the viral genome, allowing the infection to persist without detection and clearance by the host immune system. However, viral dissemination must occur for viral transmission. Viral genes involved in virus replication need to be transcribed and translated to produce infectious viral particles. This process of change from a dormant infection to active viral shedding is termed reactivation. It is also possible that reactivation plays a critical role for reseeding of latency reservoirs, facilitating maintenance of infection for the life-time of the host.

EBV establishes latency in the memory B cell reservoir (3, 24, 45). In the tonsils, the site of viral shedding, latent EBV can be found in both naïve, IgD^+ and IgD^- B cells (3). Memory B cells are proposed to traffic latent EBV through the blood into the peripheral tissues, and they harbor latent virus for the life-time of the host (3, 45). In EBV pathogenesis, reactivation from latency is associated with differentiation from a quiescent memory B cell to a plasma cell (29). Plasma cells isolated from EBV patients have been shown to be positive for the master lytic transcript, BZLF1, and, thus, associated with reactivation from latency *in vivo* (13, 29). X-box binding protein-1s (XBP-1s), a transcription factor necessary for plasma cell differentiation, has been shown to bind to the BZLF1 promoter, directly linking plasma cell differentiation and EBV reactivation (37, 48).

Similarly, KSHV reactivation is linked to plasma cell differentiation. Many PELs are of ambiguous origin – lacking cell surface markers clearly indicative of B or T cell lineage – yet many have rearranged VDJ genes, express surface CD138 (Syndecan-1, a surface marker of plasma cell differentiation), and Blimp-1 (B-lymphocyte induced maturation protein-1, discussed below) transcripts (8, 17, 23, 27). Data from microarray experiments revealed that PELs display a plasmablastic gene expression profile, a post-germinal center intermediate between plasmablasts and fully differentiated plasma cells (23, 27). Parallel to EBV pathogenesis, XBP-1s is capable of inducing KSHV reactivation by transactivation of the RTA (replication and transcription activator) promoter, the master transcriptional regulator of KSHV reactivation (31, 49, 58, 59). Thus, plasma cell differentiation is associated with both lymphomagenesis and reactivation of KSHV. However, due to the strict species-specific tropism typical of this viral family, studying latency and reactivation *in vivo* is limited.

Upon encounter of their cognate antigen, T cell help, and appropriate cytokines, memory B cells can differentiate first into pre-plasma memory B cells, proliferate and continue to develop into plasmablasts, and finally cease proliferation and become plasma cells, cellular factories of antibody secretion (41). Plasma cell differentiation is orchestrated by the master transcriptional regulator, B-lymphocyte-induced maturation protein-1 (Blimp-1), encoded by the gene *prdm1* (53). Ectopic expression of Blimp-1 leads to J-chain synthesis, immunoglobulin secretion, increase in cell size and granularity, as well as upregulation of the plasma cell marker *syndecan-1* (53). Blimp-1 directs plasma cell differentiation by repressing a broad range of genes involved in maintaining a

mature B cell phenotype as well as driving proliferation (40). Blimp-1 is necessary for differentiation to and maintenance of a plasma cell phenotype, but it is not necessary for the induction of plasma cell differentiation *in vivo* (25, 41, 42). Blimp-1 expression is needed for antibody secretion by all subsets of B cells including B-1 B cells (39).

The murine gammaherpesvirus 68 (MHV68 or γHV68) is a natural pathogen of wild murid rodents whose pathogenesis parallels that of EBV in many respects. MHV68, too, establishes latency in B cells as well as macrophages and dendritic cells (16, 50). Following intranasal infection, B cells are necessary for trafficking MHV68 infected cells to the spleen, leading to the establishment of splenic latency (47, 56). Proliferating B cells reactivate at a higher frequency than non-proliferating cells at the onset of splenic latency, and latent MHV68 can be preferentially detected in proliferating B cells as late as 3 months post-infection (34). Recent studies show that viral replication is necessary for the establishment of MHV68 latency in B cells, but not macrophages, implicating lytic replication or reactivation in the seeding of the B cell compartment in the peritoneal cavity following intraperitoneal inoculation (30). Similar to EBV pathogenesis, memory B cells are the long-term latency reservoir for MHV68 (26, 57).

Due to the many parallels between MHV68, EBV, and KSHV latency and reactivation in respects to B cell biology, we asked whether differentiation to a plasma cell is necessary for MHV68 reactivation *in vivo*. In order to address the role of plasma cell differentiation in MHV68 pathogenesis, we infected conditional Blimp-1 deficient mice ($prdm1^{flox/flox}$) with a transgenic strain of

MHV68 encoding for Cre recombinase (MHV68/Cre) (34, 41). We asked whether loss of Blimp-1 expression in infected cells had any effect on the establishment of, reactivation from, or maintenance of latency. Surprisingly, we observed a role for Blimp-1 in both the establishment and reactivation from latency at day 18 post-infection. This result was confirmed when mice deficient in Blimp-1 in all B cells were infected with MHV68. Additionally, serum anti-MHV68 antibody responses were significantly lower without Blimp-1 expression in infected splenocytes. Intriguingly, loss of Blimp-1 expression in infected splenocytes lead to an inability to maintain long-term latent viral genomes. We believe that low levels of chronic reactivating MHV68 lets to infection of naive B cells which contributes to maintaining the latently infected B cell reservoir and, in addition, the episodic expression of MHV68 replication-associated antigens drives the anti-viral antibody response. This data supports a model wherein Blimp-1 expression and subsequent plasma cell differentiation plays a significant role in the seeding of the latency reservoir, reactivation, and maintenance of long-term MHV68 infection.

MATERIALS AND METHODS

Mice and Infections. Mice were bred, housed, and treated according to the guidelines at Emory University School of Medicine (Atlanta, GA). *prdm1*$^{flox/flox}$ mice were a generous gift from Kathryn Calame (Columbia University) (41). *CD19-Cre*$^{+/-}$ mice were a kind gift from Klaus Rajewsky (Harvard University) (38). *prdm1*$^{flox/flox}$ *CD19-Cre*$^{+/-}$ mice were bred at Emory University, and *prdm1*$^{flox/flox}$ *CD19-Cre*$^{-/-}$ mice were used as controls for infections. Mice were sedated and infected with 1000 pfu of respective strain of MHV68 either intraperitoneally in 500 μL of cMEM or intranasally in 20 μL of cMEM. Mice were allowed to recover from anesthesia before being returned to their cages.

Virus generation. Wild-type MHV68 (WT MHV68) refers to the WUMS (ATCC VR-1465) strain of MHV68. MHV68/Cre virus was generated as previously described (34). MHV68/Cre.MR virus was generated by allelic exchange in *E.coli* as developed by Smith and Enquist as follows (1, 2, 44). The ORF27-ORF29b intergeneic region (WUMS sequence) of MHV68 was cloned into the suicide vector pGS284 to create pGS284-27-29B as previously described (28). pGS284 encodes for an ampicillin gene and a levansucrase cassette for positive and negative selection, respectively. S17λpir *E.coli* harboring the pGS284-27-29B plasmid were mated to GS500 *E.coli* (RecA^{+}) containing MHV68/Cre-Kan^{R} BAC (a virus wherein the Cre gene contains a kanamycin resistance (Kan) cassette), a intermediate virus made during the construction of MHV68/Cre (34). The MHV68 BAC contains a chloramphenicol (Cam) resistance cassette. Bacteria

were mated on Luria-Bertani (LB) agar plates without selection. The following day, cointegrants were selected by culturing the *E.coli* on LB plates containing both chloramphenicol and ampicillin. Matings were then allowed to resolve by culturing overnight in LB-Cam. The bacteria were then plated on LB plates containing Cam and 7% sucrose to select for loss of the pGS284 backbone sequence. Finally, individual colonies were replica plated on LB-Cam and LB-Kan plates, and $Cam^{R}Kan^{S}$ colonies were screened for loss of the Cre-Kan^{R} cassette by PCR. Positive clones were identified by PCRs for both loss of the Cre cassette and presence of the wild-type locus. The reversion to wild-type sequence as well as genomic integrity of the ORF27-ORF29b region were confirmed by Southern blotting, resulting in the selection of MHV68/Cre.MR BAC. MHV68/Cre.MR BAC DNA was purified using a Midi Prep Kit (Quiagen, Hilden, Germany) with a modified manufacturer's protocol.

BAC DNA was used to transfect Vero-Cre cells using the Superfect reagent (Quiagen, Hilden, Germany) as previously described (33). Following the appearance of cytopathic effect (CPE), virus was harvested and used to infect fresh Vero-Cre cells to generate high-titer cultures. Viral DNA was then purified from Vero-Cre cells infected with either MHV68/Cre or MHV68/Cre.MR as previously described (28). Viral DNA was then used to transfect Vero-Cre cells, and virus used to infect 50% confluent monolayers of Vero-Cre to generate high titer stocks. Once abundant CPE had occurred in the Vero-Cre cultures, supernatants and cells were harvested, the cells homogenized, and debris cleared by centrifugation. The resultant viral stocks were tested for mycobacterial

contamination and clean stocks were stored at -80°C. Viral titers were determined by plaque assay as previously described (28).

Virus Assays. Plaque assays were used to determine organ titer as previously described (28). Briefly, one day prior to the assay, 2 x 10^5 NIH3T12 cells were plated per well in a 6 well plate. The following day, organs frozen at -80°C in cMEM with 1.0 mM zirconia/silica beads (Biospec Products) were thawed and subjected to four rounds of mechanical disruption for 1 minute each in a Mini-Beadbeater 8 (Biospec Products). The resulting homogenates were then serially diluted 10-fold in cMEM. Media was removed from the 3T12 monolayers and replaced with 200 uL of each of the dilutions in duplicate. The plates were rocked every 15 minutes for one hour and then overlayed with 2% methylcellulose in cMEM. Approximately one week later, plates were stained with neutral red overnight, and, following aspiration of the methycellulose, plaques were scored. The limit of detection of this assay is 50 PFU per organ.

Limiting dilution assays for viral latency and reactivation were performed as previously described (21, 22). Single-copy-sensitive PCR was performed to determine the frequency of cells harboring latent viral genome. In brief, frozen samples were thawed, washed in isotonic buffer, counted, and 3-fold serial dilutions were plated in a background of 10^4 NIH 3T12 cells in 96 well plates. 12 wells were plated per dilution, and cells were lysed by protease K digestion for six hours at 56°C. Samples were then subjected to two rounds of nested PCR, and the products were resolved on 2% agarose gels. In order to measure the frequency of reactivating cells, cells were counted and plated in serial two-fold

dilutions on mouse embryonic fibroblast (MEF) monolayers in 96-well tissue culture plates. Parallel samples of mechanically disrupted cells were plated to detect preformed infectious virus. Wells were scored for cytopathic effect 14 to 21 days post-explant.

