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The interaction of murine gammaherpesvirus 68 with the innate and adaptive immune systems of the host

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Abstract of the Dissertation

The interaction of murine gammaherpesvirus 68 with the innate and adaptive immune systems of the host

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Herpesviruses establish life-long infections that represent a détente with the immune system of the host. These large enveloped DNA viruses have two distinct phases of their life cycle. During lytic infection, most viral genes are expressed and infectious virions are produced for dissemination. In contrast, viral gene expression is tightly controlled during the latent stage of infection, and the virus is not easily targeted by the immune system. Gammaherpesviruses evade clearance by the immune response to gain access to B lymphocytes, the primary reservoir of latent infection. In the context of immune suppression, control of the infection is lost, increasing the risk of virus-driven malignancies. Our laboratory seeks to understand the mechanisms by which gammaherpesviruses overcome innate and adaptive immune clearance and hijack immune cells for latency. We use a natural pathogen of murid rodents, murine gammaherpesvirus 68 (MHV68), to probe the interactions between the virus and the host. T cells of the adaptive immune system that recognize both lytic and latent viral antigens control

long-term infection. I determined that T cells lacking the negative regulators Suppressor of TCR signaling-1 and -2 (Sts-1 and Sts-2) better respond to gammaherpesvirus-infected cells in vitro. However, hyper-responsiveness in the T cell compartment did not impact pathogenesis *in vivo*. With regard to innate immunity, the inflammasome is an intracellular surveillance system that can detect pathogen-associated molecules. I found that Caspase1-mediated inflammasome signaling did not contribute to the control of MHV68 pathogenesis; but *in vitro* investigations uncovered a reduction in the pro-inflammatory molecule IL-1beta upon infection of primary macrophages. Lastly, I sought to examine the role of IKKalpha-dependent non-canonical NF-kappaB signaling pathway during gammaherpesvirus pathogenesis. Although IKKalpha-mediated signaling was dispensable for virus production, IKKalpha signaling was dampened upon extrinsic signaling during lytic infection. In summary, my studies determined that enhancement of T cell responses did not lead to improved clearance of the virus and that MHV68 infection evades activation of pro-inflammatory responses and alternative NF-kappaB signaling. These studies emphasize the complexity of gammaherpesvirus interactions with the innate and adaptive immune arms of the host and uncover novel mechanisms by which a gammaherpesvirus subverts these responses.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

BAC – Bacterial artificial chromosome

BM – Bone marrow

BMDM – Bone marrow-derived macrophages

CIY – MHV68-Cre-IRES-YFP

CLN – Cervical lymph node

CoIP - Coimmunoprecipitation

CPE – Cytopathic effect

DC – Dendritic cell

dsDNA – double stranded DNA

EBNA1 – Epstein-Barr nuclear antigen 1

EBV – Epstein-Barr virus

ELISA – Enzyme-linked immunosorbent assay

H2B – Histone 2B

HHV-4 – Human herpesvirus 4, Epstein-Barr virus

HHV-8 – Human herpesvirus 8, Kaposi's sarcoma-associated herpesvirus

Hpi – hours post infection

IFNα – Interferon alpha

IFNβ – Interferon beta

IFNγ – Interferon gamma

IKKα – Inhibitor of kappa B kinase alpha

IKKαSA – MHV68-IKKα S176A S180A IRES-YFP

IKKβ – Inhibitor of kappa B kinase beta

IKKγ – Inhibitor of kappa B kinase gamma

IN – Intranasal

IP – Intraperitoneal

IRES –Internal ribosome entry sequence

ITAM – Immunomodulatory tyrosine activation motif

IY – MHV68-IRES-YFP

Kbp – Kilobase pairs

KSHV – Kaposi's sarcoma associated herpesvirus

LCL – Lymphoblastoid cell line

LMP1 – Latent membrane protein 1

lncRNA – long non-coding RNA

LDA – Limiting dilution coculture assay

LD-PCR – Limiting dilultion polymerase chain reaction

LTβ – Lymphotoxin beta

LTβR – Lymphotoxin beta receptor

MEF – Mouse embryonic fibroblasts

MFI – Mean fluorescence intensity

MHV68 – Murine gammaherpesvirus 68

miRNA – micro RNA

MLN – Mediastinal lymph node

MOI – Multiplicity of infection

NEMO – see IKKγ

NF-κB – Nuclear factor kappa B

NK – Natural killer cell

NLR – NOD-like receptor

NLS – Nuclear localization signal

ORF – Open reading frame

PBMC – Peripheral blood mononucleocytes

PEC – Peritoneal exudate cell

PFU – Plaque forming units

PTLD – Post transplant lymphoproliferative disorder

RFLP – Restriction fragment length polymorphism

RT-PCR – Reverse transcriptase PCR

RTA – Replication and transcription activator

SCLN – Superficial cervical lymph node

Th1 – T helper 1

Th2 – T helper 2

TNFα – Tumor necrosis factor alpha

UTP – Uracil triphosphate

UTR – untranslated region

vGPCR – Viral G-protein coupled receptor

vUTPase – Viral UTPase

WT – Wild type

YFP – Yellow fluorescent protein

Chapter 1. Introduction

I. Overview of Gammaherpesviruses.

Gammaherpesviruses have been identified in many vertebrates and some invertebrates. Each gammaherpesvirus has extremely specific host tropism, limiting infection to a family of animals. There are two known human gammaherpesviruses. EBV (Epstein-Barr virus, formerly identified as Human Herpesvirus-4) is the prototypical member of the lymphocryptoviruses. KSHV (Kaposi's sarcoma-associated herpesvirus, formerly identified as Human Herpesvirus-8), is the prototypical member of the rhadinoviruses. EBV infects over 95% of adult humans, while KSHV infection varies by geographical location and HIV infection status. Each gammaherpesvirus establishes a life-long infection of the host. In immunocompetent hosts, long-term virus infection is maintained in a fraction of memory B cells as a latent viral episome.

In immunocompromised patients, infection with either gammaherpesvirus is associated with a greatly increased incidence of malignancies. EBV infection is associated with the development of Burkitt's lymphoma, Hodgkin's lymphoma, and nasopharyngeal carcinoma. KSHV infection is the etiological agent of Kaposi's sarcoma and is associated with primary effusion lymphoma and Multicentric Castleman's Disease.

EBV can lead to a condition known as post-transplant lymphoproliferative disorder (PTLD). The coadministration of immunosuppressive drugs to prevent organ or graft rejection impairs T cell control of the latent infection (1), leading to the emergence of EBV-infected cells with a less restricted latency program (2) that can drive proliferation of virally infected cells (3). Even in the immunocompetent host, the presence of latent gammaherpesvirus infection is

associated with higher incidence and increased severity of lymphomas, multiple sclerosis and systemic lupus erythematosus (2, 4-7). An improved understanding of the mechanisms by which the gammaherpesviruses establish life-long infection and evade immune clearance is critical for the development of therapies for gammaherpesvirus-associated diseases.

As a subfamily of the *herpesviridae,* the *gammaherpesvirinae* are characterized by an encapsidated double-stranded DNA genome that encodes 70-80 open reading frames (8-10). In addition to protein coding genes, the gammaherpesviruses encode functional miRNAs, long non-coding RNAs, and in the case of the murine gammaherpesviruses, tRNA-like molecules linked to miRNAs (11-13). Herpes virions are surrounded by a lipid envelope that contains numerous glycoproteins that mediate entry into the cell. Another characteristic of the herpesvirus virion is the tegument, a structured proteinaceous layer located between the capsid and the lipid envelope. Tegument proteins are delivered into the cytoplasm of the infected cell immediately upon infection and many play crucial roles in early infection (14-17).

A hallmark of herpesvirus infection, including that of the gammaherpesviruses, is the ability to switch between two distinct phases; lytic infection and latency. Lytic infection is characterized by expression of a majority of viral genes in a regulated cascade of gene expression, replication of viral DNA as linear concatemers, and production of infectious virions. Latency is defined by extremely restricted viral gene expression, the maintenance of the viral genome as a circular episome tethered to the cellular genome (18-20), and the ability to switch from latent infection to productive virus infection, a process known as reactivation. Gammaherpesviruses infect a wide range of cell types, including epithelial cells (21, 22), endothelial cells (23), monocytes (24), and lymphocytes (25, 26). The predominant cellular

reservoir of latency is lymphocytes; the human gammaherpesviruses target the mature memory B cell (26, 27).

Gammaherpesviruses employ an array of mechanisms to avoid immune clearance during both lytic and latent infection. Among the strategies the gammaherpesviruses employ are interference with the sensing of DNA and RNA components, TLR signaling, inflammasome signaling, and MHC class I presentation of viral epitopes. Gammaherpesviruses also secrete soluble cytokine receptors in addition to virally-encoded immunomodulatory cytokines. These subversion tactics contribute to evasion of the immune system that allows for persistent life-long infection.

A. Murine gammaherpesvirus 68 as a model gammaherpesvirus.

In vitro study of the human gammaherpesviruses in *de novo* infected peripheral blood and tumor cell lines have defined the role of many viral and host factors during infection. However, the strict host tropism of the human gammaherpesviruses restricts the *in vivo* study of their interactions with the host. To study host-pathogen interactions in the context of a whole animal, we use murine gammaherpesvirus 68 (MHV68, formerly identified as murid herpesvirus 4), a natural pathogen of murid rodents. MHV68 was originally isolated from bank voles in the former Soviet republic of Czechoslovakia (28), and has since been identified in yellow-necked mice in England (29), indicating that MHV68 may be endemic in European rodent populations. MHV68 productively infects and establishes latency in all tested strains of *mus musculus*. The genome of MHV68 is ~128kbp and encodes for an estimated 80 ORFs (8, 30).

1. **Genomic similarities.** MHV68 shares genomic, biologic, and pathologic properties with the human gammaherpesviruses. MHV68 is classified as a *Rhadinovirus* along with KSHV. MHV68 has homologs of multiple essential genes arrayed in similar genomic organization to KSHV (8). While many proteins involved in lytic replication are largely conserved amongst the gammaherpesviruses, each gammaherpesviruses encodes unique genes. Many have been implicated in the establishment and maintenance of latency. These disparate proteins often target common host pathways that enable the viruses to establish a lifelong infection in the face of robust immune surveillance (8). MHV68 contains both unique genes and miRNAs (31).

2. **Pathogenic similarities.** MHV68 also shares pathological similarities with the human gammaherpesviruses. Although the natural route of MHV68 transmission is not known, it establishes a productive lytic infection upon infection of the nasal mucosa (32) or epithelial and endothelial cells of the lung (26, 33). From the nasal or respiratory tract, MHV68 replicates in the epithelia and spreads to the draining lymph nodes. Initial infection causes localized tissue damage in the lung that undergoes normal repair (34), and the initial infection is cleared from the lungs by 12 days post infection in BALB/c and C57B/6 mice (35, 36). The virus is trafficked by B cells from the draining lymph nodes to the spleen (37) (**Fig. 1.1**).

Upon gaining access to the spleen, MHV68 establishes latency in germinal center B cells (38). MHV68 infection is associated with a non-specific expansion of both the B and T cell populations in the spleen that results in splenomegaly and a mononucleosis-like syndrome with parallels to EBV infection (39). Levels of latency in the spleen contract three to four weeks post infection, and the primary reservoir of viral latency shifts to the memory B cells. WT animals

typically control latent infection, however they develop malignancies between six months and three years post infection at a higher frequency than uninfected mice (40). Additional immunosuppression via cyclosporine A administration or impairment of CD8+ T cell responses increases the prevalence of malignancies, mirroring the development of malignancies in immunosuppressed humans (40-42).

MHV68 replicates to high titer upon infection of murine fibroblasts, dendritic cells, and macrophages. B cells can be *de novo* infected *in vitro,* albeit with low efficiency, and latent B cell lines have been established (43-45). Together, these systems allow for examination of multiple aspects of the viral life cycle *in vitro*.

The MHV68 system allows for the mutation of host or viral genes, allowing for *in vivo* analysis of determinants of viral pathogenesis. The MHV68 genome has been cloned into a bacterial artificial chromosome system (BAC), allowing for straightforward mutagenesis of the viral genome in *E. coli* (46, 47). Addition of a constitutively expressed YFP, lytic promotor-driven luciferase, or a beta-lactamase fusion to LANA into an MHV68-BAC vector has allowed for identification of infected cells by flow cytometry and tracking the development of infection *in vivo* (38, 48, 49). Likewise, the mouse system offers a wide array of germline and inducible knockouts of host genes to identify host determinants of infection and to facilitate a better understanding of the virus-host interactions that occur during multiple stages of infection.

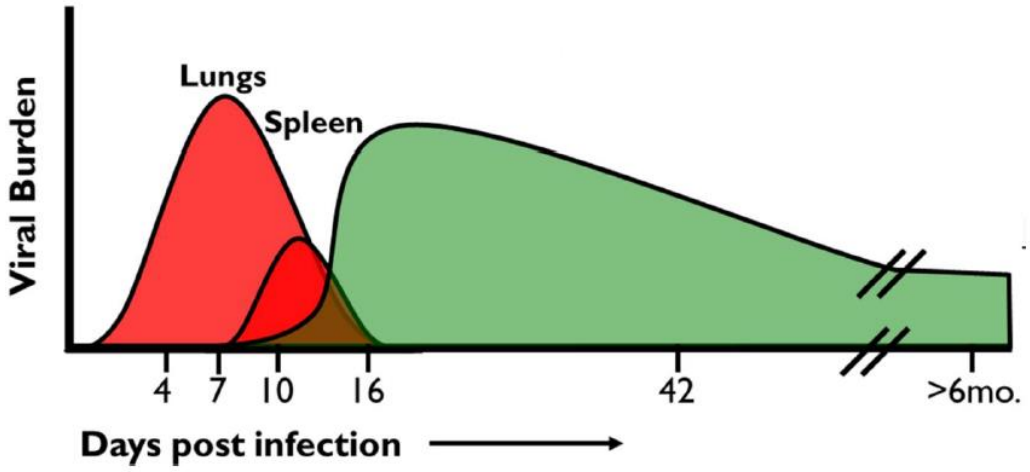

Figure 1.1 Viral pathogenesis in mice. MHV68 replicates in the lung after intranasal infection. Productive lytic infection (Red) peaks 7 days after infection and is cleared by 12 days after infection. MHV68 traffics to the spleen and undergoes a low level of lytic infection prior to infecting and establishing latency (Green) in the B cell lymphocyte reservoir. Peak latency occurs 16-18 dpi, and latency is maintained throughout the life of the animal.