Flow cytometry. Splenocytes were incubated in 96-well round bottom plates with rat anti-mouse CD16/32 (Fc block) prior to staining (BD Biosciences). Cells were stained with the following antibodies: Vβ4-FITC, GL7-FITC, IgG-FITC, IgE-FITC, IgA-FITC, CD44-PE, CD95-PE, IgD-PE, CD4-PerCP, CD67-PECy7, CD62L-APC, CD19-APC, and CD8-Pacific Blue (BD Biosciences). Samples were fixed with 1% formalin and analyzed on a LSR II flow cytometer (BD Biosciences). Data was analyzed using FlowJo software (TreeStar, Inc., San Carols, CA).

Enzyme-linked immunosorbent assay (ELISA) for MHV68-specific antibody. ELISAs to measure the MHV68-specific antibody response were performed as previously described (19). Briefly, plates were coated with viral antigen fixed in 1% paraformaldehyde to detect MHV68-specific antibody. To create a standard curve, each plate contained wells coated with 2 μg/mL of donkey anti-goat IgG (Jackson ImmunoResearch) in coating buffer (0.1 M Na_2CO_3, 0.2% NaN_3 pH 9.6) for 2 hours at 37°C. Wells were blocked in PBS/3% BSA for 2 hours at 37°C. Wells were washed wash buffer (PBS/0.1% Tween) three times before addition of samples or standards. Three-dilutions of 1 μg/mL mouse IgG in ELISA diluent (BD Biosciences) were plated in duplicate to create a standard curve. Appropriate dilutions of serum samples from infected mice were

made in ELISA diluent and plated on wells coated with viral antigen. Plates were incubated for 3 hours at 37°C or overnight at 4°C. Wells were washed three times, and bound antibody was detected with horseradish peroxidase-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch) diluted 1:5,000. Plates were incubated for 2 hours at 37°C, washed three times, and incubated with 100 µL of 1:1 solution of developer (BD Biosciences). Reactions were stopped after 10-30 minutes with 50 µL of 2 N H_2SO_4 per well. Plates were analyzed on a Synergy HT (BioTek) with KC4 software (BioTek).

Statistical Analyses. All data analysis was conducted using GraphPad Prism software. Error bars in graphs depict the standard error of the mean. Limiting dilution assays were analyzed by subjecting the data to nonlinear regression analysis with a sigmoidal dose-response algorithm for best-fit. Poisson distribution predicts that the frequency at which 63.2% of the wells are positive for an event (PCR product or cytopathic effect) is the frequency at which there is at least one positive event in the population. Statistical significance of flow cytometry and ELISA data was determined by two-tailed, unpaired Student's T test with a confidence level of 95%.

RESULTS

Blimp-1 expression is dispensable for lytic replication in the lung and spleen. In the *prdm1*$^{flox/flox}$ mice, exons 5-8, encoding the zinc finger motifs of Blimp-1, are flanked by loxP sites (41). Expression of Cre-recombinase in cells leads to deletion of the sequence between the loxP sites, and loss of Blimp-1 protein expression (41). Recently, our lab generated and characterized a recombinant MHV68, MHV68/Cre, which harbors a Cre-recombinase expression cassette inserted into a phenotypically neutral locus in the MHV68 genome (34). To assess the role of plasma cell differentiation in acute replication, *prdm1*$^{flox/flox}$ mice were infected with 1000 pfu of MHV68/Cre or MHV68/Cre.MR either intranasaly to examine acute replication in the lungs, or intraperitoneally to examine acute replication in the spleen. Viral titers in the lungs (Fig. 1A) and spleens (Fig. 1B) at days 4 and 9 post-infection were quantified by plaque assay. We observed no significant differences in virus replication in the lungs at either day 4 or day 9 post-infection (Fig. 1A) – although there was significant mouse-to-mouse variation in viral titers observed at day 9, presumably due to rapid clearance of the virus at this relatively late acute time point (i.e., asynchronous clearance of acute virus replication). Similarly, we observed no significant difference in viral titers in the spleens of MHV68/Cre infected mice (457.5 ± 720.4 pfu) as compared to MHV68/Cre.MR mice (735.0 ± 1003.0 pfu) at day 4 post-infection (Figure 1B). There was a statistically significant difference in the splenic viral titers at day 9 post-infection, with the Cre-recombinase expressing virus exhibiting slightly higher levels of viral replication [MHV68/Cre (642 ±

579.0 pfu) and MHV68/Cre.MR (169.4 ± 140.7 pfu)], but we think this <4-fold difference is unlikely to be significant to viral pathology (Figure 1B). We conclude from this that plasma cell differentiation plays little or no role in acute virus replication.

Blimp-1 expression is necessary for the efficient establishment of latency in the spleen. We next investigated the role of plasma cell differentiation in the establishment of, and reactivation from, latency. *Prdm1*$^{flox/flox}$ mice were infected intranasaly with 1000 pfu of either MHV68/Cre or MHV68/Cre.MR, and MHV68 latency and reactivation in the spleen was measured by limiting-dilution analyses at day 18 post-infection. Somewhat surprisingly, splenic latency was reduced almost 11-fold in the *prdm1*$^{flox/flox}$ mice infected with MHV68/Cre (1/5,687 splenocytes harbored latent viral genomes) compared to those animals infected with MHV68/Cre.MR (1/523) (Figure 2A). In line with the decreased efficiency in the establishment of latency, *prdm1*$^{flox/flox}$ mice infected with MHV68/Cre exhibited decreased virus reactivation compared to *prdm1*$^{flox/flox}$ mice infected with MHV68/Cre.MR (1/109,065 splenocytes in MHV68/Cre infected mice versus 1/15,931 splenocytes in MHV68/Cre.MR mice) (Figure 2B). This result suggested an important role for plasma cell differentiation during the establishment of MHV68 latency – perhaps reflecting a role for virus reactivation from latently infected B cells trafficking from distal sites in seeding acute replication in the spleen at early times post-infection.

Because we have previously noted from some MHV68 mutants differences in the establishment of splenic latency that are dependent on the route of

inoculation (21, 22), we infected *prdm1*$^{flox/flox}$ mice via intraperitoneal inoculation with 1000 pfu of either MHV68/Cre or MHV68/Cre.MR. As before, we again observed a defect in the establishment of latency [7-fold in the *prdm1*$^{flox/flox}$ mice infected with MHV68/Cre (1/906 splenocytes harbored latent viral genomes) compared to those animals infected with MHV68/Cre.MR (1/124)] (Figure 2C). Furthermore, infection of *prdm1*$^{flox/flox}$ with MHV68/Cre led to a 12.7-fold defect in the frequency of splenocytes reactivating from latency (1/89,493 splenocytes in MHV68/Cre infected mice versus 1/7,006 splenocytes in MHV68/Cre.MR mice) (Figure 2D). Thus, taken together with the decrease establishment of latency, we observed a modest impact on the efficiency of splenocyte reactivation (Table 1). Notably, previous characterizations of the MHV68/Cre virus in C57Bl/6 mice did not reveal any defect in establishment or reactivation from latency (34), indicating that the observed latency phenotypes in the *prdm1*$^{flox/flox}$ mice infected with the MHV68/Cre virus are due to loss of a functional Blimp-1 gene.

Mice deficient in plasma cells exhibit decreased establishment of, and reactivation from, splenic latency in vivo. The experiments described above depended upon efficient excision of exons 5-8 in the *prdm1* gene by Cre-recombinase expressed from the MHV68 genome. We were concerned that limited expression of Cre-recombinase from the viral genome might lead to a failure to efficiently excise exons 5-8 from both floxed *prdm1* alleles and thus could mask viral latency and reactivation phenotypes that are dependent upon plasma cell differentiation. To address this concern, we bred *prdm1*$^{flox/flox}$ to *CD19*$^{Cre/+}$ mice and backcrossed to *prdm1*$^{flox/flox}$ mice to select for homozygosity

at the *prdm1* locus. The resultant *prdm1*$^{flox/flox}$ *CD19*$^{Cre/+}$ mice express Cre-recombinase under the control of the CD19 promoter, and thus these mice lack Blimp-1 expression in all B cells. Previous characterization of *prdm1*$^{flox/flox}$ *CD19*$^{Cre/+}$ mice has demonstrated that they lack pre-plasma memory and plasma B cells (41).

We examined the effect of lack of plasma cells in the entire mouse on latency and reactivation by comparing wild type MHV68 infection of *prdm1*$^{flox/flox}$ *CD19*$^{Cre/+}$ and *prdm1*$^{flox/flox}$ mice. Following intranasal inoculation with 1,000 pfu of MHV68, at day 18 post-infection there was a similar frequency of latently infected splenocytes in both groups of mice (Figure 3A). However, there was a 21-fold decrease in the frequency of splenocytes capable of reactivation from latency in the *prdm1*$^{flox/flox}$ *CD19*$^{Cre/+}$ mice (Figure 3B). This decrease in the frequency of reactivating splenocytes is reflected in a dramatic decrease in the efficiency of reactivation (0.4% versus 7% in the *prdm1*$^{flox/flox}$ mice) (Table 1). These data demonstrate a requirement for Blimp-1 for MHV68 reactivation from splenocytes.

Following intraperitoneal infection with 1000 pfu of MHV68, we observed a 12-fold decrease in the establishment of latency in *prdm1*$^{flox/flox}$ *CD19*$^{Cre/+}$ mice (1/1073 splenocytes) as compared to the control *prdm1*$^{flox/flox}$ mice (1/90 splenocytes) (Figure 3C). Similar to infection of *prdm1*$^{flox/flox}$ with the transgenic MHV68/Cre virus, we observed a further five-fold reduction in the frequency of splenocytes capable of reactivating from latency in the *prdm1*$^{flox/flox}$ *CD19*$^{Cre/+}$ mice (1/129,320) versus the control animals (1/7664) for a combined 17-fold reduction in reactivation frequency (Figure 3D). Overall, there was only a modest

decrease in reactivation efficiency (0.8% versus 1.2%) in the *prdm1*$^{flox/flox}$ *CD19*$^{Cre/+}$ mice (Table 1). Finally, following intraperitoneal inoculation we did not observe any significant difference in the frequency of PECs capable of establishing or reactivating from latency in the *prdm1*$^{flox/flox}$ *CD19*$^{Cre/+}$ mice (Figure 3E and F).