3. Lytic infection in the mouse. EBV and KSHV are contracted through the mucosal surfaces of the body, particularly the oral mucosa. Initial infection by either gammaherpesvirus is often subclinical. EBV infection can lead to infectious mononucleosis, especially when acquired in adolescence or early adulthood (50), during which replication can be detected in the oropharyngeal epithelium (22). Although MHV68 has been used as a model system for over three decades, the route of transmission for MHV68 is not clearly defined. MHV68 can readily infect the nasal and respiratory mucosa upon intranasal infection (51), however it is unclear if this occurs in the wild. MHV68 and the closely related MHV72 may undergo vertical transfer from mother to pup, as viral DNA can be detected in the breast milk of infected dams and stomachs of the pups (52, 53). However, these studies did not demonstrate the establishment of latency in pups, and other studies observed no vertical transmission (54). Co-housing of latently infected animals with naïve animals is insufficient for horizontal transmission (55), although this may relate to a lack of normal murine behaviors such as scent-marking in laboratory housing.

Recent studies support a sexual mode of transmission for MHV68. Epidemiologic studies of wild mice found the highest prevalence of infection in the heaviest, most sexually active male mice (56). High titers of virus inoculum applied to the vagina of an infected mouse led to intranasal infection of naïve mice (32). Intermittent virus replication has been reported in the genitalia of intranasally infected male and female mice during latent infection (54). MHV68 was transmitted from a female mouse reactivating at the genitalia to a naïve male, and can occur from an infected male to a naïve female with lower efficiency (54).

4. **Upper respiratory tract infection.** In standard pathogenesis studies, mice are infected with MHV68 via the intranasal or intraperitoneal routes. Intranasal infection can deliver the viral inoculum into the upper airways without the use of anesthetic (51), or deep into the lungs with anesthetic (33). Upon inoculation of the upper respiratory tract infection, MHV68 first infects the neuroepithelium of the rostral nasal cavity (32). Infection of the neuroepithelium is dependent on viral heparin sulfate-binding glycoproteins (32, 57). From here, infection progresses to the draining superficial cervical lymph nodes (SCLNs) via dendritic cells, after which the virus transits to the spleen (48, 58). Upper respiratory tract infection leads to similar levels of latency in the spleen as that obtained from deeper lung infection; however, there is a kinetic delay in viral colonization of the spleen (48). It is important to note that there are multiple levels of immunologic barriers poised at this entry route that may contribute to the delay in infection. Interestingly, subcapsular sinus macrophages filter the afferent lymph nodes, and may become infected by MHV68 (59). This infection produces few infectious progeny virions, and may serve to impair herpesvirus infection.

5. **Lower respiratory tract infection.** Deep lung infection makes use of an autonomic deep inhalation occurring after light anesthesia. A large bolus of liquid can be inhaled deep into the lung, infecting both the upper respiratory tract and the lung epithelium, but not the trachea nor bronchi (48). Intranasal inoculation leads to viral attachment to type 1 alveolar epithelial cells and initial infection in alveolar macrophages (60), which collect particulate in the alveolar lumen (61). From these macrophages, infection spreads to type 1 alveolar epithelial cells (60). Viral titer increases in the lungs, peaking at 7 dpi, before being cleared by 12 dpi (34). There is significant infection of macrophages in the lungs by this method,

and the virus primarily drains to the mediastinal lymph node (MLN) via a dendritic cell-independent route (58). The virus then transits to the spleen in a B cell-dependent manner (37). Interestingly, despite the significant expansion of virus in the lungs and inflammatory infiltrate (35), there is evidence of significant tissue repair that largely restores lung morphology (34, 35, 62). The majority of MHV68 pathogenesis studies have used the lung infection route.

6. **Dissemination to secondary lymphoid tissues.** Little is known about the transit of the human gammaherpesviruses from their initial sites of acute infection to secondary lymphoid tissue. While it is accepted that infection occurs at mucosal barriers, infected individuals only exhibit symptoms many weeks after infection (50), complicating study of early infection. Infection of mice with murine gammaherpesvirus 68 has given us insight into how gammaherpesviruses gain access to the B cell memory reservoir. Transit of the gammaherpesvirus to the spleen differs depending on the route of infection. Infection of the upper respiratory tract results in infection of the neuroepithelium (32). From the site of initial infection, MHV68 infects dendritic cells and is trafficked to the draining lymph nodes to undergo additional rounds of replication and infect B cells (36, 58, 63).

Upon infection of the lower respiratory tract, MHV68 makes use of macrophages instead of dendritic cells for migration to the draining lymph nodes (58). In the absence of B cells, seeding to the spleen is significantly decreased, and mice do not develop an infectious mononucleosis-like syndrome (37, 63). Even in the absence of B cells, latency is detectable in the spleen at late times post infection, suggesting alternative routes of accessing distal reservoirs of latency (64). MHV68 latency in non-B cells such as macrophages requires the function of v-cyclin to drive reactivation and maintain the viral reservoir (65). It is not clear if

the B cells traffic to the spleen to infect splenic cells or if they merely release infectious virions into the blood.

Recently the Stevenson laboratory has undertaken a careful immunofluorescence analysis of infected spleen sections to reveal a complex series of cellular transitions that enable virus access to the germinal center B cell (66). Blood entering the spleen in a central arteriole first passes through the macrophage-rich marginal sinuses. These macrophages are the first splenocytes to become infected by MHV68, providing an entryway into the white pulp and expanding lymphocyte reservoir (66). Infection of these macrophages leads to productive lytic infection of virions that are better suited to infect B cells (57). These virions latently infect adjacent marginal zone B cells (66). These marginal zone B cells then carry the virus against the flow of blood to the emerging germinal centers (66). Marginal zone B cells appear to differentiate into plasma cells (66), which is a general trigger for gammaherpesvirus reactivation (67, 68). Here the marginal zone B cells associate with and deposit virions onto the follicular dendritic cells (66). Without becoming infected themselves, the dendritic cells seed the expanding population of germinal center B cells with MHV68, where the bulk of latent infection is established (26). Although the germinal center B cell response subsides after 14-16 dpi, latency is maintained in germinal center B cells as well as memory B cells (69) before finally contracting to the memory B cell reservoir for long-term persistence (70).

These trafficking studies have been carried out in mice, and the specific steps in human may differ. The human spleen has subtly different architecture than the murine spleen. In humans, the marginal sinuses and associated macrophages reside more proximal to the follicle in a region called the perifollicular zone, rather than in the more distal marginal zone where the

marginal zone B cells reside in the mouse (71). While the path to the germinal center may differ slightly between species, MHV68 studies suggest that access to the memory B cell involves transition through multiple cell types and may be driven in part by the developing adaptive immune response to infection.

7. Latency maintenance. Although MHV68 initially gains access to the germinal center B cell reservoir, it is predominantly found in memory B cells during long-term latency (27, 69, 72, 73). Access to and establishment of latency in the memory B cell reservoir provides the gammaherpesvirus with a long lived cellular reservoir that can reside in lymphoid tissue (74) or circulate through the body (74-76). However, memory B cells are not static; they undergo homeostatic proliferation and may terminally differentiate into plasma cells.

There are a number of events that trigger gammaherpesvirus reactivation. Although the *in vivo* stimuli for the human gammaherpesviruses are poorly understood in the immunocompetent host, *in vitro* EBV infected cells can be reactivated using chemical or biological agents (77). Studies of other herpesviruses reveal they reactivate in response to physical (78) or mental stresses (79), and there is indication that reactivation is linked to coinfection (80, 81). MHV68 has been used to further evaluate reactivation *in vivo.* A critical event in induction of reactivation of all the gammaherpesviruses is the maturation of the B cell into a plasma cell (67, 82-86). This phenomenon appears to be driven by the transcription factors IRF-4 and XBP-1, which are expressed upon B cell maturation into plasma cells (87). The human gammaherpesviruses are dependent on XBP-1 (67, 82-85), while MHV68 requires IRF-4 (68). Deletion of the plasma cell lineage causes a reduction in reactivation as well as a contraction of the latent reservoir during long-term infection (86). MHV68 encodes M2, a

latency associated gene (31), that promotes B cells differentiation into a plasma cell-like phenotype (68). *In vivo* injection of TLR agonists into a latently infected animal drives reactivation, and results in a higher latent viral load (88). Stimulation of either CD40 or TLR9 can also drive reactivation in cell culture (89). Co-infection of MHV68 infected mice with the pathogenic helminths *H. polygyrus* or *S. mansoni* induces reactivation, although this appears to be caused by IL-4 release induced by helminth infection that reduces IFNγ levels, which are necessary for control of viral latency through the repression of expression of the lytic transactivator RTA (81). Immune activation may starve infected memory B cells of survival signals, triggering their reactivation (90). The necessity of reactivation for maintenance of viral load and transmission to other hosts is well supported, but the mechanisms that govern reactivation, both constitutively and during co-infection, are not well understood.

II. Innate immune control of infection.

A. Intracellular immune control of infection.

1. Inflammasome. The failure of innate immune cells to clear an infectious gammaherpesvirus spurred additional investigation into the mechanisms by which the virus evades recognition. The inflammasome is an intracellular surveillance system that can respond to both damage-associated and pathogen-associated molecular patterns (DAMPs and PAMPs). Numerous cytoplasmic and nuclear sensors detect pathogen-associated molecules such as cytoplasmic dsDNA (91-94), flagellin (95), and toxins (96), as well as damage-associated molecules such as exogenous ATP, uric acid, and dysregulated GTPases (97). Upon sensor activation, some of these molecules complex with the scaffolding protein ASC and procaspase-1, while others can directly associate with procaspase-1. This association enables procaspase1 to undergo cleavage into activated caspase-1. Active caspase-1 in turn processes the potent pro-inflammatory molecules proIL-1β and proIL-18 into their mature forms, which are rapidly secreted (98-100) **(Fig. 1.2)**. Importantly, a second signal, provided by IL-1R, TNF-R, or TLRs, may be required to upregulate production of pro-IL-1β and pro-IL-18 (101). IL-1β is a central pro-inflammatory cytokine capable of being produced by virtually any cell in the body (102). IL-1β is a potent pyrogen (103), licensing the production of a variety of inflammatory mediators to control cell responses ranging from cell recruitment, division, and proliferation to systemic blood pressure (102). IL-18 shares some functions with IL-1β, such as inducing TNF-α production, but also plays a unique role in licensing the release of interferon-γ (IFNγ) from other cell types and aiding in the maturation of Th1 T cells (102). By initiating these critical

inflammatory responses, the inflammasome plays an important role in innate immune control of numerous pathogens, including double-stranded DNA viruses (104, 105).

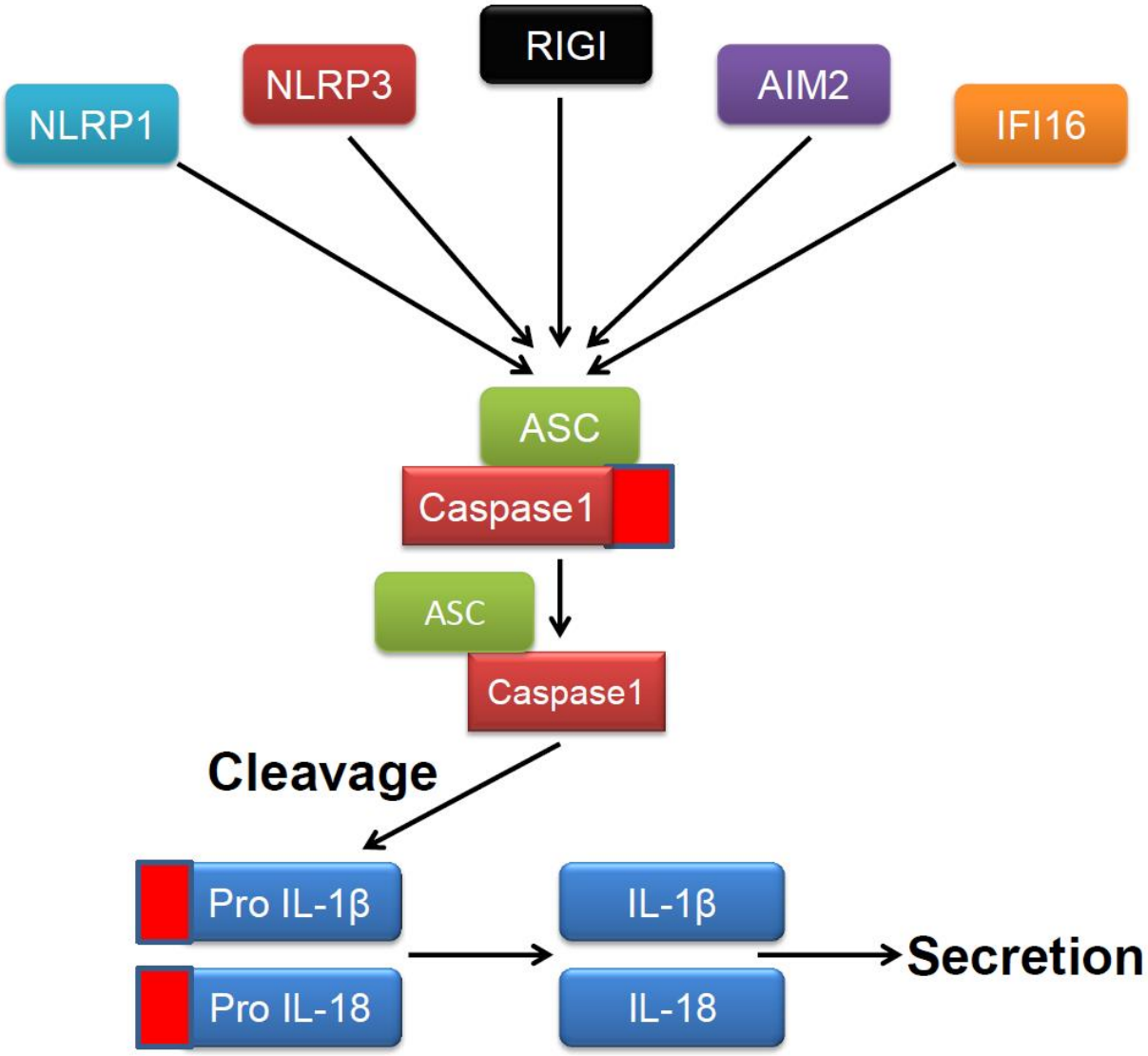

Figure 1.2 The inflammasome signaling pathway.

Inflammasome activation occurs upon activation of one of numerous intracellular sensors. Most activated sensors then associate with the scaffolding protein ASC, which mediates assembly with procaspase-1 and multimerization. Procaspase-1 undergoes cleavage into caspase-1, which activates the proteolytic function of caspase-1. Activated caspase-1 can cleave pro-IL-1β and pro-IL-18 into their active forms, which are subsequently secreted.

2. Gammaherpesvirus interactions with the inflammasome. The gammaherpesviruses can be detected by certain intracellular sensors upon viral infection. Infection of human monocytic or endothelial cell lines by KSHV induces caspase-1 cleavage and IL-1β secretion (106). This response is dependent on the cytoplasmic and nuclear dsDNA sensor IFI16. Inflammasome activation is also be detected in both cell lines and primary tissue latently infected with KSHV (107). Cell lines latently infected with EBV also have activated inflammasomes, as indicated by the presence of cleaved caspase-1, IL-1β, and IL-18 (108). The outcome of inflammasome activation by the host and subversive strategies of the gammaherpesviruses was investigated during our studies below.

The gammaherpesviruses employ various mechanisms to impair inflammasome activation. KSHV encodes the NLR homolog ORF63, which is capable of impairing inflammasome signaling through the NLRP3 and NALP1 sensors (17). ORF63 is packaged into the tegument of the virus (15), and may function immediately upon viral entry into a cell. EBV mediates the impairment of inflammasome signaling using a different strategy. The EBV-encoded miRNA BART15 targets the host miRNA binding site in the 3'-UTR of NLRP3 and represses its expression (109). MHV68 delivers the ORF64 deubiquitinase as a tegument protein that mediates inflammasome evasion upon infection (105), seemingly by efficiently shuttling the capsid to the nucleus for delivery of viral DNA. Mutation of ORF64 deubiquitinase function leads to mislocalization of viral DNA and inflammasome activation. This protein may be responsible for the ability of MHV68 infection to impair inflammasome-dependent signaling upon extrinsic stimulation as described in Chapter 3 below (110).

B. Cellular immune control of infection.

1. Natural killer (NK) cells. Studies of the early immune response to MHV68 infection have revealed a dependence on specific cells and cytokines of innate and adaptive immunity to control infection, while other factors are dispensable. Natural killer cells constitute an important antiviral defense mechanism that recognizes cells low levels of surface marker expression, such as MHC, and targets them for killing by degranulation or Fas-FasL interactions. This allows NK cells to restrict the pathogenesis of a large range of viral infections, including that of the human gammaherpesviruses (111). Despite the potent down-regulation of MHCI by both KSHV and MHV68 (112) and recruitment of NK cells to the site of infection (113), NK cells are dispensable for control of wild type viral replication in the lungs, clearance, and recruitment of CD8+ T cells (113, 114). Recent work indicates viral infection leads to upregulation of the inhibitory receptor CEACAM on infected alveolar epithelial cells (115), which can protect cells against NK-mediated killing (116).

2. Macrophages. Macrophages are another prominent innate immune cell dispensable for the control of WT MHV68 infection in the lung. Upon infection, macrophages are recruited to the lungs in large numbers (117). Deletion of CCR2, the receptor for the macrophage chemokine CCL2, impairs macrophage recruitment to the lung following multiple viral infections (118), including MHV68 (117). Despite the paucity of macrophages recruited to the lungs, replication kinetics were normal, with a peak around 6 dpi and clearance by 12 dpi. This contrasts with the observation that macrophage depletion after upper respiratory tract infection leads to increased viral titer in the draining lymph nodes (59), indicating that the two routes of infection are subject to different immune controls. Although macrophages did not

have a role in direct control of lytic replication and clearance, CCR2 deficient animals had increased neutrophil recruitment to the lungs and increased severity in lung pathology (117), suggesting that macrophages contribute to limiting damage to the host.

C. Cytokine-mediated control of infection.

Early immune responses play an important role in control and clearance of lytic infection (119). A number of cytokines and chemokines are produced in response to acute MHV68 infection. The pro-inflammatory cytokines IL-2, IL-6, and IFNγ are upregulated in the lungs, as is the anti-inflammatory cytokine IL-10 (120-122). Disruption of these cytokines has various effects on viral clearance. IL-6 is a pleiotropic cytokine responsible for functions ranging from B cell differentiation, T cell expansion and maturation, and acute phase responses (123, 124). Impairment of IL-6 commonly results in susceptibility to infection (123), however mice lacking IL-6 have normal progression of MHV68 lytic infection and equivalent latent viral loads as wild type mice (120, 125). IL-10 plays a central role in immune polarization towards a Th2 response, and can repress protective immune responses against viral pathogens (126). EBV encodes a homolog of IL-10 (127, 128), while the *rhadinoviruses* KSHV and MHV68 do not. MHV68 infection of mice lacking IL-10 results in increased expression of IL-12 and lower viral latency in the spleen (122). Although MHV68 does not encode its own vIL-10, the latency protein M2 can drive IL-10 production to promote proliferation *in vitro* and latency maintenance *in vivo* (129). IL-12 plays a role in Th1 T cell polarization and IFNγ secretion (130), and it has been observed that expression of IL-10 or the virally-encoded vIL-10 impairs killing of infected cells by NK cells, interferes with CD4+ T cell activity, and blocks CD8+ recruitment to impair clearance of infected cells (129, 131).

1. **Type 1 interferon signaling during MHV68 infection.** Interferons (IFNs) are critical for immune control of MHV68. Mice bearing deletions of the interferonα/β receptor suffer persistent lytic lung infection accompanied by hemorrhage and necrosis that causes

significant mortality after even a modest infectious dose (132). Low dose infection leads to an approximately 50% mortality rate following increased viral replication in the lungs and abnormal replication in the liver and spleen (133). Surprisingly, interferon α/β receptor knockout mice that survive the acute phase of infection establish normal levels of latency, but have increased levels of reactivation (133). These mice generate higher levels of viral-specific CD8+ T cells, but these cells are skewed towards a short lived effector phenotype, express higher levels of PD-1, and produce lower levels of TNFα and IFNγ upon stimulation with MHV68 peptide (134). KSHV-infected cells, including *de novo*-infected endothelial cell lines, tumor cell lines, and PBMCs from KSHV-infected individuals, have reduced reactivation in the presence of interferon-α (135, 136). The complexity of the interaction between gammaherpesvirus infection and type I interferon signaling highlights the efficiency with which the gammaherpesviruses subvert the immune system to successfully establish a latent infection.

Studies *in vitro* reveal that gammaherpesvirus infection is impaired by the expression of interferon-stimulated genes prior to infection (137). However, type I interferon (138) and the cell response to exogenous application of interferon is dampened during MHV68 infection, suggesting that infection modulates interferon signaling within a cell (139, 140). Indeed, a major outcome of the ORF37-mediated host cell shutoff phenomenon observed in gammaherpesvirus infection (141, 142) is the impairment of interferon signaling (142). MHV68 encodes additional proteins that specifically modulate downstream interferon signaling. The viral vUTPase (ORF54) induces the degradation of the interferon-α/β receptor, allowing for efficient establishment of latency (143). The viral ORF36 binds to interferon response factor-3 (IRF-3) in the nucleus of infected cells, suppressing interferon-mediated gene transcription

(144). MHV68 infection also integrates interferon signaling to limit lytic infection. Interferon stimulation of infected cells reduces the expression of the viral Replication and Transcriptional Activator (RTA) gene (140), which is responsible for inducing transcription of many lytic genes (145). Analysis of the viral genome revealed that the latency-associated gene M2 contains an interferon-stimulated response element in its promoter that serves to dampen expression of M2 in response to interferon signaling (146). Removal of this sequence leads to uncontrolled expression of M2, persistent acute replication, and increased mortality (146).

2. Role of Type II interferon in early gammaherpesvirus infection.

Gammaherpesvirus infection is also strongly influenced by signaling mediated by the type II interferon interferon-γ (IFNγ), although the loss of interferon-γ signaling is not as debilitating the loss of type I IFN. *In vitro,* bone marrow-derived macrophages newly infected with MHV68 and treated with IFNγ have striking defects in replication and early viral gene expression (147). Conversely, latently infected KSHV cell lines have increased replication in response to IFNγ treatment (148, 149). C57BL/6 mice lacking IFNγ clear lytic infection with slightly slower kinetics, and suffer no recrudescence during long-term infection (150), whereas interferon-deficient Balb/c mice have increased viral titers, delayed clearance, and appreciable mortality from pneumonia, especially after high dose infection (151) (152). Balb/c mice secrete higher levels of IFNγ and other chemokines during lung infection (153), suggesting it may play a more important role in viral clearance in some mouse strains. Further studies revealed that in B-cell deficient CD8+ T cell depleted mice, CD4+ T cells are sufficient for clearance of lytic replication. When these mice are also depleted of IFNγ, viral clearance is delayed (154). Likewise, restoration of IFNγ in IFNγ-knockout mice restores control of viral replication (155). IFNγ plays a

role in viral clearance; however its impact is dependent on the genetic background of the host. Further investigation of the mechanisms by which IFNγ affects the immune response in these different backgrounds would add to our understanding of immune clearance and control of gammaherpesvirus infection.

3. Loss of IFNγR signaling causes chronic pathology. While most studies of the gammaherpesviruses focus on their interactions with the immune system at the sites of latency maintenance, the IFNγR-KO mice exhibited interesting pathology in sites distal from the B cell reservoir. Examination outside the traditional reservoirs of latency reveals that IFNγR-deficient mice suffer from arterial vasculitis (156-159) and hepatic bile duct inflammation (160) during long-term MHV68 infection. These inflammatory pathologies stem from persistent MHV68 reactivation (161-163) in these locations, leading to the recruitment of IFNγ-secreting Vβ4+ CD8+ T cell (164) and polarization of macrophages into an M2 phenotype (165). These data suggest control of reactivation is similarly controlled by the immune system in the lymphoid tissue and at distal sites. Unlike IFNγ knockout mice, IFNγR-KO mice have reduced levels of splenic latency by 30 dpi (62). In addition, these mice suffer from spleen pathology that involves heightened granulocyte infiltration, fewer B and T cell, and fibrous depositions in the spleen, all of which are dependent on CD8+ T cell function (62). These mice also suffered from inflammatory lesions in the lungs that led to the development pulmonary lymphomas in over 80% of infected mice (159). It is not clear why this phenotype diverges from the acute pneumonia and death observed in IFNγ knockout mice on a Balb/c background.

III. Adaptive immune control of infection.

A. B cell-mediated control of viral infection.

In addition to being the primary reservoir of latency, B cells contribute to immune control of gammaherpesvirus infection. Studies in B cell-deficient mice reveal high levels of viral latency and reactivation in the peritoneum (166), but the lack of B cells also impairs the development of Vβ4 T cells, which secrete IFNγ and control viral reactivation (64, 167). Passive transfer of immune sera into B cell deficient μMT mice reduces the establishment of viral latency (168). Infection of mice lacking CD28, a costimulatory molecule necessary for T cell support of germinal center B cells, reveals only slightly reduced latency establishment and normal antibody response (169). However, this antibody response rapidly decreased over time and was poorly neutralizing. Depletion of T cells from CD28-/- mice led to recrudescence of the virus that could be inhibited by passive transfer of immune sera, indicating that humoral immunity contributes to the control of latent infection (169). In addition, immunization of mice with a reactivation-deficient virus generates immune sera that can prevent superinfection by a WT virus (170). Interestingly, neutralizing antibody can prevent infection of cell types that do no express Fc receptors, but increases infection of cell types expressing Fc receptors, suggesting MHV68 also hijacks humoral immunity to enable infection (171). To tease apart the contributions of B cell and T cell interactions from the generation of anti-viral antibody, McClellan et al. (172) infected mice with a germline rearrangement of antibody variable region causing all B cells to be specific to hen egg lysozyme. While virus-specific antibody was not necessary for control of latent infection, depletion of CD8+ T cells increased latency and reactivation in the peritoneum and depletion of either CD4+ or CD8+ caused increased latency

and reactivation in the spleen (172). The humoral immune response and T cell mediated responses have both unique and overlapping functions that together control MHV68 reactivation from multiple reservoirs.

B. T cell activation. T cells constitute one of the two arms of the adaptive immune response and are largely responsible for cytotoxic killing of cells and coordinating the immune response to infection. The three major populations of T cells; CD4+, CD8+, and γδ T cells, differ based on the composition of their T cell receptor and their costimulatory molecules. CD4+ T cells are restricted to recognition of antigens displayed within an MHC class II molecule. CD8+ T cells recognize antigen displayed within an MHC class I molecule on the target cell. γδ T cells express functional T cell receptors, but do not express the costimulatory receptors CD4 or CD8 and do not require MHC presentation of antigen for activation (173).