A possible explanation as to why we observe equal levels of latency following intranasal inoculation but not intraperitoneal inoculation may hinge on little-understood route-dependent differences in the immune response to MHV68. *prdm1*$^{flox/flox}$ *CD19*$^{Cre/+}$ mice have enlarged germinal centers in response to antigenic stimulation; perhaps the equal amounts of latent virus in the two groups of animals reflects an increase in latency in the germinal center B cells in the *prdm1*$^{flox/flox}$ *CD19*$^{Cre/+}$ mice following intranasal inoculation (41). However, following intraperitoneal inoculation, the dynamics of seeding splenic latency may be different, perhaps failing to elicit as potent a germinal center reaction in the *prdm1*$^{flox/flox}$ *CD19*$^{Cre/+}$ mice, leading to the observed deficient in the establishment of latency. It is certainly notable that the frequency of splenocytes reactivating virus in wt MHV68 infected *prdm1*$^{flox/flox}$ mice was very similar whether the mice were infected via intranasal or intraperitoneal inoculation, even though there was a significant greater frequency of viral genome positive splenocytes following intraperitoneal inoculation compared to intranasal inoculation (Table 1). In addition, as discussed above, there are mutant strains of MHV68 which have revealed profound differences in pathology that are route-dependent (21, 22). Notwithstanding these issues, taken together these studies provide evidence that Blimp-1 (and presumably plasma cell

differentiation) plays an important role in both the establishment of latency, as well as reactivation from latency *in vivo*, perhaps pointing to a complex role of virus reactivation from B cells and seeding of latency under certain circumstances.

B cell responses to MHV68 are diminished in the absence of Blimp-1. To follow up on the analysis of MHV68 latency in specific reservoirs, we next assessed whether there were alterations in the host immune response to MHV68 infection in the absence of Blimp-1 expression in infected cells. To this end, we examined the B and T cell responses following intraperitoneal MHV68 infection at days 18, 42, and 90 post-infection by multicolor flow cytometry. At day 18 post-infection, at the peak of latency, we observed a significant decrease in the frequency of activated ($CD69^{high}$) B cells in the $prdm1^{flox/flox}$ mice infected with MHV68/Cre as compared to those infected with the marker rescue virus, MHV68/Cre.MR (Figure 4, A and B). Notably, under these experimental conditions Blimp-1 is absent in only those B cells that are MHV68 infection (<1% of B cells). Thus, global B cell activation is influenced by the inability of MHV68 infected B cells to differentiate into plasma cells.

In the absence of B cell activation, we hypothesized that the frequency of B cells capable of entering a germinal center reaction and undergoing class-switch recombination would be reduced. Indeed we observed a lower frequency of $CD95^{high}GL7^{high}$ B cells in the $prdm1^{flox/flox}$ mice infected with MHV68/Cre versus MHV68/Cre.MR (Figure 4, A and C). A lower frequency of B cells underwent class-switch recombination becoming IgD^{low} $IgG/A/E^{+}$ in the $prdm1^{flox/flox}$ mice

infected with MHV68/Cre (Figure 4, A and C). However, by day 42 post-infection, there was no significant difference in the B cell responses in the *prdm1*$^{flox/flox}$ mice infected with MHV68/Cre or MHV68/Cre.MR, and the B cell responses were largely quiescent as previously observed (data not shown) (19, 28). Interestingly, we have observed similar decreases in B cell responses in other instances where there are defects in the establishment of latency and reactivation from latency, such as loss of MyD88 signaling, inhibition of NFκB signaling, or infection with a M2-null virus (19, 28) (Siegel and Speck, unpublished data).

Vβ4$^+$ CD8$^+$ T cells expand to wild-type levels in the absence of Blimp-1. Infection of C57Bl/6 mice with MHV68 leads to an expansion of a TCR-specific population of CD8$^+$ T cells that share the Vβ4 chain of the TCR (15, 52). Notably, the Vβ4$^+$ CD8$^+$ T cell population fails to contract over time, and we have previously shown that the M1 ORF is required for the expansion of this population of T cells *in vivo* (14). We have shown that M1 limits the degree of reactivation *in vivo* though induction of IFNγ, and B cells are required for the Vβ4$^+$ CD8$^+$ T cell expansion (6, 11). Thus, we hypothesized that during an infection where reactivation is decreased from B cells, we might observe a defect in the expansion of this T cell subset if M1 expression is tied to MHV68 reactivation. However, over the course of the infection, we observed no significant decrease in the frequency of Vβ4$^+$CD8$^+$ T cells in the *prdm1*$^{flox/flox}$ mice infected intraperitoneally with MHV68/Cre (Figure 5A). This data suggests that

Blimp-1-mediated reactivation is not necessary for the expansion of the Vβ4$^+$ CD8$^+$ T cell population in the spleen.

We next accessed the CD4$^+$ T cell response to MHV68 infection by measuring the frequency of effector CD4$^+$ T cells in the spleen (CD62L^{low}CD44^{high}). We observed a 20% decrease in the frequency of effector CD4$^+$ T cells in the *prdm1*$^{flox/flox}$ mice infected with MHV68/Cre intraperitoneally at day 18 post-infection (51% in MHV68/Cre versus 63% in MHV68/Cre.MR mice) (Figure 5B). However, during a long-term MHV68 infection, there was no significant difference in the effector CD4$^+$ T cell response in the two groups of animals, and the CD4 response was largely quiescent in both groups by day 90 post-infection (Figure 5B). We observed no difference in the CD8$^+$ effector T cell populations in the two groups of mice throughout the time-course of infection (data not shown).

Blimp-1 is necessary for the prolonged antibody response to MHV68 in vivo. MHV68 infection leads to a delayed increase in MHV68-specific serum IgG beginning at day 20 post-infection that is maintained for more than 100 days post infection (46). We asked if the loss of Blimp-1 expression was associated with a decrease in the antibody response to MHV68. Beginning at day 42 post-infection, we observed a significant four-fold decrease in the levels of MHV68-specific IgG in the *prdm1*$^{flox/flox}$ infected with MHV68/Cre intraperitoneally versus those infected with MHV68/Cre.MR (Figure 6). Importantly, the lower antibody titers in the *prdm1*$^{flox/flox}$ MHV68/Cre infected animals persisted until the end of the time-course at day 90 post-infection.

Importantly, as noted above, this experimental approach only leads to a loss of Blimp-1 in infected B cells, which represent <1% of B cells in the infected mouse. Previously, a transient decrease in MHV68-specific IgG was observed in MyD88$^{-/-}$ animals, but, as the infection progressed, antibody levels became equivalent to wild-type (19). We observed no such return of antibody titers, suggesting that perhaps differentiation to a plasma cell phenotype and the associated reactivation from latency is needed to maintain the high levels of MHV68-specific IgG observed in wild-type mice.

Loss of Blimp-1 expression in infected splenocytes leads to a significant decrease in latency at day 90 post-infection. We next examined the requirement for Blimp-1 expression for the maintenance of MHV68 latency at late time-points (day 42 and day 90) post-infection. Mice were inoculated intraperitoneally with 1000 pfu of MHV68/Cre and MHV68/Cre.MR, splenocytes were harvested at 42 and day 90, and latency was measured by limiting-dilution PCR. At day 42 post-infection, there was no significant difference in the frequency of latently infected splenocytes between the two groups of mice [1/8953 splenocytes harbored latent MHV68/Cre as compared to 1/3295 splenocytes in MHV68/Cre.MR animals, a roughly three-fold difference in the frequency of latently infected splenocytes] (Figure 7A). Interestingly, we observed a significant decrease in the frequency of latently infected splenocytes at day 90 post-infection in the mice infected intraperitoneally with MHV68/Cre (Figure 7B). Only 1/19,210 splenocytes from MHV68/Cre mice carried latent MHV68 as compared to 1/2350 splenocytes from MHV68/Cre.MR animals, an

eight-fold decrease in latency (Figure 7B). These results are significant because previously characterized mutations affecting latency and reactivation have resulted in an eventual return to wild-type levels of latency at late times post-infection (19, 21). These data suggest that Blimp-1 is necessary for the maintenance of MHV68 latency, presumably through episodic reactivation necessary for reseeding the latency reservoir.

DISCUSSION

In this report we demonstrate that Blimp-1 expression plays a role in both the establishment of latency as well as reactivation from splenic latency. Importantly, abrogation of Blimp-1 expression in infected splenocytes led to a loss of long-term latency in the spleen and a significant decrease in the humoral response to MHV68. Together, these results suggest that periodic reactivation from the plasma cell compartment is necessary for long-term latency maintenance as well as the protracted, high serum levels of MHV68-specific antibodies observed in MHV68 infected mice.

Following infection, *prdm1*$^{flox/flox}$ mice had no defect in acute viral replication, yet there was a significant decrease in the establishment of and reactivation from latency. Recently, our lab generated a system to track cells infected with MHV68 *in vivo* by inserting a YFP transgene into the ORF27-29b intergenic region (MHV68/YFP) (12). Flow cytometry analysis of MHV68/YFP intranasally infected mice showed that a significant proportion of infected splenocytes at day 14 post-infection were plasma cells (ca. 13%) (12). Additionally, MHV68 infected plasma cells were sorted from mice and shown to reactivate from latency with greater efficiency than total splenocytes (Liang et al., submitted for publication). Without Blimp-1 expression in infected splenocytes, MHV68 latency waned by day 90 post-infection. Additionally, viral replication in B cells is critical for maintenance of MHV68 latency (30). Together, these data support the hypothesis that MHV68 reactivation occurs in the plasma cell compartment, and that Blimp-1 expression plays a role in MHV68 reactivation.

We propose that a burst of reactivation is necessary for efficient seeding of splenic latency. In addition, episodic reactivation from plasma cells leads to infection of naïve B cells and reseeding of the latency reservoir.

Notably, murine gammaherpesvirus68 itself carries genes capable of facilitating reactivation (21, 54). One such gene, designated M2, is a latency-associated protein that plays a role in both the establishment of latency and reactivation from latency (21, 22, 32). M2 expression in primary murine B cells leads to B cell proliferation, survival, and differentiation to a pre-plasma memory B cell phenotype (43). M2-mediated proliferation is dependent on IL-10, and M2 expression in B cells leads to copious secretion of IL-10 *in vitro* (43). Additionally, infection of mice with an M2-null strain of MHV68 leads to a significant reduction in serum IL-10 levels at the onset of latency (43). IL-10 interrupts human B cells in the germinal center and triggers them to differentiate into plasma cells (10). Experiments utilizing an YFP-tagged M2-null strain of MHV68 revealed that M2 is necessary for MHV68 latency in the plasma cell compartment (Liang et al., submitted for publication). These studies directly link M2, a specific MHV68 viral protein necessary for reactivation from latency, to the manipulation of plasma cell differentiation *in vivo*. Notably, M2 expression in BCL1 B cells lead to upregulation of Blimp-1, XBP-1s, and IRF4 transcripts, directly linking M2 and plasma cell differentiation (Liang et al., submitted for publication). We hypothesize that M2 mediates reactivation by manipulating B cell differentiation to a plasma cell phenotype, a cell type permissive for reactivation.

Although highly compromised, reactivation was not completely eliminated even in the plasma cell-deficient animals. Likewise, sorted plasma cells from mice infected with MHV68 accounted for much, but not all, of reactivation observed upon *ex vivo* culturing (Liang et al., submitted for publication). We hypothesize that there are pathways to MHV68 reactivation independent of Blimp-1 and plasma cell differentiation. For instance, stress of the host B cell or reactivation from alternative latency reservoirs (e.g., macrophages and dendritic cells) also likely contributed to the observed reactivation, especially at the early onset of latency (day 18 post-inoculation). These alternative pathways of reactivation are echoed in the humoral response to MHV68 which, although significantly lower, is not diminished in the absence of Blimp-1 expression in infected B cells. The antibody response likely reflects the low-levels of persistent viral reactivation that cannot be measured by *ex vivo* limiting-dilution reactivation assays, but, nonetheless, continues to occur.

How is this periodic reactivation likely triggered *in vivo*? Stimulation of B cells though the BCR, TLRs, and CD40, as well as exposure to IL-21, IL-2, IL-6, IL-5, and IL-10 have been shown to lead to *prdm1* expression [reviewed in (7)]. Stimulation of latently infected splenocytes with anti-IgM/IgG and anti-CD40 leads to an increase in reactivation *ex vivo*, implying that direct BCR stimulation of latently infected memory B cells with their cognate antigen could lead to plasma cell differentiation and reactivation (35). However, the likelihood of a memory B cell encountering the specific antigen that their BCR recognizes is very low and, as such, herpesvirus latency would most likely be lost. We hypothesize that rather than an encounter with a specific antigen, stimulation of memory B

cells with a heterologous pathogen through their toll-like receptors (TLRs) leads to maintenance of latency through plasma cell differentiation. Indeed, humoral memory is known to be maintained through TLR signaling (4). *In vivo* stimulation with TLR ligands leads to an increase in MHV68 reactivation, seeding more latently infected splenocytes, "refilling" the latency reservoir (18). Additionally, MyD88-deficient mice have a significant defect in reactivation from latency (19). Mice deficient in TLR signaling have a decreased ability to form germinal centers, express Blimp-1, and secrete antibody in a T-dependent fashion (36). EBV LMP2a has been shown to enhance B cell sensitivity to TLR9 stimulation (55). We hypothesize that superinfection of MHV68 latently-infected mice with another virus or bacteria is likely to lead to stimulation of TLRs on memory B cells with a higher frequency, triggering plasma cell differentiation and reseeding of latency.

Plasma cell differentiation triggers reactivation in both EBV and KSHV. The transcription factor X-box binding protein-1s (XBP-1) is required for plasma cell differentiation, and XBP-1s expression in EBV and KSHV latently infected cell lines leads to reactivation (37). Expression of XBP-1s in latent KSHV-infected B cells leads to transactivation of the RTA promoter and expression of the lytic protein K8 (59). XBP-1s binds directly to the Rta promoter leading to induction of KSHV reactivation (58). In latently infected epithelial cells and lymphoblastoid cells, XBP-1s in combination with protein kinase D (PKD) induces BZLF1 and BRLF1, two proteins capable of activating the entire EBV lytic cascade (5). Furthermore, as previously mentioned, XBP-1s binds the BZLF1 promoter, directly linking plasma cell differentiation and EBV reactivation (48).

It is unknown whether there is a similar mechanism of induction of Rta expression in MHV68 that is activated by transcription factors associated with plasma cell differentiation. Due to the parallel requirement for plasma cell differentiation in EBV, KSHV, and MHV68 reactivation, it is likely that a similar mechanism exists to drive MHV68 immediate-early gene expression leading to virus reactivation.

In conclusion, Blimp-1 expression in MHV68-infected splenocytes plays a role in both the establishment of latency, as well as reactivation from latency. Furthermore, we demonstrate that mice lacking the entire plasma cell compartment have a phenotype similar to those mice wherein only infected B cells cannot differentiate into plasma cells. Notably, we provide evidence to support a model in which periodic reactivation from latency is needed for long-term maintenance of MHV68 genomes and the sustained MHV68-specific humoral response. Moreover, these studies show another parallel aspect of gammaherpesvirus pathogenesis conserved across species.

FIGURES

Figure 1.

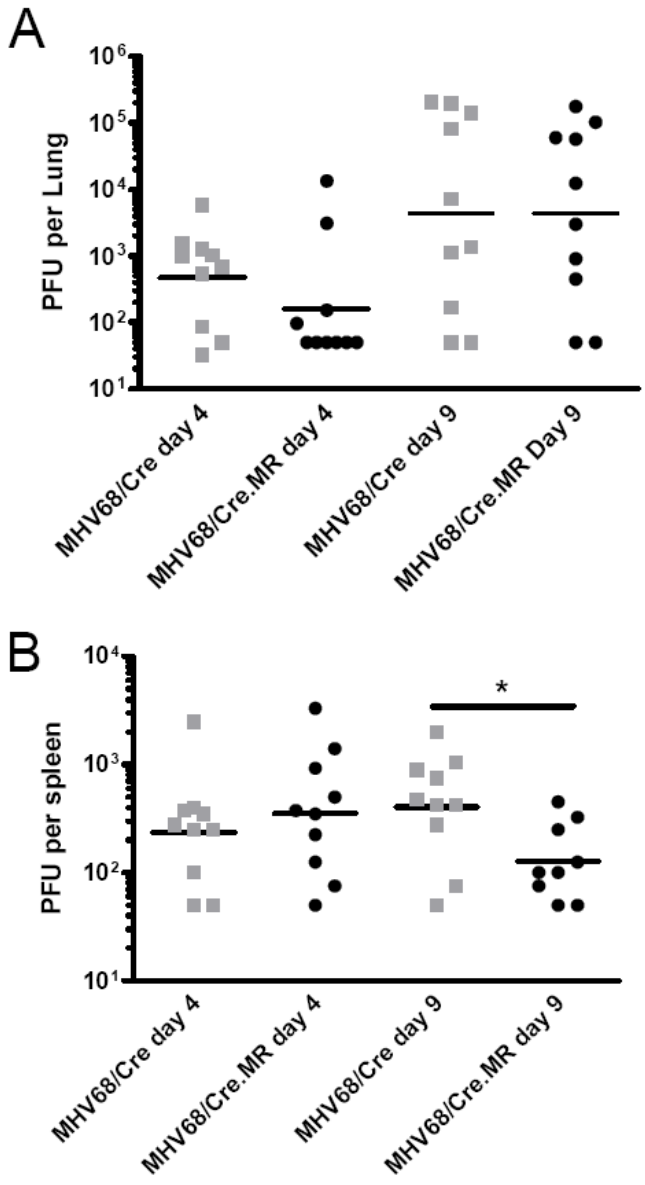

Figure 2.

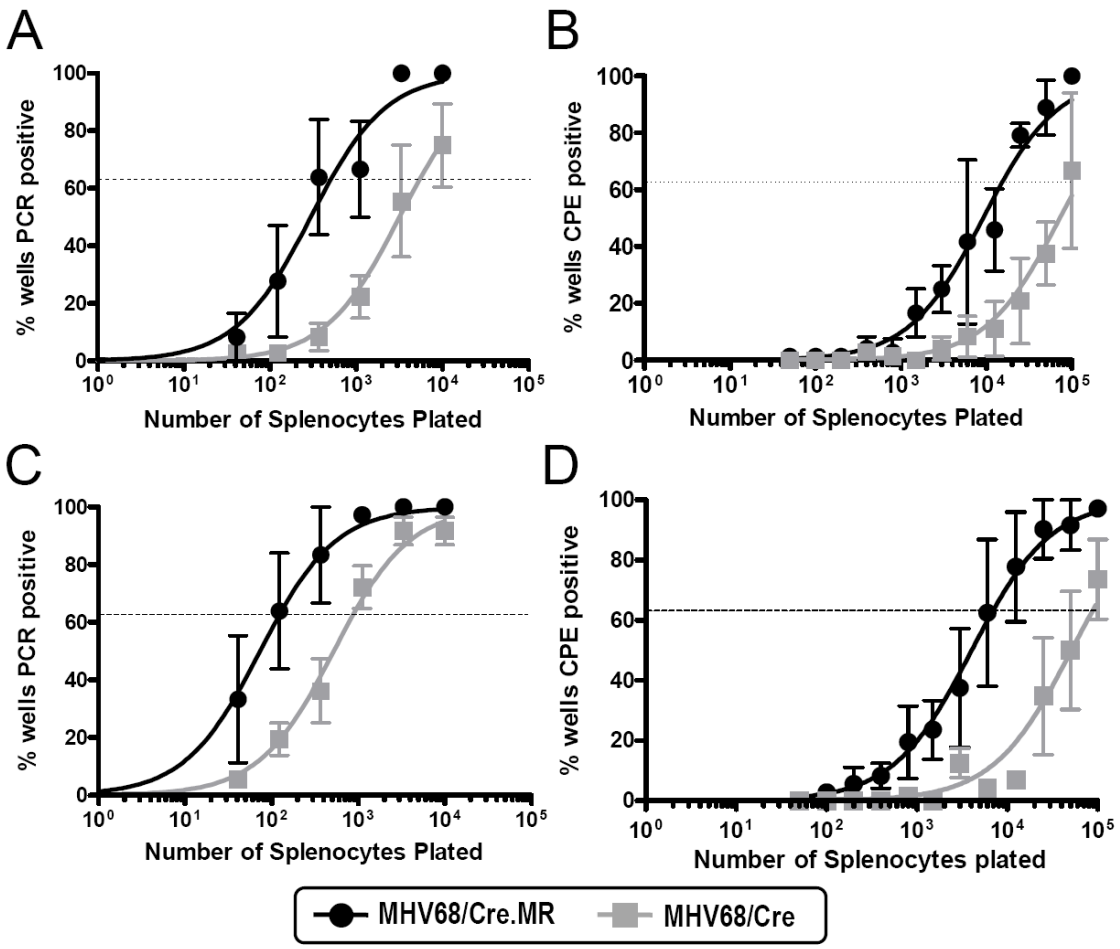
A
B
C
D
% wells PCR positive
% wells CPE positive
Number of Splenocytes Plated
Number of Splenocytes plated
MHV68/Cre.MR
MHV68/Cre

Figure 3.