Activation of T cells can have far-reaching consequences for the target cell and the host immune system, not limited to lysing of the target cell, secretion of proinflammatory cytokines, and support of a continued immune response. T cell activation requires both strong binding to cognate antigen and costimulatory signals (174). Activation of the T cell receptor initiates with engagement of T cell receptor (TCR) by an MHC molecule on the target cell (175-177) **(Fig. 1.3)**. If the TCR has low affinity for the MHC-loaded antigen, the binding is weak and transient, transducing no signal. However, if the TCR has high affinity for the antigen, tight binding is established, satisfying the first requirement for T cell activation, and the T cell receptor remains engaged with the MHC for up to 24 h (175, 178). This interaction can occur with as few as 1-10 MHC/TCR complexes for CD8+ T cells, while CD4+ T cells require greater numbers (179). Binding allows for the assembly of the immunologic synapse around the TCR, which is a specialized lipid raft enriched in TCR coreceptors and costimulatory molecules (176). Many costimulatory receptors, such as CD27, CD28 and HVEM, are constitutively expressed on the naïve T cell (180), however the cognate ligands CD80, CD86, are typically expressed at low abundance on the

target cell and only upregulated during infection (181, 182). Successful ligation of these costimulatory molecules with their ligands provides the second requirement for TCR activation, driving the recruitment of pro-growth pro-survival signaling molecules to the immunologic synapse (177). The activated TCR transmits its signal via immunoreceptor tyrosine-based activation motifs (ITAMs) on the CD3ζ chain (183). CD3ζ is phosphorylated by the protein tyrosine kinases lck and fyn that associated with the TCR and CD4 or CD8 (184-186). The phosphorylated CD3 recruits ZAP-70 and syk, phosphoproteins that can bind to LAT and SLP-76, two large scaffolding proteins that mediate much of the downstream effects of TCR activation (187-190). LAT can bind PLCγ1, PI3K, and GRB2, whereas SLP-76 can bind vav1, nck, and itk (177). Successful assembly of this signaling complex licenses numerous changes in the T cell, including calcium efflux from the endoplasmic reticulum, cytoskeletal rearrangement, activation of integrins, and gene transcription (177). It is important to note that there are multiple levels of negative regulation of T cell signaling that serve to control T cell activation, preventing overactivation and disease pathology (177, 191) (Additionally discussed in Chapter 2).

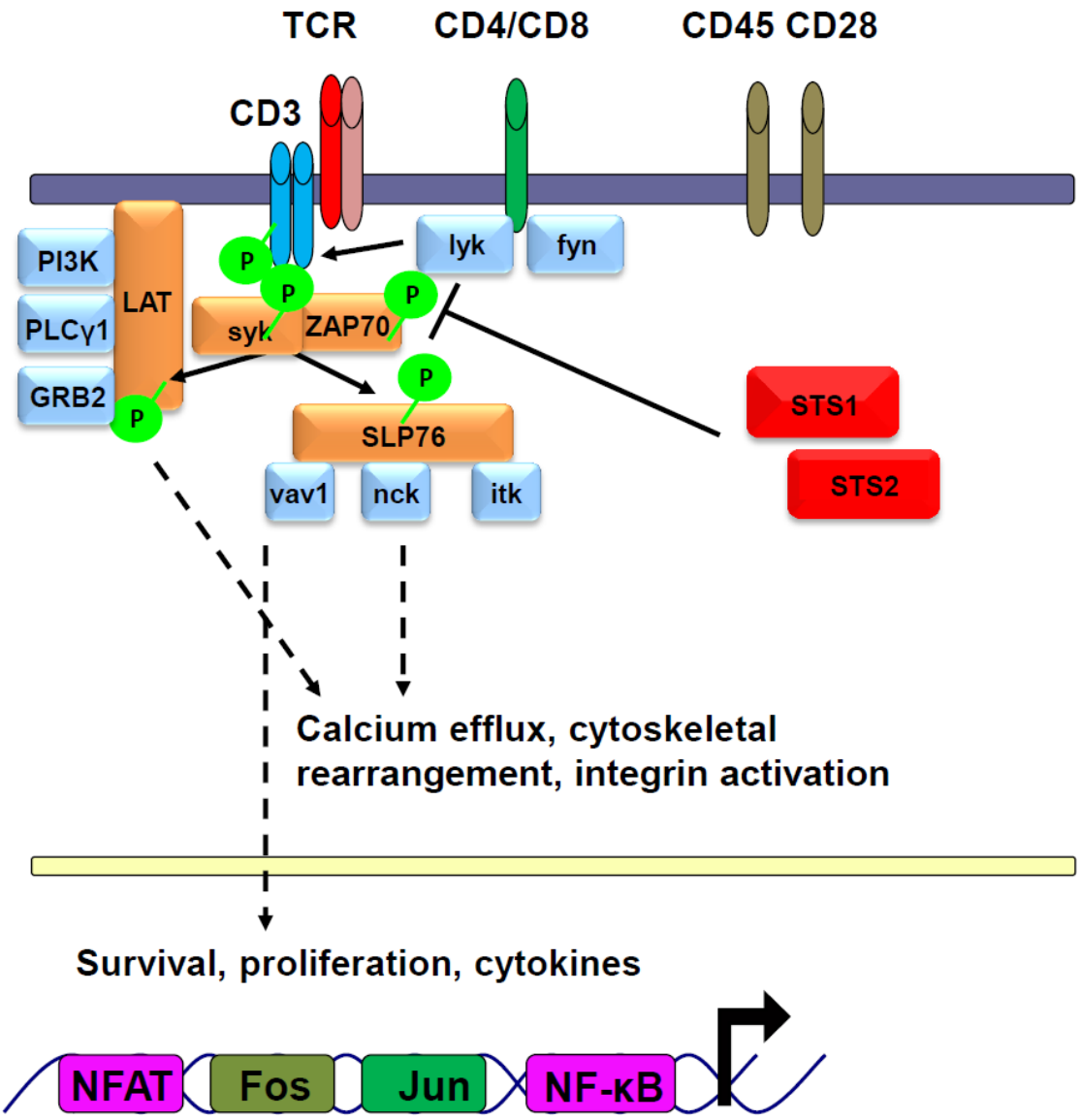

Figure 1.3 T cell receptor signaling. Activation of the T cell receptor results in the association of multiple cell surface receptors at the immunologic synapse. The kinases lyk and fyn associate with the intracellular tail of CD4 or CD8 and phosphorylate the ITAM motifs on CD3. Phosphorylated CD3 recruits the kinases syk and ZAP70, which phosphorylate the adaptor molecules SLP76 and LAT. These adaptors bind multiple mediators of downstream signaling, leading to cytoskeletal rearrangement, integrin activation, and gene transcription. Negative regulators, such as Sts-1 and Sts-2, dephosphorylate signaling proteins to limit TCR activation.

C. The T cell response to lytic infection. Numerous studies have identified roles for both CD8+ and CD4+ T cells in clearance of acute lytic infection in the lungs (34, 36, 192, 193). In both C57/B6 and Balb/c mice, depletion of CD4+ T cells delays viral clearance in the lungs and reduces systemic IFNγ, but early infection is otherwise normal (34, 36, 192). CD8+ T cells are recruited to the lung at equal frequencies and generate similar numbers of epitope-specific T cells in the lungs of CD4+ T cell deficient mice (194). In the absence of CD8+ T cells and B cells, CD4+ T cells clear lung infection in an IFNγ-dependent manner (154, 155). During long-term infection, loss of CD4+ T cells compromises immune control, leading to recrudescence in the lungs, progressive pathology, and death (36, 193).

Cytotoxic CD8+ T cells also play an important role in control of early gammaherpesvirus infection; however, their loss leads to a much different phenotype than that of CD4+ T cell-deficient animals. In Balb/c mice, depletion of CD8+ T cells after lung infection causes persistent lytic infection in the lung as well as the spleen and is accompanied by significant clinical pathology and increased necrosis of the lung (34). This phenomenon is strain-background dependent, as C57BL/6 mice depleted of CD8+ T cells suffer only delayed clearance from the lung accompanied by increased viral latency in the spleen (192). In both backgrounds simultaneous depletion of CD4+ and CD8+ T cells leads to persistent virus replication and death (34, 192). It is not known why CD4+ T cells of C57BL/6 animals, but not Balb/c animals, are able to control early infection in the absence of CD8+ T cells. Regardless, the different phenotypes observed in CD4+ or CD8+ T cell deficient animals indicate that each of these populations contribute to direct cytotoxic elimination of infected cells and cytokine signaling, and cooperate to control gammaherpesvirus lytic replication.

D. T cell control of latent gammaherpesviruses. While T cells play an important role in initial clearance of viral infection, they are also crucial for control of latent infection. It is telling that much of the burden of human disease caused by the gammaherpesviruses occurs during T cell immunosuppression (195-197). As such, there has been significant effort in uncovering the functions that T cells play in controlling gammaherpesvirus latency, and the mechanisms by which gammaherpesviruses evade them.

1. CD8+ T cells. CD8+ T cells play an important role in immune surveillance and control of gammaherpesvirus latency. CD8+ T cells generate cytotoxic responses against EBV or KSHV antigens (198-202). Humans generate T cells primarily against lytic antigen in EBV and KSHV infections, and this response persists for several months to several years (90, 200, 202-204). CD8+ T cells specific to MHV68 are observed in the infected mice as soon as one week after infection (205-207). This response consists of multiple immunodominant and subdominant T cells that shift over time (206). Mice first develop lytic epitope-specific T cells (208), and these subsets may be persistently stimulated by low level reactivation, delaying the development of a latent antigen-specific response (209). Nonetheless, subdominant T cells against latent antigen expand over time, displacing lytic epitope-specific T cells as the immunodominant population (206, 210-213). Latency establishment appears to be the primary inducer of latent antigen-specific T cell expansion, which helps to control infection (212). Hastening the development of a latent antigen-specific T cell population by adoptive transfer of M2-specific T cells has no effect on the levels of lytic replication, but reduces the initial establishment of latency (212). Interestingly, this did not affect levels of long-term latent virus, suggesting that the virus can evade clearance even with preexisting immunity (212).

During latency, CD8+ T cells serve to control viral reactivation in two important sites of latency: the spleen and the peritoneum (214). In the absence of CD8+ T cells, latency and reactivation occur at higher levels in both reservoirs. While the precise mechanism by which CD8+ T cells accomplish this control is no clear, there is evidence that perforin and IFNγ contribute to control of virus in these reservoirs (161, 162). Humans with an IFNγ polymorphism associated with low cytokine expression experience higher rates of PTLD (215). In mice, IFNγ secretion is linked to iNOS expression in target cells and a reduction of reactivation (216). Animals deficient or depleted of IFNγ have significantly increased reactivation from peritoneal exudate macrophages, even though they harbor only marginally higher levels of latently infected cells (161, 162, 214). Intriguingly, there are much higher levels of lytic viral mRNA in the PECs of IFNγ KO mice, which suggests IFNγ signaling contributes to the control of viral gene expression (161). Supplementation of IFNγ *in vitro* can reduce reactivation from PECs (161). There are minimal differences in latency and reactivation in the spleen of IFNγ-deficient animals (214). Conversely, animals lacking perforin, a cytotoxic molecule secreted from CD8+ T cells, fail to control viral reactivation in the spleen, leading to increased levels of latency and higher reactivation from latency (214). Perforin KO animals had slightly higher levels of reactivation, but no change in the levels of latency in the PEC compartment. While perforin and IFNγ are highly secreted from activated CD8+ T cells, they can also be secreted by CD4+ T cells and NK cells. NK cells are dispensable for control of MHV68 infection (114), but IFNγ is necessary for CD4+ T cell mediated control of lytic infection in B-cell deficient and WT mice (154, 155). Further studies are needed to dissect the contributions of each T cell subtype towards generating complete immune surveillance.

2. MHV68 infection induces the expansion of a non-antigen specific Vβ4+ CD8+ T cell population. An important aspect of CD8+ T cell control of MHV68 infection is the MHC-independent expansion of T cells bearing the Vβ4 T cell receptor subunit (39, 217). This expansion is delayed from initial infection, only occurring after 2 weeks post infection as latency is established in the spleen (217). This incubation period mimics the delayed onset of mononucleosis observed after EBV infection of humans (50). The expansion of these T cells is not dependent on recognition of specific viral antigens (206, 213), but rather stems from the superantigen-like function of the virally encoded M1 gene (218). Unlike most superantigens, this phenomenon does not target CD4+ T cells and occurs independently of MHC-peptide loading (218). Vβ4+ T cells secrete high levels of IFNγ (218), a cytokine that suppresses reactivation in the PEC reservoir (161, 219). Generation of the Vβ4 T cell subset requires the indirect action of CD4+ T cell support of B cell activation, as M1 is secreted from infected B cells (164, 167, 220). In the WT animal, M1 promotes expansion of the Vβ4+ subset and maintenance of latency, but in IFNγR$^{-/-}$ mice this expansion contributes to extensive tissue damage (218, 219). Overall, the CD8+ T cell subset plays important roles in the control of latent infection in multiple reservoirs.

3. CD4+ T cells. CD4+ T cells play a critical role in successful control of gammaherpesvirus infection. Activated, virus-specific CD4+ T cells can be detected early during latent infection and persist for the life of the host (205, 221-223). Initial latency establishment occurs normally in the absence of CD4+ T cells (192). Unlike CD8+ T cell KO animals, latency in the splenic reservoir fails to contract in CD4+ T cell KO mice; these mice suffer a progressive increase in reactivation in the lungs, eventually leading to death (36). This defect can be rescued with anti-CD40 antibody treatment in a CD8+ T cell dependent manner, indicating that CD4+ T cells provide important costimulatory signals to CD8+ T cells (224, 225). The CD8+ T cell population is maintained at WT levels in the absence of CD4+ T cells, and at late times post infection, CD8+ T cells respond normally to infected cells *in vitro,* as indicated by expression of activation markers, IFNγ expression, and *in vitro* cytotoxicity (193, 194, 226). However, the loss of CD4+ T cells also leads to higher levels of exhaustion markers in the CD8+ compartment (225), as well as the expansion of a subpopulation of FOXP3- CD122+ PD-1+ regulatory CD8+ T cells capable of secreting IL-10, an immunosuppressive cytokine that impairs control of viral latency in the lungs (193). Blocking PD-1 signaling in CD4+ T cell deficient animals rescues control of the virus (225). Virus-specific CD8+ T cells continue to expand in the spleen of CD4+ T cell-deficient mice, but are recruited to sites of recrudescence in very low numbers (194). Boosting an infected animal with a vaccinia virus expressing MHV68 lytic epitopes expands the virus-specific CD8+ T cell reservoir in the absence of CD4+ T cells, but even this expanded population does not protect the animals against recrudescence (227). The lack of CD4+ T cell help greatly impairs the maintenance of CD8+ T cells specific to the dominant viral epitope, while CD8+ T cells against subdominant epitopes are less impaired (228).