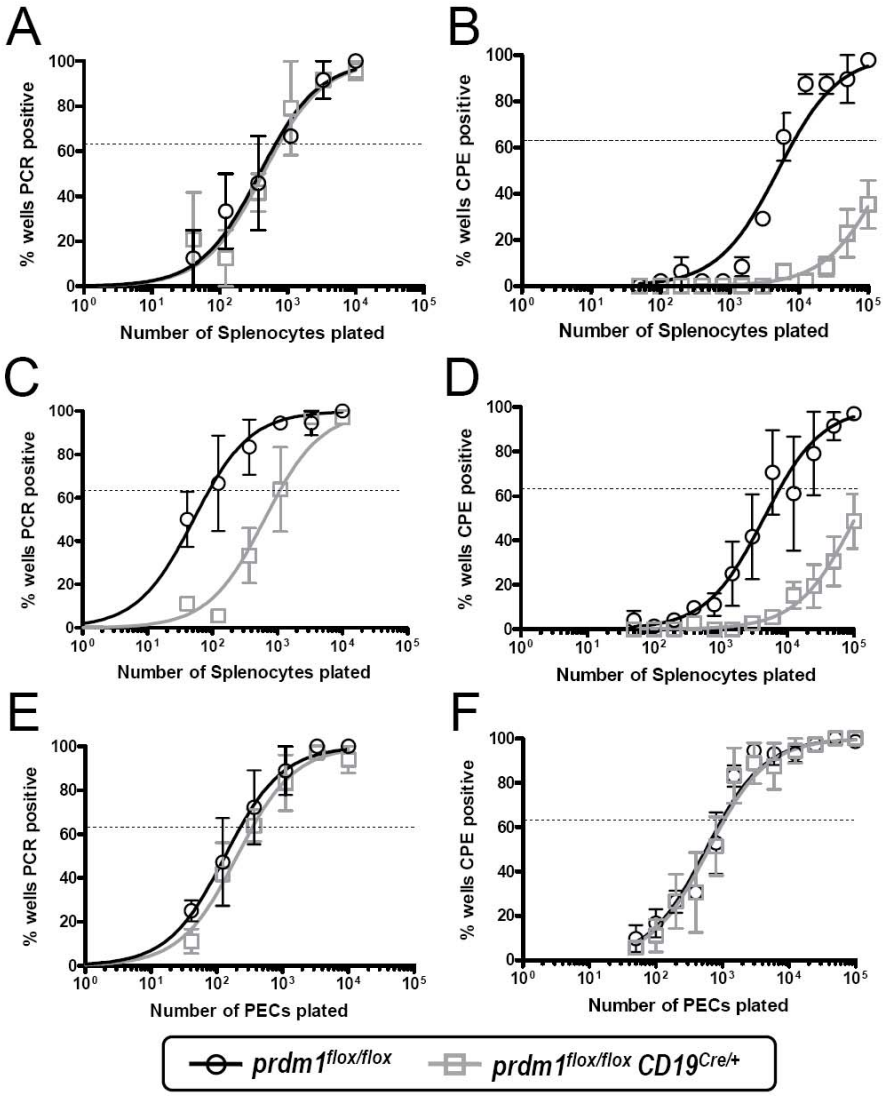
A
B
C
D
E
F
% wells PCR positive
% wells CPE positive
Number of Splenocytes plated
Number of PECs plated
prdm1flox/flox
prdm1flox/flox CD19Cre/+

Figure 4.

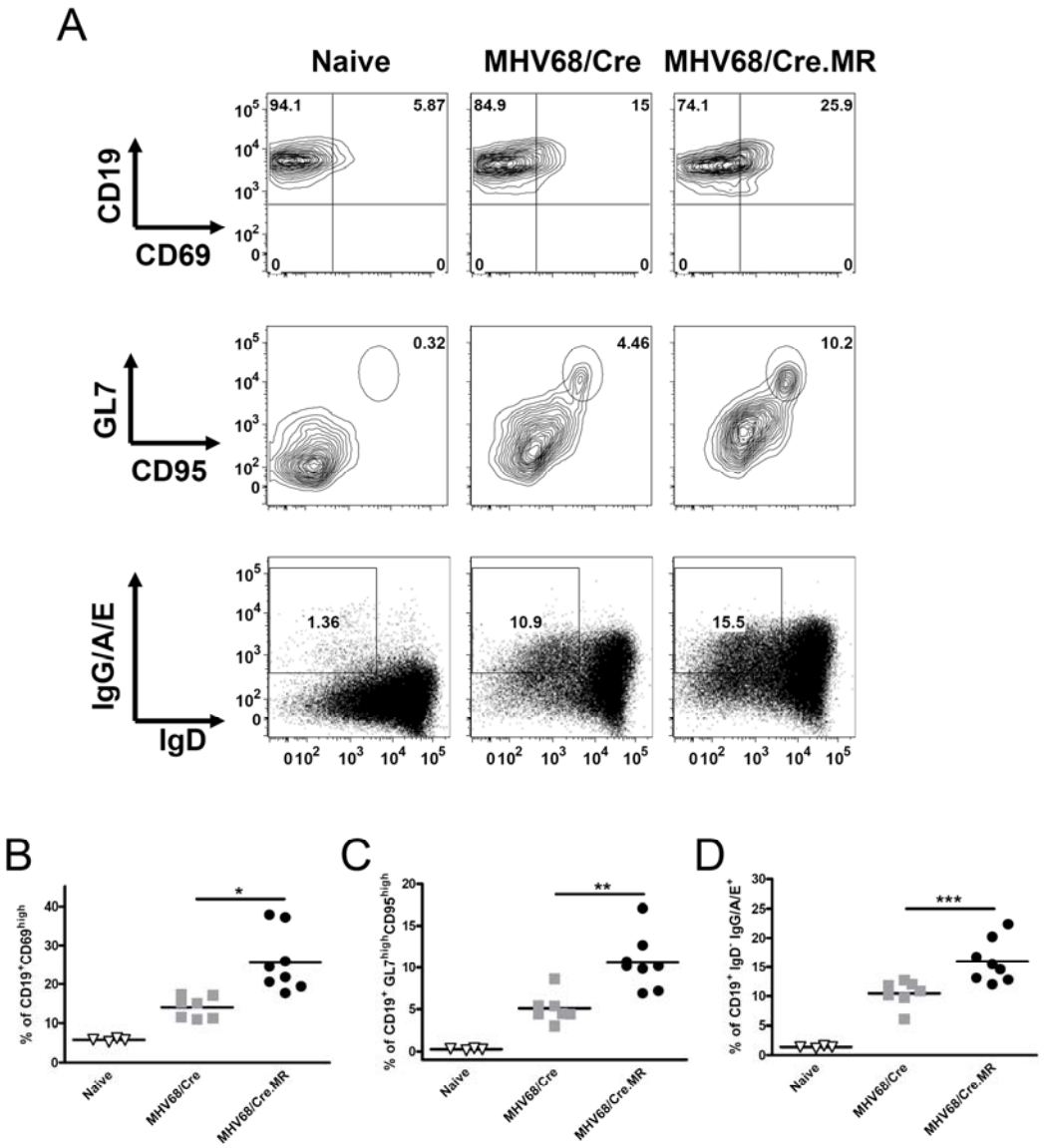
A
Naive
MHV68/Cre
MHV68/Cre.MR
CD19
CD69
94.1
5.87
84.9
15
74.1
25.9
GL7
CD95
0.32
4.46
10.2
IgG/A/E
IgD
1.36
10.9
15.5
B
% of CD19+CD69high
Naive
MHV68/Cre
MHV68/Cre.MR
C
% of CD19+ GL7highCD95high
D
% of CD19+ IgD- IgG/A/E+

Figure 5.

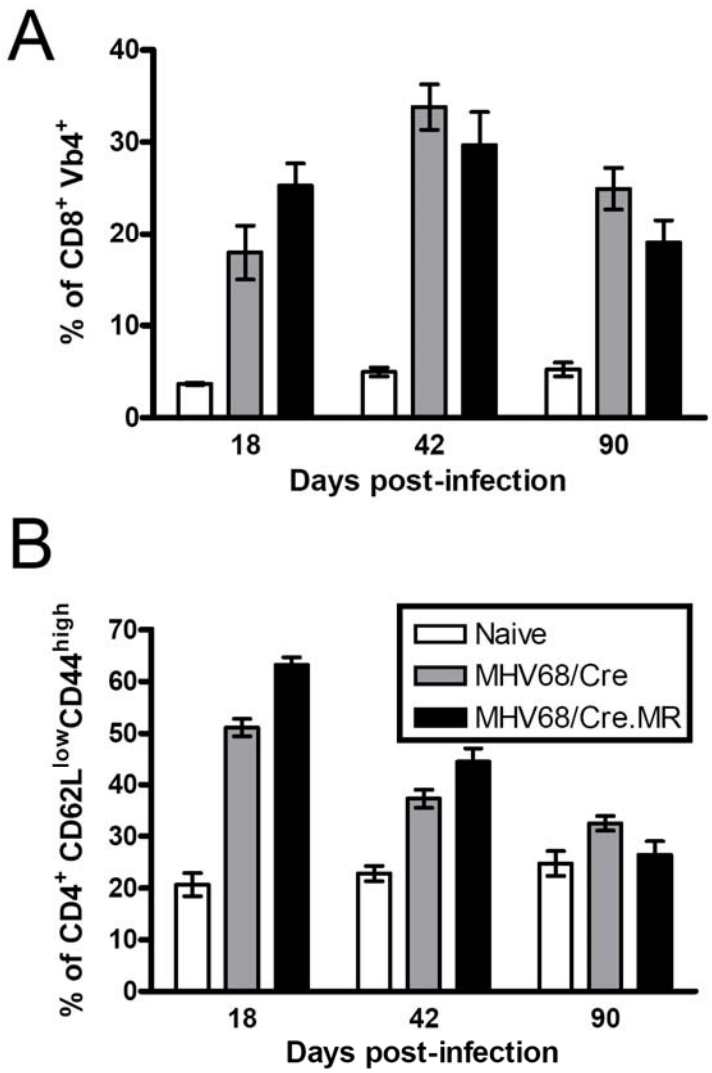
A
40
30
20
10
0
% of CD8+ Vb4+
18
42
90
Days post-infection
B
70
60
50
40
30
20
10
0
% of CD4+ CD62Llow CD44high
Naive
MHV68/Cre
MHV68/Cre.MR
18
42
90
Days post-infection

Figure 6.

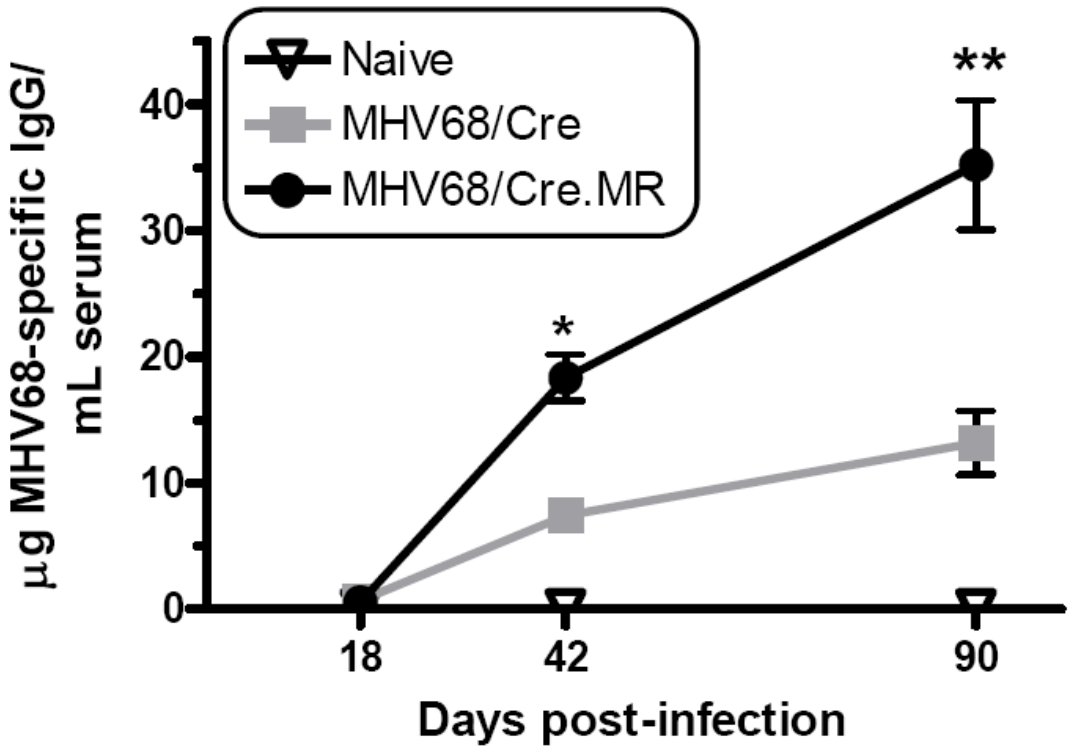
Naive
MHV68/Cre
MHV68/Cre.MR
μg MHV68-specific IgG/ mL serum
0
10
20
30
40
*
**
18
42
90
Days post-infection

Figure 7.

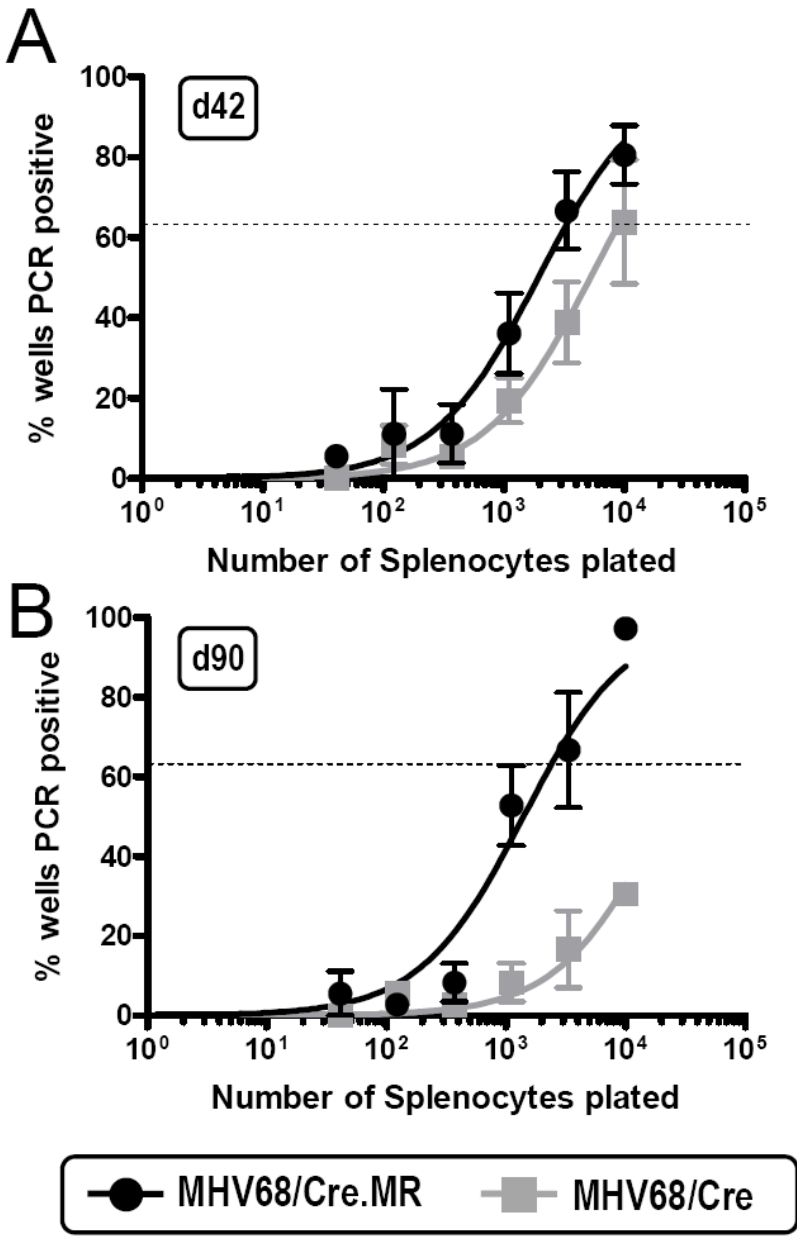
A
d42
% wells PCR positive
100
80
60
40
20
0
10^0
10^1
10^2
10^3
10^4
10^5
Number of Splenocytes plated
B
d90
% wells PCR positive
100
80
60
40
20
0
10^0
10^1
10^2
10^3
10^4
10^5
Number of Splenocytes plated
MHV68/Cre.MR
MHV68/Cre

Table 1. Frequency of viral genome positive and reactivating cells days 16-18 post-infection.

Table 1. Summary of MHV68 latency and reactivation in Blimp-1 conditional mice

Mice	Virus	Intranasal - splenocytes			Intraperitoneal - splenocytes			Intraperitoneal - PECs		
		Freq viral genome + [a]	Freq virus reactivation [b]	Reactivation efficiency [c]	Freq viral genome + [a]	Freq virus reactivation [b]	Reactivation efficiency [c]	Freq viral genome + [a]	Freq virus reactivation [b]	Reactivation efficiency [c]
Prdm1$^{flox/flox}$	MHV68-Cre.MR	1/523	1/15,931	3.3%	1/124	1/7,006	1.8%	1/131	1/378	34.5%
Prdm1$^{flox/flox}$	MHV68-Cre	1/5,687	1/109,065	5.2%	1/906	1/89,483	1.0%	1/792	1/887	89.2%
Prdm1$^{flox/flox}$	wt MHV68	1/666	1/8,653	7.7%	1/90	1/7,664	1.2%	1/239	1/969	24.7%
Prdm1$^{flox/flox}$ x *CD19*$^{Cre/+}$	wt MHV68	1/731	1/182,759	0.4%	1/1,073	1/129,320	0.8%	1/357	1/1,069	33.4%

[a] Frequency of cells harboring viral genome as determined by limiting dilution nested PCR for presence of MHV68 gene 50 sequences.

[b] Frequency of cells spontaneously reactivating virus upon explant into tissue culture determined by limiting dilution analysis.

[c] Reactivation efficiency determined as the percentage of viral genome positive cells that spontaneously reactivate upon explant.

FIGURE LEGENDS

Figure 1. Blimp-1 is dispensable for acute virus replication in the lung and spleen. *Prdm1*$^{flox/flox}$ mice were infected either intranasaly **(A)** or intraperitoneally **(B)** with 1000 pfu of either MHV68/Cre or MHV68/Cre.MR. Lungs and spleens were harvested at days 4 and 9 post-infection and viral titers determined by plaque assay (see Materials and Methods). A statistically significant difference in titers between the groups of mice at day 9 post-infection in the spleen was observed (* p = 0.0292). Data represents results compiled from two independent experiments with five mice per group.

Figure 2. A functional Blimp-1 gene is required in infected splenocytes for both efficient establishment of latency, as well as reactivation from latency, at day 18 post-infection. *Prdm1*$^{flox/flox}$ were infected either intranasaly **(A & B)** or intraperitoneally **(C & D)** with 1000 pfu of either MHV68/Cre or MHV68/Cre.MR. At 18 days post-infection, the frequency of cells establishing latency and capable of reactivating from latency was determined by limiting dilution assay. **(A & C)** Limiting dilution determination of the frequency of splenocytes harboring latent MHV68 in *prdm1*$^{flox/flox}$ mice infected with either MHV68/Cre or MHV68/Cre.MR. **(B & D)** Limiting dilution determination of the frequency of splenocytes spontaneously reactivating MHV68 upon explant from *prdm1*$^{flox/flox}$ mice infected with either MHV68/Cre or MHV68/Cre.MR. Data

represents results compiled from three independent experiments with three to four mice per group.