In the human host, it is well appreciated that loss of CD4+ T cell control in HIV+ patients leads to EBV and KSHV reactivation and the development of malignancies (198, 229). Upon explant from an infected patient, tonsillar B cells will reactivate KSHV, but only in the absence of CD4+ T cells (230). Curiously, CD4+ T cell-mediated repression of KSHV reactivation did not require virus-specific T cells and did not lead to target cell lysis in explant cultures (230). While the effect observed in this study was dependent on cell-cell contact, another study revealed that *in vitro* infection of peripheral B cells with EBV can be steered towards a more restricted latency type by IL-21 and soluble CD40L secreted by CD4+ T cells (231). Taken together, these data indicate that CD4+ T cells contribute to gammaherpesvirus latency through direct, non-cytotoxic interactions with infected cells and indirect cytokine-mediated interactions, in addition to providing helper functions to CD8+ T cells and B cells.

IV. Evasion of the immune defenses by gammaherpesviruses.

Despite the extensive immune activation in response to gammaherpesvirus infections, latency is inevitably established and maintained for the lifetime of the host. The gammaherpesviruses accomplish this by employing an extensive toolkit of immunomodulatory and immune evasion molecules, as well as interfering with cell signaling within the infected cell and modulating viral gene expression.

A. Control of viral gene expression.

Intracellular pathogens are largely protected from phagocytes, complement, and serum antibody. CD8+ T cells are primarily responsible for eliminating pathogens in this niche by recognizing pathogen-derived peptides displayed by host MHC class I molecules. One aspect of immune evasion by the gammaherpesviruses stems from the drastic differences in gene expression between lytic and latent infection. Gammaherpesviruses avoid epitope display by limiting viral gene expression during latency (31, 232-236).

EBV latency has 3 distinct gene transcription profiles (237). Type III latency is the most active form of latency, expressing Latent membrane protein 1 (LMP1), LMP2A, LMP2B, Epstein-Barr nuclear antigen leader protein (EBNA-LP), EBNA2, EBNA3A, B, and C, and EBNA1. EBNA-LP, EBNA2, EBNA3A, and EBNA3C play critical roles in establishment of latency in primary B cells (237). This profile appears to best mimic the expression profile of B cells upon initial infection (238), and is observed in lymphoblastoid cell lines derived from *de novo* infected PBMCs (232). Type II latency involves the transcription of the three transmembrane proteins LMP1, LMP2A, and LMP2B, that drive host cell survival in addition to EBNA1. This type of latency is common in Hodgkins lymphoma and nasopharyngeal carcinoma (237). In type I latency, gene expression is

restricted to non-coding RNAs and the EBNA1 protein that tethers the viral genome to the host genome (232). EBNA1 also manipulates cellular miRNAs to promote repression of reactivation (239). This profile is most commonly associated with long-term latency in infected memory B cells and some Burkitt's lymphomas (240, 241). KSHV and MHV68 appear to have a more 'active' program during latency. Gene expression profiles of latently infected B cells reveal that KSHV expresses six proteins and multiple non-coding mRNAs during latency (236). MHV68 appears to have an intermediate level of gene expression, expressing three to four genes and multiple non-coding mRNAs during latency (11, 31, 242, 243). By restricting gene expression, the gammaherpesviruses minimize detection by the adaptive immune system.

B. Manipulating host gene expression.

Despite restricted protein expression during latency, the gammaherpesviruses make use of multifunctional proteins, non-coding mRNA, and miRNAs to affect the host. EBV drives a number of signaling changes by associating with cellular transcription factors. Upon *de novo* infection of PBMCs *in vitro,* EBNA2 induces rapid upregulation of the cellular genes c-myc, CD21, and CD23 (25, 244) by binding with the host transcription factor RBPJκ (245, 246). This binding also induces the expression of the bcl-2 family member bfl-1, which can promote survival in the otherwise bcl-2-deficient germinal center B cell (247). EBV targets cell survival pathways with numerous effectors. Bfl-1, as well as the bcl-2 family member mcl-1, are induced by LMP1-induced NF-κB signaling (248, 249). LMP2a drives expression of bcl-2 and bcl-X_L to promote survival (250, 251). EBV downregulates signaling molecules downstream of the TGF-β receptor, impairing this pro-apoptotic signal (252, 253). To facilitate cell growth, EBV shuts off p53 surveillance of genome integrity and cell cycle through binding the p53 negative regulator MDM3 with the viral protein EBNA-LP, stabilizing the MDM3-p53 complex (254).

To better understand the extent of cellular transcriptional changes, Marquitz et al. (255) have performed RNA-seq analysis of *in vitro* infected cell lines and determined that EBV infection induces numerous proliferation genes, pro-survival genes, and transcription factors. EBV also alters the expression of the DNMT epigenetic maintenance proteins, causing broad changes in both viral and cellular gene expression (256). Even during type I latency, with gene expression limited to EBNA1 and non-coding mRNAs, EBV can promote survival. EBNA1, in addition to tethering the viral genome to the host's (257), induces transcription of survivin, a pro-survival protein (258). EBNA1 promotes cellular gene transcription through binding the

promotors of pro-survival genes (259). EBV can also modulate the expression of specific genes or pathways via the expression of 40 mature miRNAs (13, 255, 260). The viral BART long non-coding RNA (lncRNA) is highly expressed during latency (261), and can also mediate the inhibition of multiple cellular genes (255).

KSHV employs a number of similar strategies to facilitate maintenance in the host cell. Latent infection leads to broad changes in gene expression mediated by viral proteins. Expressing LANA alone in EBV-negative Burkitt's lymphoma cell lines induces upregulation of a number of interferon-stimulated genes with potential transcriptional activity (262), although the outcome of this effect is not yet known. LANA also interacts with p53 and suppresses its transcriptional activity, inhibiting cell death pathways (263-265). KSHV also encodes non-protein modulators of transcription, including 12 miRNAs (266). Hundreds of cellular and viral targets are predicted for KSHV and EBV miRNAs (267, 268). One KSHV-encoded miRNA, miR-K12-11, mimics the cellular miRNA miR-155, which promotes a program of cell proliferation by downregulating genes involved in cell growth restriction (269). This miRNA also inhibits downstream mediators of TGF-β signaling, preventing cytostasis (270). KSHV miRNAs also downreguate TWEAKR to prevent TWEAK-mediated apoptosis (271), downregulate pro-apoptotic caspases (272), and effect broader changes in gene expression through modulation of DNMT (273). KSHV encodes the lncRNA PAN RNA, however unlike EBV, the KSHV lncRNA function seems to be mainly in lytic infection where it is expressed at high levels and prevents LANA-mediated shutoff of viral genes (274).

The effects of MHV68 infection on latently infected cells have not been as extensively studied as those of the human gammaherpesviruses, but it is clear that viral infection leads to a

number of changes in host cell signaling. MHV68, like the other gammaherpesviruses, encodes a viral bcl-2 functional homolog during latency (31). The v-bcl-2 can bind the pro-apoptotic molecules Bak and Bax, blocking apoptosis in the infected cell (275, 276). V-bcl-2 promotes infection of immature and transitional B cells (277), which compete for limited amounts of BAFF to survive early maturation in the spleen (278, 279). The v-bcl-2 also supports latency maintenance through the inhibition of beclin-1, which inhibits autophagy (276, 280, 281). This mechanism is more potent than the autophagy inhibiting effect of endogenous bcl-2 (280, 281), revealing not only molecular mimicry by the virus but also an adaptation in function. MHV68 LANA can activate p53 during lytic infection to promote cell survival and support viral infection (282).

C. Hijacking host cell signaling.

The gammaherpesviruses alter signaling through multiple host pathways to promote long-term latency. The germinal center reaction serves as a selection process whereby B cells sample epitopes presented to them derived from infectious epitopes. B cells with high affinity to the displayed epitopes receive strong pro-survival signals and proliferate, while B cells that do not bind or bind weakly undergo apoptosis (283, 284). Gammaherpesviruses establish latency in the rapidly dividing germinal center B cell population. Infection may skew the fate of the target cell by the action of virus-encoded pro-survival genes and repressing pro-apoptotic host genes. For example, EBV expresses the potent pro-survival molecules LMP1 and LMP2a, which mimic cellular CD40 (285-287) and BCR (288), respectively. When expressed together in transgenic mice, these molecules can drive the infected B cell through the germinal center reaction regardless of its avidity to any epitope, preventing elimination of the infected cell by apoptosis (289). However, it is not clear if LMP1 and LMP2A activity is as potent during EBV infection (90, 290). Infected memory B cells from EBV-infected people appear to have undergone germinal center maturation, including somatic hypermutation, class switching, and antigen selection (291).

KHSV encodes K1 and K15 that mimic the function of the BCR (292). Replacement of EBV LMP2A, which also acts as a constitutively active BCR, with K1 and K15 in a recombinant EBV rescues the ability of LMP2A-KO EBV to establish immortalized LCL lines (292). KSHV expresses v-FLIP during latency (234), which activates the canonical and non-canonical NF-κB signaling pathways, providing additional pro-survival signals (293-296). KSHV also activates proliferation programs through the expression of a miR-K12-11, a cellular miRNA mimic (297).

MHV68 supports the survival of the infected B cell by mimicking cell signaling via the latency-associated M2 protein. Expression of M2 activates the vav1 and vav2 kinases, which participate in the transduction of the B cell receptor activation signal (298). This signaling is pro-survival (299) and supports B cell proliferation and differentiation (129, 298, 300), which may facilitate MHV68-infected B cell transit of the germinal center. To further promote cell proliferation, MHV68 LANA can associate with the transcriptional regulators brd2 and brd4 to promote the transcription of the G1 and S phase cyclins (301). LANA also impairs canonical NF-κB signaling through degradation of RelA (302), which has been proposed to enable passage of the infected cell through the dark zone of the germinal center (303). Establishment of MHV68 latency in B cells with a memory phenotype has been observed in CD40-deficient animals, suggesting that this critical pro-survival signal is not essential to ultimately gain access into the memory B cell reservoir (70).

D. Gammaherpesviruses evade immune clearance.

While gammaherpesviruses employ multiple strategies to manipulate their host cell, they also have mechanisms to evade host recognition and clearance. Class I MHC presents epitopes on the cell surface derived from proteosomal breakdown products of cytoplasmic proteins (304). One shared strategy of the gammaherpesviruses is the downregulation of MHC receptors. KSHV represses MHC class I transcription through the action of vIRF1 during latency (305) and can target class I MHC molecules for proteasomal degradation during lytic infection through K3 and K5 (306). MHV68 mK3 degrades the nascent MHC class I molecule and the TAP chaperone, preventing antigen presentation (307, 308). All the gammaherpesvirus LANA/EBNA1 proteins studied to date share the ability to impair their own processing in the infected cell, preventing recognition of the latent cell (309-311). EBV EBNA1 limits its translation through secondary structure in its mRNA, minimizing protein display in MHC class I (312, 313).

MHC class II molecules serve a similar function as MHC class I, but derive their peptides from phagolysosomes for presentation to CD4+ T cells (314). As such, their expression is restricted to antigen presenting cells. Latent infection with a gammaherpesvirus can impair MHC class II expression and function, but not to the same extent as class I. vIRF3 and LANA of KSHV and LMP2A of EBV inhibit MHC class II expression at the transcriptional level during latency (315-317).

Latently infected cells also modulate their immune environment through the secretion of IL-10. IL-10 is an immunomodulatory cytokine that suppresses APC and T cell functions (318), and it promotes the proliferation of activated B cells (319). During EBV latency, expression of LMP1 on the infected cell drives the production of regulatory T cells that produce IL-10 (320).

EBV+ Burkitt's lymphoma cells produce higher amounts of both the human and the virally-encoded IL-10, preferentially recruiting CD4+ T cells instead of CD8+ T cells (321, 322). Although MHV68 does not encode a viral IL-10, the secreted latency protein M2 induces IL-10 production from latently infected B cells (129, 300), and is important for the differentiation of infected B cells to plasma cells (300).

A unique immune evasion strategy employed by MHV68 but not the human gammaherpesvirus is the secretion of the chemokine binding protein M3. M3 is a latency-associated gene product that interacts with numerous murine and human chemokines to prevent binding to their cognate receptors (323-326). While M3 can prevent lymphocyte chemotaxis (326), deletion of M3 did not affect lytic replication in the lungs after infection (327). Infection of *Mus musculus* differs from infection of wild wood mice (*Apodemus sylvaticus)*, where M3 serves to significantly reduce levels of multiple cytokines in the lung to support lytic replication, and plays a role in efficient maintenance of long term latency (328). The myriad of effects that latent gammaherpesviruses have not only on their host cell, but also on the immune response to infection culminate in life-long maintenance and immune evasion. The methods by which each gammaherpesvirus accomplish these changes are subtly different, but well-tailored to their niche in the host.

V. NF-κB signaling

NF-κB signaling is a potent pro-survival pro-inflammatory signaling pathway that plays a critical role in B cell activation and survival. NF-κB signaling consists of two distinct pathways that are largely activated by separate sets cell surface receptors; the IKKβ-dependent canonical pathway, and the IKKα-dependent non-canonical pathway. The gammaherpesviruses encode numerous proteins that modulate or activate NF-κB signaling during both lytic and latent infection (296, 329-332). The canonical signaling pathway is important for the establishment of latency by MHV68 (333, 334). A complete understanding of NF-κB signaling in gammaherpesvirus infection is not yet known and requires further study. Here I describe the two arms of NF-κB signaling and their downstream effects in depth.