Figure 3. Global loss of plasma cells in MHV68 infected mice leads to a defect in both the establishment of latency, as well as reactivation from latency, in the spleen, but has no impact on latency in the peritoneal cavity. *Prdm1*$^{flox/flox}$ *CD19*$^{Cre/+}$ mice, deficient in plasma cells, and *prdm1*$^{flox/flox}$ were inoculated intranasally **(A & B)** or intraperitoneally **(C-E)** with 1000 pfu of wild-type MHV68. At day 18 post-infection, splenocytes and PECs were subjected to limiting dilution analyses for establishment of latency and reactivation from latency as described in Materials and Methods. **(A)** The frequency of splenocytes harboring latent MHV68 was not significantly reduced in the *prdm1*$^{flox/flox}$ *CD19*$^{Cre/+}$ mice infected intranasally. **(B)** A lower frequency of splenocytes capable of reactivation from latency was also observed in the *prdm1*$^{flox/flox}$ *CD19*$^{Cre/+}$ mice following i.n. infection. **(C)** The frequency of latently infected splenocytes was reduced in *prdm1*$^{flox/flox}$ *CD19*$^{Cre/+}$ mice infected intraperitoneally. **(D)** There was a further reduction in the frequency of splenocytes reactivating from latency in *prdm1*$^{flox/flox}$ *CD19*$^{Cre/+}$ mice infected intraperitoneally. **(E & F)** No significant difference in the establishment of latency or reactivation from latency was observed in PECs isolated from MHV68 infected *prdm1*$^{flox/flox}$ or *prdm1*$^{flox/flox}$ *CD19*$^{Cre/+}$ mice. Data represents results from two to three independent experiments with three to five mice per experimental group.

Figure 4. The B cell response to MHV68 infection is lower in the absence of a functional Blimp-1 gene in infected cells. *Prdm1*$^{flox/flox}$ mice were infected with 1000 pfu of MHV68/Cre or MHV68/Cre.MR. At day 18 post-infection, spleens were harvested and single cell suspensions were stained with CD19-APC, CD69-PECy7, CD95-PE, and GL7-FITC or CD19-APC, IgD-PE, and IgG/A/E-FITC and analyzed by flow cytometry. **(A)** Representative flow cytometry data from the median mice per group. Cells were gated on a live lymphocyte gate as determined by forward and side scatter followed by discrimination of B cells by selecting CD19^{+} lymphocytes. **(B)** Fewer B cells from *prdm1*$^{flox/flox}$ mice infected with MHV68/Cre were activated (CD69^{high}). **(C)** A lower frequency of B cells from *prdm1*$^{flox/flox}$ MHV68/Cre infected animals entered the germinal center reaction (CD95^{high}GL7^{high}). **(D)** There was a significant decrease in the frequency of isotype-switched (IgD^{low}IgG/A/E^{+}) B cells in *prdm1*$^{flox/flox}$ mice infected with MHV68/Cre. Data represents results from two independent infections with three to four animals per group with two naïve controls per experiment. (* p = 0.0027, ** p = 0.0015, and *** p = 0.0043).

Figure 5. T cell responses in *prdm1*$^{flox/flox}$ mice infected with MHV68/Cre. *Prdm1*$^{flox/flox}$ mice were infected intraperitoneally with 1000 pfu of MHV68/Cre or MHV68/Cre.MR, and splenocytes were harvested at days 18, 42, and 90 post-infection. Cells were stained with Vβ4-FITC, CD44-PE, CD4-PerCP, CD62L-APC, and CD8-Pacific Blue and analyzed by flow cytometry. **(A)** The Vβ4^{+} CD8^{+} T cell population expands to wild-type frequencies in prdm1$^{flox/flox}$ mice infected with MHV68/Cre. **(B)** Fewer effector CD4^{+} (CD62L^{low}CD44^{high}) T

cells were observed in prdm1$^{flox/flox}$ mice infected with MHV68/Cre at day 18 post-infection. At day 42 and 90 there was no significant difference in the $CD4^+$ T cell response in the two groups of mice. Data represents results of two independent experiments with three to four mice per infection with two naïve controls per experiment. (* p = 0.0001).

Figure 6. MHV68-specific antibody responses are decreased in *prdm1$^{flox/flox}$* mice infected with MHV68/Cre. *Prdm1$^{flox/flox}$* mice were infected with MHV68/Cre and MHV68/Cre.MR, and serum MHV68-specific IgG levels were measured by quantitative ELISA. Data represents results of three independent experiments with three to four infected mice per group with two naïve controls per experiment. (* p < 0.0001, ** p = 0.0010).

Figure 7. A functional Blimp-1 gene is necessary for long-term maintenance of MHV68 latency. *Prdm1$^{flox/flox}$* mice were infected intraperitoneally with 1000 pfu of MHV68/Cre or MHV68/Cre.MR and frequency of latently infected splenocytes was determined by limiting dilution PCR at days 42 **(A)** and 90 **(B)** post-infection. Data represents results of three independent experiments with three to four mice per group.

CHAPTER IIII

SUMMARY, FUTURE DIRECTIONS, and CONCLUSIONS

Examination of host/pathogen relationships in immunology is often confounded with an inability to examine both sides of the problem. Not all hosts are readily malleable, and few pathogens can be easily manipulated in the laboratory. Thus, MHV68, as a BAC cloned virus whose natural pathogen is the mouse, provides a unique system wherein to examine questions of gammaherpesvirus pathogenesis from both the host and pathogen perspective. The studies included in this dissertation sought to examine the role of a unique MHV68 gene, M2, in B cell biology, as well as the role of host plasma cell differentiation in MHV68 reactivation.

M2 and IL-10

Previous work has shown that M2 is necessary for the establishment of latency and reactivation *in vivo* (62, 68, 97). M2 is necessary for the efficient exit of latently infected B cells from the germinal center into the class-switched, memory B cell compartment (63, 142). Biochemical data indicates that M2 may manipulate the Vav-Fyn-Rac1 pathway to activate B cells (98, 116, 125). Mutational analysis of M2 has indicated that M2 likely manipulates multiple signaling pathways in order to facilitate MHV68 latency and reactivation (63). To directly address the role of M2 in B cells in a system capable of differentiation, we chose to express M2 in primary murine B cells by retroviral transduction.

In this dissertation, we demonstrated that the M2 latency-associated gene manipulates B cell biology in order to facilitate the establishment of and reactivation from latency (141). We showed that M2 expression in primary murine B cells leads to increased B cell proliferation and survival. M2 expressing B cells secreted more IgG, IL-2, IL-6, MIP-1α, and IL-10. Importantly, M2-mediated B cell proliferation was significantly reduced in IL-10$^{-/-}$ B cells and this proliferation could be rescued with exogenous recombinant IL-10. Mice infected with an M2-null strain (MHV68/M2.Stop) of MHV68 had significantly reduced levels of serum IL-10 at the onset of latency as compared to the marker rescue infected mice. Concomitant with the reduction of serum IL-10, there was an enhanced MHV68-specific CD8^{+} T cell response in the animals infected with MHV68/M2.Stop.

There are several key questions arising from this study remaining to be addressed. Firstly, does expression of M2 in B cells drive differentiation *in vivo*? We attempted to answer this question by generation of M2-expressing retroviral chimeric mice (Siegel and Speck, unpublished). Ly5.1^{+} bone marrow was transduced with MSCV M2-IRES-Thy1.1 or MSCV-M2.Stop-IRES-Thy1.1 (negative control) and adoptively transferred into lethally irradiated Ly5.2^{+} hosts. While mice receiving bone marrow transduced with the control retrovirus had transduced B and T cells in the periphery, all cells transduced with M2 died in the bone marrow of the chimeras by three weeks post-adoptive transfer (Siegel and Speck, unpublished). While Ly5.1^{+} lymphocytes were observed, there were no Thy1.1^{+} lymphocytes in the bone marrow or the spleens of the mice given M2-transduced bone marrow. We attempted to make an inducible system by

transducing bone marrow from tamoxifen-inducible Cre (Cre-Esr1) with a retrovirus carrying an inverted M2 ORF flanked by unidirectional LoxP sites. Thy1.1^{+} cells were present in the animals prior to administration of tamoxifen. Upon induction of M2 expression by intraperitoneal administration of tamoxifen, mice became visibly sick with ruffled fur and wasting (Siegel and Speck, unpublished). A few of the mice became severely anemic, but there was no uniform change in the serum cytokine profile. After induction of M2, no Thy1.1^{+} cells were observed in the periphery (Siegel and Speck, unpublished). We concluded from these studies that M2 over-expression is highly toxic *in vivo* and, therefore, expression in the context of MHV68 infection must be tightly regulated. Therefore, we turned to a system of transducing primary murine B cells *in vitro* to determine the biological consequences of M2 expression.

The second critical line of investigation is to determine the molecular mechanism of M2-mediated IL-10 secretion and B cell differentiation. Recently, the transcriptional profile of M2 transduced B cells has been studied by microarray (Rangaswamy and Speck, unpublished data). This data is highly preliminary and has not been confirmed by real-time reverse-transcriptase PCR, but it allows one to hypothesize with more confidence as to the biochemical pathways altered by M2. M2 expression in primary B cells lead to the upregulation of genes involved in TLR signaling and the JAK/STAT pathway (Rangaswamy and Speck, unpublished data). It is key to note that in order to transduce the primary murine B cells, they were stimulated overnight with LPS, a TLR4 agonist. LPS is known to stimulate B cells to differentiate into plasma cells; perhaps by enhancing TLR4 signaling, M2 is mediating its effects.

Intriguingly, LMP1 is able to bind JAK3, activate STAT-3, and induce IL-10 (137). K1 is also capable of inducing IL-10 in B cells by an unknown mechanism (87). The mechanism of M2-induced IL-10 production could possibly use the same signaling pathways as LMP1. Stat3 is phosphorylated by IL-10 binding to the IL-10 receptor, and p-Stat3 dimers, in turn, activate the IL-10 promoter in human B cells (11). Stat3 signaling is critical for IL-10 induced proliferation of the pro-B cell line Ba/F3 (124). Deletion of Stat3 in murine B cells leads to an inability of class-switched IgG B cells to differentiate into plasma cells following T cell-dependent stimulation (49). Culturing human tonsillar B cells with CD40L, IL-2, and IL-10 leads to upregulation of Blimp-1 transcripts; blocking the Stat3 pathway in BCL1 cells inhibits *prdm1* induction (85, 121). We hypothesize that M2 modulates Stat3 signaling to both induce IL-10 and cause the murine B cells to respond more potently to IL-10, upregulate Blimp-1, and differentiate into pre-plasma memory B cells. Alternatively, M2 may manipulate IL-10 induction of Stat1 and/or Stat5 signaling events or other components of the IL-10 signaling pathway (115).

An alternative mechanism is that M2 increases the amount of IL-10 receptor on the surface of the B cell. IL-10 receptor consists of two chains of two subunits, IL-10Rα and IL-10Rβ (reviewed in (115)). LPS stimulation leads to upregulation of IL-10R transcription in mouse fibroblasts (181). Human neutrophils upregulate IL-10Rα in response to LPS and have enhanced IL-10 responsiveness; perhaps M2 is likewise upregulating the IL-10 receptor (31). This question could be easily addressed by FACS sorting of transduced B cell populations, preparation of mRNA, and reverse transcriptase real-time PCR to

quantitate IL-10Rα and IL-10Rβ transcripts. Protein expression of the IL-10 receptor would be most accurately measured by western blotting of lysates from transduced populations.

Future experiments into the biochemical mechanism of M2-mediated IL-10 signaling will hinge on recapitulation of the primary B cell phenotype in a tissue-culture system. The key to understanding this process will likely be development of an M2-inducible B cell line in a cell line that is capable of undergoing B cell differentiation such as BCL1.3B3 (165). A20 B cells produce only a modest, two-fold increase in IL-10 secretion when transfected with M2 and are terminally differentiated (IgG_{2a}^{+}), and, thus, are probably not the ideal cell line to study this question. Once an inducible B cell line has been generated that secretes IL-10 when M2 protein is made, it can be used to further explore the biochemical pathways activated by M2 expression. We have yet to confirm the phosphorylation state of M2 *in vitro*, and these experiments could also be conducted using an M2-inducible cell line.

An inducible cell line would also be utilized in demonstrating M2-mediated activation of the IL-10 promoter. To date, our luciferase experiments have used a construct encoding the IL-10 promoter as mapped in the human 8226.1 B cell line and have shown no M2-mediated induction of the IL-10 promoter (Siegel and Speck, unpublished) (11). Perhaps M2 expression leads to alternative promoter usage or the murine IL-10 promoter is significantly different than the human promoter. 5' and 3' RACE analysis could be conducted in the M2-inducible cell line to map the IL-10 promoter in this context. Once the IL-10 promoter is mapped, the minimal elements needed for IL-10 induction can be

determined and TESS analysis used to predict transcription factor binding sites. Binding can then be confirmed by electromobility shift and chromatin immunoprecipitation assays (EMSA and ChIP, respectively). Direct binding of M2 to the IL-10 promoter is unlikely, as the M2 protein has no DNA binding motifs, however, that does not preclude M2 from being capable of recruiting DNA binding factors to the IL-10 promoters. EMSAs and ChIP of the IL-10 promoter in B cells could be performed with antibodies to M2 to determine if M2 is recruited to the promoter.

Blimp-1 and Murine Gammaherpesvirus68 pathogenesis

In this dissertation, we have also studied the role of Blimp-1 in MHV68 latency and reactivation *in vivo*. Differentiation to a plasma cell triggers reactivation from latency both in EBV and KSHV (24, 86). The master lytic transactivator proteins of both gammaherpesviruses contain XBP-1s binding sites, and XBP-1s is critical in the transformation of a B cell into the antibody producing factory that is a plasma cell (120, 155, 186). Due to the many biological similarities between EBV, KSHV, and MHV68 pathogenesis, we hypothesized that plasma cell differentiation would be critical in MHV68 latency and reactivation.

To this end, we studied infection of conditional Blimp-1 (B lymphocyte-induced maturation protein-1) mice ($prdm1^{flox/flox}$) with a Cre-expressing strain of MHV68 (MHV68/Cre). Blimp-1 expression is necessary for plasma cell differentiation *in vivo* (139). Deletion of Blimp-1 during MHV68 acute replication had no effect on lytic titers from the spleen. However, following

intraperitoneal or intranasal infection of *prdm1*$^{flox/flox}$ mice with MHV68/Cre, we observed a significant decrease in both the establishment of and reactivation from latency at day 18 post-infection. We also noted a significantly diminished B cell response to MHV68/Cre with decreased frequencies of activated, germinal center, and class-switched B cells at the onset of latency. At 42 days post-infection, there was an equal frequency of latently infected splenocytes in *prdm1*$^{flox/flox}$ mice infected with MHV68/Cre and MHV68/Cre.MR. Notably, at 90 days post-infection, *prdm1*$^{flox/flox}$ mice infected intraperitoneally with MHV68/Cre had a dramatically reduced frequency of latently infected splenocytes as compared to the marker rescue infected animals. Over time, MHV68/Cre infected animals also had significantly lower levels of MHV68-specific IgG. Infection of mice that lacked plasma cells entirely (*prdm1*$^{flox/flox}$ *CD19*$^{Cre/+}$) revealed that plasma cells are necessary for reactivation following intranasal infection and establishment and reactivation in the spleen following intraperitoneal infection. This data supports a model wherein periodic differentiation of memory B cells into plasma cells leads to both reseeding of the latency reservoir and maintenance of the high-titer serum anti-MHV68 antibodies.

One of the key questions arising from this study is mechanistically how plasma cell differentiation leads to MHV68 reactivation. XBP-1s directly binds and activates the RTA and BRLF1 promoters in KSHV and EBV respectively (155, 186). The MHV68 ORF50 encodes Rta, the master lytic transactivator of MHV68 reactivation (113). There is a positionally conserved XBP-1s binding site within the proximal Rta promoter of MHV68 as well, so it is plausible that MHV68 uses

the same conserved XBP-1s mediated mechanism to induce reactivation (Gray and Speck, unpublished). Additionally, Blimp-1 expression could directly trigger reactivation; TESS analysis shows a Blimp-1 binding site in the recently mapped distal Rta promoter (Gray and Speck, unpublished data) (58). The distal Rta promoter is LPS responsive in a macrophage cell line (58). Intriguingly, when MHV68 latently-infected A20s are stimulated to reactivate with PMA, they secrete 5-fold higher levels of IL-10 (Siegel and Speck, unpublished data).

Future experiments into the mechanism of plasma cell induced reactivation could focus on the conserved XBP-1s site and the predicted Blimp-1 binding site. Firstly, both promoters of Rta could be tested for activity in BCL1.3B3 cells before and after induction of plasma cell differentiation. If promoter activity is found to be responsive to plasma cell differentiation, each site could be mutated within the promoter. If mutation of the Blimp-1 and/or XBP-1s binding sites abrogates luciferase activity, binding of the transcription factors can be confirmed by EMSA and ChIP. Finally, if the site(s) are found to be of biological relevance *in vitro*, their relevance *in vivo* could be determined by mutation within MHV68, infection of mice, and determination of the frequency of cells capable of reactivation *ex vivo*.

Another key problem raised by these data is determination of the biological stimuli for reactivation *in vivo*. It has been demonstrated that LPS or CpG stimulation leads to reactivation and reseeding of the latency reservoir; however, this system is artificial (54). MHV68 replication in B cells in the peritoneum is crucial for maintenance of latency in the B cell compartment, supporting the hypothesis that this latency pool is highly dynamic and dependent

on recrudescence (89). Future work could explore the effect of heterologous infection or vaccination on MHV68 reactivation both in wild-type mice and in *prdm1*$^{flox/flox}$ mice infected with MHV68/Cre. This system would examine more physiologically relevant triggers of reactivation and test their relevance to plasma cell mediated recrudescence.

Both the Calame and Nutt groups observed Blimp-1^{+} cells that are not fully differentiated into plasma cells similar to the *in vitro* M2-transduced B cells (6, 73). Recently, infection of mice with a YFP expressing strain of MHV68 revealed that a significant percentage of infected splenocytes are plasma cells (29). An M2-null, YFP expressing strain of MHV68 (MHV68/M2.Stop.YFP) revealed that without M2, MHV68 latency does accumulate in the germinal center as previously reported (142) (Liang et al., manuscript in submission). Notably, YFP^{+} plasma cells were absent in the mice infected with MHV68/M2.Stop.YFP, indicating that M2 expression leads to full differentiation into a plasma cell *in vivo* (Liang at al, manuscript in submission). Additionally, M2 expression in BCL1 B cells lead to upregulation of Blimp-1, XBP-1s, and IRF-4 transcripts as well as an increase in cell size (Liang et al., manuscript in submission). M2 interacts with Vav1, and Vav expression in marginal zone B cells is needed for efficient induction of Blimp-1 transcripts, plasma cell differentiation, and antibody secretion following TLR stimulation (149). Therefore, mechanistically, M2 could induce plasma cell differentiation through binding Vav and enhancing TLR stimulation.

A primary question remains as to why M2-transduced B cells differentiate only to pre-plasma memory B cells *in vitro* instead of fully becoming plasma

cells. Perhaps further stimulation is needed *in vitro* to mimic the T cell help (CD40 ligation) and cytokines (IL-4, IL-5) present in a germinal center environment *in vivo* that forms in response to MHV68 infection. Future experiments of signaling events in M2-transduced primary murine B cells could be conducted using phosphorylation-specific antibodies to various signaling molecules. We hypothesize that M2-transduced B cells will respond more strongly to BCR and anti-CD40 ligation, supporting the theory that M2 enhances B cell signaling by interacting with Vav. Examination of signaling in M2-transduced B cells following BCR ligation could lead to further insight into the molecular mechanisms of latency establishment and reactivation.

Conclusions

Together, the studies of M2 and Blimp-1 point to a role for reactivation in seeding and maintaining the latency reservoir through manipulation of B cell differentiation. We hypothesize that M2 expression in MHV68 infected B cells leads to IL-10 and Blimp-1 expression which together influence the host B cell to differentiate into a plasma cell. IL-10 is key in this process because it is known to influence B cell development towards a plasma cell fate. During the establishment phase of latency, plasma cell mediated reactivation is critical for efficient seeding of the latency reservoir. Once latency has been established in memory B cells, M2 enhances TLR signaling in response to heterologous pathogens, enabling differentiation from memory B cell to plasma cell. MHV68 reactivation from the resultant plasma cell infects fresh naïve B cells, refilling the

pool of latently infected cells. The subsequent frequent, low level exposure to MHV68 lytic antigen ensures prolonged levels of serum anti-MHV68 antibody.

Gammaherpesviruses have evolved several strategies to exploit the same pathways to establish and reactivate from latency – both on the side of the host and on the side of the pathogen. This dissertation work provides a link between these two aspects of MHV68 pathogenesis, namely, that M2 manipulates B cells in vitro to resemble pre-plasma memory B cells and Blimp-1 expression is necessary for MHV68 pathogenesis. Although M2 shares no direct homology to another gammaherpesvirus protein, it is possible that the human herpesviruses, too, carry a gene that is capable of influencing B cell differentiation towards a plasma cell fate. Understanding the molecular mechanism of how the M2 and Blimp-1 pathways are intertwined will, hopefully, inform on more universal themes of gammaherpesvirus biology.

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