A. Canonical signaling pathway.

The canonical NF-κB signaling pathway serves a broad range of functions within the body, contributing to immune activation in response to cytokines and pro-inflammatory signaling in response to pathogen detection. These cell surface receptors include the IL-1R and the TNFR family of receptors which bind cytokines, TLRs that recognize pathogen-associated molecular patterns, and the B and T cell receptors, which provide pro-survival signals (Fig. 1.4). Each receptor transduces its signal with an overlapping set of effector molecules.

1. TLR signaling. Upon binding to their target ligands, the TLRs homo- or hetero-dimerize, bringing their intracellular TIR domains into close proximity (335). Myd88 is first recruited to the TLR through the action of the TIR domain-containing adaptor proteins TIRAP and MAL (336). Activated Myd88 then associates with IRAK4 (337, 338), which in turn recruits IRAK1, forming a complex known as the Myddosome (339). The association of the IRAK proteins

leads to the phosphorylation of IRAK1 and its dissociation from the Myddosome (340). IRAK1 binds and activates TRAF6, inducing the TRAF6-mediated ubiquitination of the TAK1 protein complex (337, 341). Activated TAK1 phosphorylates IKKβ (342), activating the IKK complex and the canonical NF-κB transcription factors.

2. IL-1R signaling. The IL-1 receptor family senses IL-1α and IL-1β, which are pro-inflammatory molecules that are released upon detection of cellular damage or pathogens (98). The IL-1 receptors homodimerize upon binding IL-1. The intracellular tail of the IL-1R shares significant homology to that of the TLRs, including the presence of a TIR domain, which enables them to engage common downstream signaling molecules. After ligand binding, the IL-1Rs recruit Myd88 via the adaptor TIRAP, allowing the Myddosome to form and activate IRAK1 (335). This activates TRAF6, which then ubiquitinates and activates TAK1 (341), and culminates in the phosphorylation of IKKβ.

3. B cell receptor and T cell receptor signaling. The B cell and T cell receptor signaling pathways share common factors that induce NF-κB activation. The SRC family kinases (SFKs) are the first mediators of signaling within the lymphocyte. The SFKs syc and lck are recruited to the TCR upon engagement, while the BCR recruits blk, fyn, and lyn (343). These kinases phosphorylate ITAM motifs on the intracellular tails of the BCR or TCR (343), leading to recruitment and phosphorylation of ZAP70 and syk in T cell and B cells, respectively. ZAP70 and syk then phosphorylate large adaptor proteins that nucleate numerous downstream signaling pathways including NF-κB. The BCL10/MALT1/CARMA signaling complex is first activated by these adaptors (343-345). Nucleation of this complex begins with the phosphorylation of CARMA by BCR or TCR-activated PKCs (346, 347). This causes the recruitment of BCL10, TAK1,

and IKKγ/NEMO (348). BCL10 is a promiscuous mediator of signaling, and can associate with multiple signaling molecules including MALT1 (349), CARMA proteins (350-352), and TRAF6 (353). CARMA phosphorylates BCL10 (350), which then associates with MALT1 (349). Together, BCL10 and MALT1 activate TRAF6 and TRAF2, causing ubiquitination and activation of TAK1 (341), ubiquitination of IKKγ, and phosphorylation of IKKβ (342, 349, 353).

4. TNFR signaling. The TNF family of signaling molecules includes multiple trimeric signaling peptides and their receptors. Binding of TNFα, the canonical signaling molecule in this family, to the TNFR1 leads to the recruitment of TRADD to the Death domain (DD) on the cytoplasmic tail of TNFR(354). TRADD serves as an adaptor protein for RIP1 and TRAF2 (355-357). Although RIP1 can directly bind TNFR and TRAF2, these interactions are weak in the absence of TRADD (358). RIP1 and TRAF2 cooperate to recruit and activate the IKK complex. RIP1 plays a critical role in recruitment of the IKK complex through binding to IKKγ/NEMO (359), but does not mediate activation (360). TRAF2 cannot recruit the IKK complex, but is responsible for ubiquitinating TAK1 (341, 361, 362). The proteins cIAP1 and cIAP2 are necessary for ubiquitination and activation of TRAF2 and RIP1 (363). TRAF2 and RIP1 mediate the ubiquitination of TAK1, leading to activation of the IKK complex after TNFR signaling (364).

5. IKK complex and NF-κB transcription factors. The IKK complex consists of the two closely related kinases, IKKα and IKKβ, and the scaffolding protein IKKγ/NEMO (365-367). TAK1 binds to the IKK complex and activates IKKβ via two phosphorylation events on its activation loop (337, 368). This activation is dependent on IKKγ/NEMO, which is necessary for the association of the IKK complex with upstream signaling components (369). Phosphorylation of IKKβ is critical for activation of the canonical NF-κB subunits (365, 368), while IKKα is

dispensable (368). The IκB family of proteins are inhibitors of NF-κB signaling. IκB proteins serve to sequester NF-κB transcription factors in the cytoplasm by binding to their Ankyrin-repeat domains and masking their nuclear localization signals (NLSs) (370). These inhibitory proteins are phosphorylated by the IKKs (371, 372) and induced to undergo rapid ubiquitination and degradation (373). This releases the canonical NF-κB subunits p65/RelA, p50/NFκB1, and c-Rel, allowing for homo or heterodimerization and translocation to the nucleus. These subunits have preferential binding partners, with p65 and c-Rel heterodimerizing with p50, or p50 homodimerizing with itself (374). These dimers have subtly different target sequences, and can associate with other transcription factors to control their target genes (375-378). NF-κB signaling activates multiple target genes related to cell survival and inflammation (379).

Uncontrolled inflammation can cause deleterious effects to the host, and as such NF-κB signaling is subject to multiple modes of negative regulation. Canonical NF-κB activation initiates many levels of negative regulation of NF-κB signaling. NF-κB-driven transcription of IκBα turns off NF-κB signaling by binding to the transcription factors and removing them from the nucleus (380). Transcription of the deubiquitinase A20 by NF-κB leads to the deubiquitination of the upstream signaling molecules TRAF6 and RIP1, terminating signaling (381). The IKKs can also activate the CYLD deubiquitinase, which suppresses TRAF2 ubiquitination to turn off signaling (382). Additionally, activated NF-κB subunits can be the target of ubiquitin ligases that target them for proteasomal degradation (383). The IKKα kinase can also target p65 for phosphorylation, which aids in ubiquitin-mediated proteasomal degradation (384, 385).

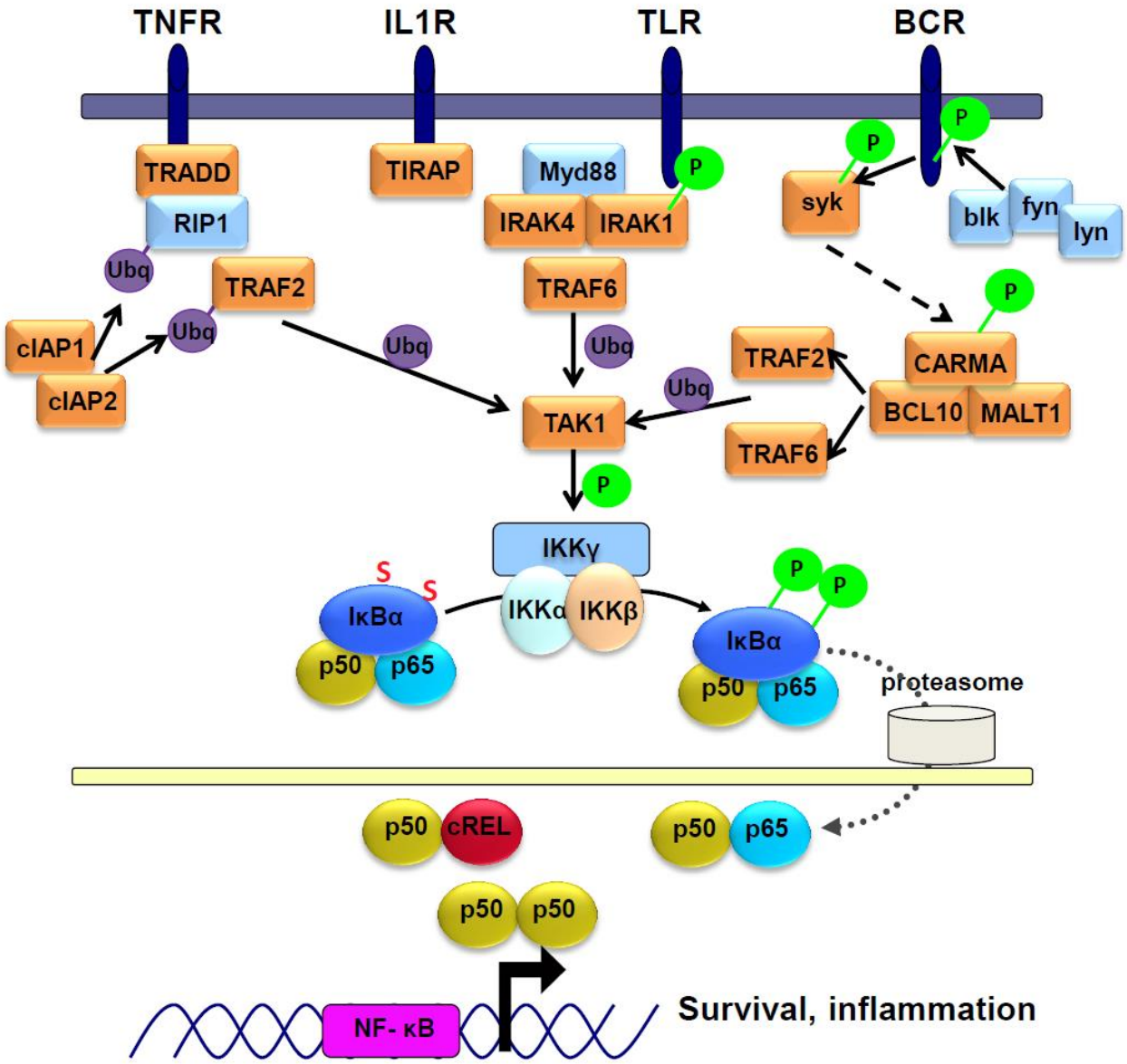

Figure 1.4 Canonical NF-κB signaling. Canonical NF-κB signaling is activated by the binding of extracellular ligands. Each receptor family differs in the upstream signaling molecules used to mediate signaling, but they share activation of TRAF2 and TRAF6. The TRAFs ubiquitinate TAK1, causing TAK1 to be activated and associate with IKKγ. TAK1 can then phosphorylate IKKβ, which in turn phosphorylates IκBα. Phosphorylated IκBα is degraded by the proteasome, releasing the canonical transcription factors p65, p50, and cREL. These translocate into the nucleus and activate pro-survival and pro-inflammatory genes.

B. Non-canonical/Alternative signaling pathway

The non-canonical/alternative NF-κB signaling makes up a related signaling pathway that mediates organogenesis, cell maturation, and pro-survival signaling. Alternative pathway signaling is activated by specific members of the TNFR family, such as CD40, BAFFR, and TNFRSF3 (LTβR) (386, 387) **(Fig. 1.5)**. Like the receptors of the canonical pathway, these alternative pathway receptors do not have enzymatic function. In contrast to the canonical pathway receptors, activated receptors of the non-canonical NF-κB pathway directly associate with TRAF proteins (388, 389) through multiple TRAF binding sites on their intracellular tails (390). Assembly of TRAF2, TRAF3, and cIAP1/2 onto the cytoplasmic tail leads to the ubiquitination of cIAP1/2 by TRAF2 (391), followed by ubiquitination of TRAF3 and its subsequent degradation (391-393). In resting cells, NF-κB-inducing kinase (NIK), which is the major IKKα-activating kinase in cells, is constitutively active, but is present in very low levels due to its constitutive degradation by cIAP1/2 (394), dependent on TRAF3 and TRAF2 (392, 393). Relief from constitutive degradation by receptor engagement and TRAF3 degradation allows for activated NIK accumulation over the course of three to five hours, leading to signal propagation (392, 395). TRAF3 binds to both NIK and CD40 via the same protein surface, so activation may recruit TRAF3 away from NIK, forcing cIAP1/2 to use TRAF3 as a substrate for ubiquitin (396). Accumulation of NIK allows for the phosphorylation of IKKα on two serines in its activation loop (397-399). This licenses IKKα to phosphorylate p100 (400). Full length p100 can act similarly to IκBα, sequestering NF-κB subunits in the cytoplasm. Upon phosphorylation, p100 undergoes partial proteolysis, and the remaining p52 domain can associate with the NF-κB transcription factor RelB and translocate to the nucleus to initiate gene transcription (378, 401, 402).

Activated IKKα also serves to negatively regulate the non-canonical pathway signaling through phosphorylation and destabilization of NIK (403).

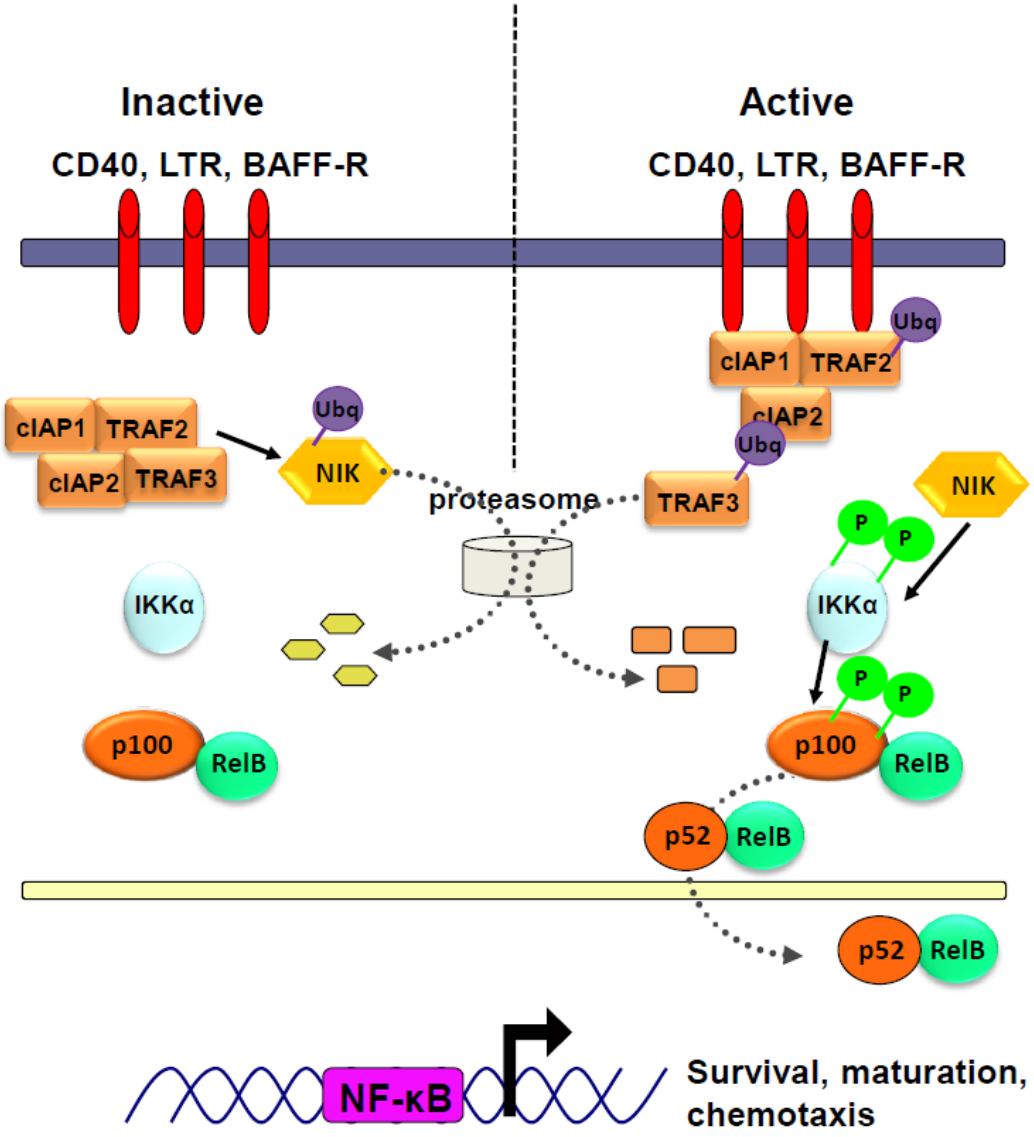

Figure 1.5 Non-canonical NF-κB signaling. Non-canonical NF-κB signaling is initiated by the binding of cell surface receptors to their ligands. Trimerization of the receptors recruits TRAF2, cIAP1, and cIAP2 to the receptor. This changes the specificity of cIAP1/2 ubiquitin ligation from NIK to TRAF3. TRAF3 is ubiquitinated by this complex and degraded by the proteasome. The absence of TRAF3 prevents degradation of NIK, leading to its accumulation. NIK can phosphorylate IKKα. Activated IKKα phosphorylates p100, inducing its partial degradation by the proteasome. The remaining subunit, p52, associates with RelB and translocates into the nucleus where it mediates transcription of pro-survival genes.

C. Crosstalk between the canonical and non-canonical pathways.

While extensive research has defined the signaling pathways for each arm of NF-κB signaling, there are numerous levels of crosstalk between these two pathways. The IκBδ domain and Rel-homology domain of p100 bind to various canonical subunit dimers in addition to RelB, sequestering them in the cytoplasm (404). This provides an avenue for alternative pathway signaling to activate canonical subunits, as these factors are released upon non-canonical induced p100 degradation to synergize with canonical signaling to increase transcriptional activity (404). Canonical NF-κB signaling can influence alternative pathway signaling through the induction of RelB and p100 transcription (395, 405). Additionally, p50 can compete with p52 for binding to RelB, impairing p52/RelB signaling (405). Similarly, RelA can bind p100 and induce its degradation, leading to the formation of p52/RelA dimers (406). The use of common upstream signaling molecules and the crossover regulation of the transcription factor dimers likely contribute to the shared pool of target genes for both arms of the NF-κB pathway (379).

D. Gammherpesvirus manipulation of NF-κB signaling.

NF-κB signaling plays a central role in many critical cell pathways, including apoptosis, cell survival, and inflammatory signaling. It also is highly activated in B cells, which serve as the primary reservoir of gammaherpesvirus latency. Both the human and murine gammaherpesviruses integrate NF-κB signals and interfere with NF-κB signals at different points during latency. During lytic infection or reactivation, the gammaherpesviruses manipulate NF-κB signaling. KSHV expresses vFLIP upon *de novo* infection. vFLIP can bind to IKKγ/NEMO, inducing a conformational change that activates IKKβ (294). IKKβ activation causes IκBα degradation and activates p65 via phosphorylation on two serine residues, both of which serve to promote latent infection of KSHV (407). MHV68 also hijacks IKKβ activation to phosphorylate p65 in coordination with RTA (408).The rapid phosphorylation of p65 induces its degradation, depleting p65 from the cell between two and eight hpi (409). Activation of IKKβ during lytic infection can target the viral RTA protein for phosphorylation, which promotes lytic gene transcription (410). MHV68 can also degrade RelA by LANA-induced ubiquitination and degradation of p65, preventing NF-κB-dependent gene expression (302). NF-κB activation of p65 can have a deleterious effect on gammaherpesvirus lytic replication, blocking lytic gene transcription and viral replication (411).

EBV, KSHV, and MHV68 each encode cell surface proteins capable of activating NF-κB signaling. EBV LMP1 is a potent mimic of CD40 that activates both IKKα and IKKβ (329, 412, 413). LMP1 constitutively associates with the TRAF signaling proteins, driving downstream activation of NF-κB to transcribe pro-survival molecules in latently infected cells (414-417). EBV LMP2A also constitutively activates canonical NF-κB signaling (330), providing pro-survival

signals that can substitute for BCR activation in B cell survival (288). Survival of cells latently infected with KSHV requires activation of NF-κB signaling as well (418). KSHV expresses vFLIP during latency, driving both canonical and alternative NF-κB activation in order to promote cell survival and proliferation, as well as induce alterations in morphology (296, 331, 419-421).

KSHV and MHV68, but not EBV, express a viral G-protein coupled receptor (vGPCR) that drives NF-κB signaling. KSHV vGPCR is constitutively active (422) and is primarily expressed during lytic replication (423). KSHV vGPCR can include the expression of proinflammatory chemokines and cytokines (424). Despite its constitutive activity, KSHV vGPCR is a promiscuous cytokine receptor, and binding to chemokines can stimulate or repress vGPCR function (425). While genetically similar (8), MHV68 vGPCR is functionally and transcriptionally divergent from KSHV vGPCR. MHV68 vGPCR is expressed with late kinetics during lytic infection and also during latency (426). Overexpression of vGPCR transforms 3T3 murine fibroblasts (426). However, MHV68 vGPCR is only active upon binding to murine chemokines containing ELR motifs, and only weakly promotes NF-κB signaling (332).

The importance of NF-κB signaling for the establishment of latency has been shown *in vivo* for MHV68. Mutation of the IKKβ-targeted serines in IκBα prevents its degradation and blocks canonical NF-κB signaling (427). MHV68 was mutagenized to constitutively express this molecule from a neutral locus. Blocking NF-κB signaling did not affect lytic replication *in vitro* or in the lungs *in vivo* (333); however, latency establishment was significantly impaired in the spleen (333). This defect could not be rescued by overexpression of the anti-apoptotic protein Bcl-2, suggesting it was a defect in latency rather than a defect in cell survival (333). This defect is maintained into long term in both splenocytes and the lungs, supporting a role for canonical

NF-κB signaling in latency establishment (333). Furthermore, in bone-marrow chimeric mice generated from WT and $p50^{-/-}$ donors, lytic replication occurs normally in the lungs, but latency establishment and reactivation are defective in the $p50^{-/-}$ population (334). This defect is exacerbated over time, with the frequency of latently infected $p50^{-/-}$ cells shrinking between 46 and 92 dpi (334). It is unclear if this is the result of an effect on viral gene expression, or if it derives from the impaired ability of $p50^{-/-}$ B cells to become activated in response to infection and participate in a germinal center response. The presence of pre-formed infectious virus in the mixed bone marrow chimeras in the lungs long after clearance suggests that NF-κB signaling plays a role in maintenance of latency (334). Importantly, although p50 is completely absent from the $p50^{-/-}$ subset, latency is not completely abolished, suggesting there are additional pathways that promote latency establishment.

E. B cell development in the spleen

B cells develop in the bone marrow where they undergo VDJ recombination and emerge with a functional heavy and light immunoglobulin chain (428). The B cells emerge into the blood stream as transitional I (T1) B cells, characterized as $CD23^{lo}$ IgM^{hi} (429), and home to secondary lymphoid organs **(Fig. 1.6)**. Successful entry to the periarteriolar lymphoid sheath of the spleen (430) exposes the nascent B cell to peripheral selection by BAFF signaling. BAFF is secreted by numerous cell types in the spleen, including myeloid, dendritic, and stromal cells (431-433). BAFF binds primarily to the BAFFR on B cells, inducing pro-survival Bcl-2 family members (434) and down-regulating apoptotic genes (435) through alternative pathway NF-κB signaling. BAFF, BAFFR, or IKKα- knockout cells mature and exit the bone marrow but undergo apoptosis upon entry to the spleen, reducing the transitional II ($CD23^{hi}$ IgM^{hi}) and mature B cell reservoirs by up to 95% (400, 429, 436, 437). Conversely, transgenic expression of BAFF in mice drastically expands the transitional and marginal zone ($CD21^{hi}$ $CD23^{hi}$) B cell reservoirs (278). Upon maturation past the T1 phase into the T2 phase, the nascent B cell relocates to the splenic follicle where it undergoes BCR-dependent selection (430) mediated by the NF-κB signaling pathway (342, 348, 349). Successful activation licenses the T2 B cell to become a marginal zone or naïve mature follicular B cell (430).

The next step of B cell maturation occurs upon detection of a cognate antigen for the BCR, accompanied by CD40 co-signaling, leading to the formation of the germinal center (438). CD40 signals through both IKKα and IKKβ (439). Mice lacking CD40 fail to form germinal centers (438). Germinal centers can form from the small population of mature B cells in $BAFF^{-/-}$ mice during infection, although these are short-lived (440, 441). Both the downstream IKKs are

necessary for passage of the B cell through the germinal center (438). A dominant negative knock-in of IKKα (IKKαSSAA) abrogates germinal center formation (437, 442), while conditional deletion of IKKβ knockout drastically impairs global B cell survival (443). It has been recently shown that conditional deletion of NIK after passage of the T1 checkpoint impairs the ability of B cells to enter the germinal center, further confirming the necessity of non-canonical signaling in the germinal center reaction (444).

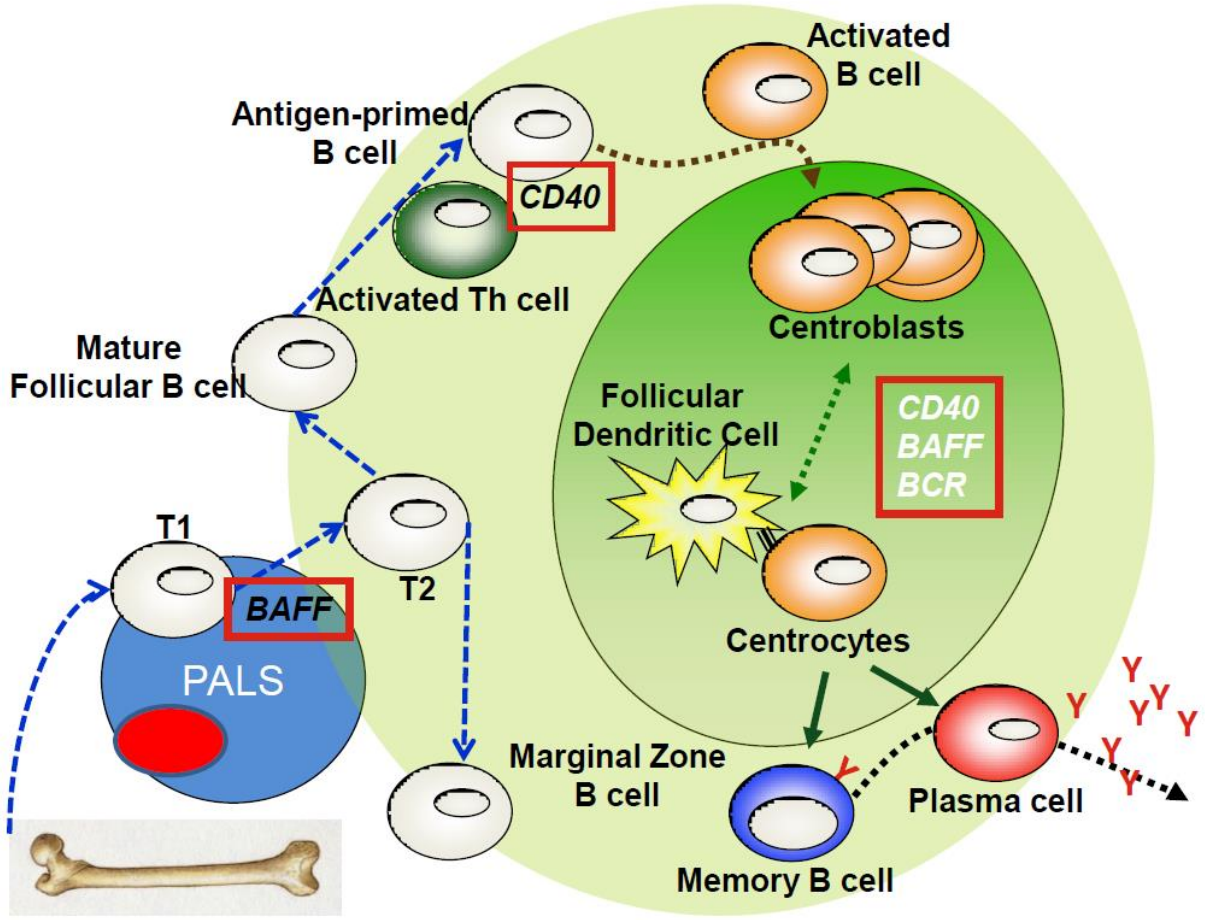

Figure 1.6 B cell maturation in the spleen. B cells exit the bone marrow and then home to secondary lymphoid organs. The B cell exits from the arteriole into the peripheral arteriole lymphoid sheath (PALS) as a T1 B cell. Here the B cell requires BAFF signaling to survive and mature to a T2 B cell. The T2 B cell can mature to a marginal zone B cell or follicular B cell. The mature follicular B cell can be activated by the detection of cognate antigen and CD40 costimulation. The activated B cell enters the germinal center, where it competes for antigen and costimulatory factors presented on follicular dendritic cells. Strong antigen binding and receipt of costimulatory NF-κB-dependent signaling through CD40 and BAFF allows the B cell to mature into a memory B cell or a plasma cell.

VI. Rationale

Over the long course of coevolution with their hosts, the gammaherpesviruses have evolved to subvert host signaling and immunity at every step of infection. Attempts at generating a vaccine against gammaherpesviruses have largely been ineffective to date, and gammaherpesvirus-driven malignancies remain difficult to treat. Gammaherpesviruses productively infect numerous cell types, including innate and adaptive immune cells, to reach the B cell. Here, the virus integrates host cell signaling to undergo latent infection whereby the virus evades clearance by the host immune response and maintains long-term infection. We seek to gain a better understanding of the mechanisms by which the gammaherpesviruses evade clearance during all phases of infection and the signals that decide whether infection is lytic or latent. Understanding how the virus carefully evades and hijacks different aspects of the immune response may allow us to target evasion mechanisms, improve immune recognition, or disrupt latency maintenance during normal infection or after the development of virus-driven malignancies. I have undertaken 3 avenues of research for this thesis. I have tested whether potentiating T cell signaling through STS1/2 deletion could improve detection and clearance of infected cells. I have investigated the contribution of Caspase1 signaling in virus control. Lastly I have developed novel approaches to determine whether IKKα signaling in infected cells drives the latency fate decision.

Chapter 2. Enhanced response of T cells from murine gammaherpesvirus 68-infected mice lacking the suppressor of T cell receptor signaling molecules Sts-1 and Sts-2

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Abstract

The human gammaherpesviruses establish life-long infections that are associated with the development of lymphomas and neoplasms, especially in immunocompromised individuals. T cells play a crucial role in the control of gammaherpesvirus infection through multiple functions, including the direct killing of infected cells, production of cytokines such as interferon-γ (IFN-γ), and costimulation of B cells. Impaired T cell function in mice infected with murine gammaherpesvirus 68 (MHV68) leads to increased reactivation and pathologies, including a higher incidence of lymphoid hyperplasia. Here we report that the absence of Suppressor of TCR signaling -1 and -2 (Sts-$1^{-/-}$/$2^{-/-}$) during MHV68 infection leads to the generation of T cells with significantly heightened responses. Transient differences in the T and B cell response of infected Sts-$1^{-/-}$/$2^{-/-}$ (Sts dKO) mice were also observed when compared to WT mice. However, these alterations in the immune response and the overall absence of Sts-1 and Sts-2 did not impact viral pathogenesis or lead to pathology. Acute lytic replication in the lungs, establishment of latency in the spleen and reactivation from latency in the spleen in the Sts dKO mice were comparable to WT mice. Our studies indicate that Sts-1 and Sts-2 are not required for the immune control of MHV68 in a normal course of gammaherpesvirus infection, but suggest that interference with negative regulators of T cell responses might be further explored as a safe and efficacious strategy to improve adoptive T cell therapy.

Introduction

The human gammaherpesviruses Epstein-Barr virus (EBV/HHV-4) and Kaposi's Sarcoma-associated Herpesvirus (KSHV/HHV-8) collectively infect over 95% of individuals, causing life-long infections that predispose infected individuals to the development of malignancies (195, 196, 445, 446). While the extent of productive replication upon primary infection with EBV or KSHV is not clear, these viruses ultimately establish a latent infection during which the genome is maintained, but few viral proteins are expressed (8, 23, 27, 447). In an immunocompetent host, immune surveillance by virus-specific T cells controls intermittent virus reactivation from latency (204, 210, 212, 230, 231). However, loss of immune control increases the risk of malignancies in viral reservoirs including B lymphocytes (EBV and KSHV), epithelial cells (EBV) and endothelial cells (KSHV) (229, 241). Reactivation and persistent infection cause disease in HIV-infected individuals (e.g Kaposi's Sarcoma), while the seeding of naïve lymphocytes leads to uncontrolled proliferative expansion in EBV- or KSHV-negative transplant recipients (e.g. post-transplant lymphoproliferative disorder, PTLD) (448, 449).

The murine gammaherpesvirus 68 is a natural pathogen of murid rodents with genetic and biological similarities to the human gammaherpesviruses (8, 450). This model pathogen has aided in the dissection of the roles of T lymphocytes during a natural host infection (451-453). Both $CD4^+$ and $CD8^+$ T cells promote clearance of productive replication in the lung during acute infection (34, 155). T cell surveillance plays a critical role in control of MHV68 during the chronic, latent phase of infection (162, 212, 454). Virus specific $CD8^+$ T cells persist for the life of the infected host (194, 208, 210, 212) and secrete effector molecules such as perforin and IFNγ that are necessary to repress reactivation from B cells and macrophages, respectively (162, 214,

455). Activated $CD4^+$ T cells are present throughout chronic infection to promote B cell responses, support $CD8^+$ T cell effector function (193, 209), and directly inhibit reactivation through the secretion of cytokines (36, 155, 223, 230, 231). T cells specific for viral antigens exposed during latency control virus expansion in the spleen, while those that recognize lytic epitopes prevent viral recrudescence in the lungs (208, 212, 223). Vaccination that drives the generation of virus-specific CD8 T cells reduces viral burden (456), as does adoptive transfer of virus-specific T cells to naïve mice prior to infection (457).

Effective T cell function requires signaling through costimulatory receptors and sustained activation of intracellular signaling pathways. Alteration of these signaling factors can reduce or enhance T cell responses, which in turn impacts control of MHV68 latency. A knockout mouse lacking both B7-family receptors CD80 and CD86 has severe defects in IFNγ secretion by T cells and the response to secondary infection, in addition to a failure to produce neutralizing antibodies and maintain long-term control of latency (458, 459). Deletion of another B7 family member, 4-1BB, impairs T cell effector function and leads to increased viral latency (460). On the other hand, mice lacking the SLAM associated protein (SAP), a negative regulator of lymphocyte signaling, have increased $CD8^+$ T cell activation in response to infection and impaired antibody production that ultimately does not alter long term control of the virus (461, 462). The role of mutations that generate gain-of-function T cells in the absence of other off-target B cell effects in the control of MHV68 infection has not been determined.

Negative feedback molecules control the duration of T cell activation by engagement of inhibitory receptors or inactivation of TCR signaling intermediates through direct binding, phosphatase activity, or ubiquitin ligase activity (191). Removal of these negative regulators can

increase TCR sensitivity (463), extend the duration of T cell effector function (464) or increase effector function (191, 464-466). Sts-1 and Sts-2 (TULA-2 and TULA-1, respectively) are intracellular phosphatases conserved in mice and humans that promote the dephosphorylation of the TCR signaling intermediates Zap-70 and syk (467-470). Sts-1 is ubiquitously expressed, whereas Sts-2 has a more restricted pattern of expression, predominantly in naïve and mature T cells (467, 468). Sts-1 and Sts-2 serve distinct but compensatory inhibitory roles in TCR signaling that impact T cell biology (468, 469). Disruption of both Sts genes lowers the threshold of T cell activation and leads to increased cytokine production and proliferation in response to TCR stimulation (469). In addition, loss of these TCR regulators confers increased incidence of autoimmunity in a model of multiple sclerosis (468).

In this study we examined the impact of Sts-1 and Sts-2 on the control of gammaherpesvirus pathogenesis. We infected Sts-1/2 double knockout mice (Sts dKO) with MHV68 and monitored immune responses and viral burden at multiple time points during the acute and chronic phases of infection. We found that the absence of the negative TCR regulators Sts-1 and Sts-2 led to increased effector T cell responses in culture that did not impact the normal course of MHV68 pathogenesis *in vivo*.

Materials and Methods

Ethics Statement. All of the animal experiments described herein were done under strict observance of the National Institute of Health guidelines. The Stony Brook University Institutional Animal Care and Use Committee approved this study (IACUC protocol #253637). All efforts were made to minimize suffering. Infection and adoptive T cell transfers were performed under isofluorane anesthesia.

Mice. Mice bearing germline Sts-1$^{-/-}$ and Sts-2$^{-/-}$ (Sts dKO) on a C57BL/6 background were bred in our facility (468). Age and sex matched C57BL/6 mice were purchased from Jackson Labs (Maine, USA). 8-12 week old animals of mixed genders were used in groups of 3-7 for most experiments. Experiments were conducted with BSL2 safety precautions.

Cell Culture. Low passage murine embryonic fibroblasts were cultured at 37^{o}C 5% CO_2 in DMEM containing 10% FBS, 2 µM L-glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin (10% CMEM). NIH murine 3T12 fibroblasts were cultured at 37^{o}C 5% CO_2 in DMEM containing 8% FBS, 2 µM L-glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin (8% CMEM). Bone marrow-derived macrophages(BMDMs)were generated by isolating bone marrow from 8-12 wk old mice from the femur and differentiation for 5d in DMEM with Glutamax (Life Technologies) containing 20% FBS and 30% L-supplement (BMM-Hi) in non-tissue culture treated plates. Cells were maintained in DMEM with Glutamax containing 10% FBS and 15% L-supplement (BMM-low) in non-tissue culture treated plates.

Virus and Infections. For intranasal infection experiments, we used the WT MHV68 isolate (WUMS strain, ATCC # VR1465) that was propagated as previously described (64). For intranasal infection, mice were lightly anesthetized using isofluorane and infected with 1,000 plaque forming units (PFU) of virus in a 20 µl bolus of 10% CMEM applied to the nose. Back titers of inoculate were performed to confirm infectious dose. For intraperitoneal infections, mice were lightly anesthetized using isofluorane and injected intraperitoneally with 1000 PFU of virus in 500 µl of 10% CMEM. For acute titers, speens were harvested from mice at 9 dpi and titered as described above. At 16 dpi, peritoneal exudate cells were isolated by injecting the peritoneum with 10 ml of 8% CMEM, followed by shaking the abdomen and then removing the media with a syringe. For growth curves, semi-confluent primary were infected at a multiplicity of infection (MOI) of 0.05 or 10 PFU with WT MHV68. For the BMDM growth curve, semi-confluent BMDMs were infected with MHV68-eYFP (38) at an MOI of 10 PFU. At the indicated times post infection, cells were freeze-thawed and serial dilutions were plated on subconfluent monolayers of NIH 3T12 murine fibroblasts in 6 well plates. Plates were rocked intermittently for 1 h and then overlaid with 3 ml of 8% CMEM + 1.5% methylcellulose. Cells were fixed after 1 week, stained, and counted. For coculture experiments, semi-confluent primary MEFs were infected at a multiplicity of infection (MOI) 10 PFU with WT MHV68 24 h prior to use. Peritoneal exudate cells were isolated by peritoneal injection of 10 ml of DMEM, agitating the abdomen, and withdrawing the peritoneal wash by syringe.

Flow Cytometry. For analysis of immune cell responses in mouse tissues, $2x10^6$ cells per sample were resuspended in 200 µl PBS + 2% FBS (FACS). Cells were blocked with TruStain fcX (Clone

93, Biolegend), washed, and stained with the following combinations: Effector T cell (CD4, CD62L, CD8, CD44), Vβ4 T cells (Vβ4, CD62L, CD8, CD3), p79 tetramer $CD8^+$ T cell (CD4, CD62L, CD8, p79 tetramer (kindly provided by the NIH Tetramer facility, TSINFVKI/H-$2K^b$), p56 tetramer $CD8^+$ T cell (CD4, CD62L, CD8, p56 tetramer (kindly provided by the NIH Tetramer facility, AGPHNDMEI /H-$2K^b$), activated B cell (CD69, CD19, CD3), germinal center B Cell (CD19, CD95, CD3, GL7). Cells were analyzed using a FACScalibur or Dxp8 FACScan (Cytek Development; BD Biosciences).

For analysis of T cell effector responses, spleens were isolated from naïve and infected mice 28 dpi. 2 x 10^6 bulk splenocytes were plated into each well of a 96-well round bottomed plate and either left untreated or treated with 1 μg/ml LEAF-purified αCD3 antibody (Clone 17A2, Biolegend), or 2 x 10^5 24 hr-infected MEFs that were gamma-irradiated with 2000 rads immediately prior to coculture. Cells were incubated overnight at 37^{o}C for 12 h in CMEM with golgiplug (BD Biosciences) and LAMP1/CD104a antibody (Clone 1D4B, Biolegend). The Fc receptors were blocked (TruStain fcX Clone 93, Biolegend) prior to staining with the following antibodies: CD8 (Clone 53-6.7, Biolegend), CD19 (Clone 6D5, Biolegend), and CD3 (Clone 145-2C11, Biolegend). Cells were also stained with APC-conjugated p79 tetramer. Upon fixation and permeabilization with the BD Cytofix/Cytoperm kit (BD Biosciences), cells were stained with IFNγ (Clone XMG1.2, Biolegend). Cells were analyzed using a Dxp8-FACScan (Cytek Development; BD Biosciences) and data was analyzed using FlowJo vX (Treestar).

Viral pathogenesis assays. For acute titers, mice were sacrificed by isofluorane overdose at the indicated days post infection, the left lung was removed, frozen at -80^{o}C, thawed, and disrupted

in 1 ml of 8% CMEM using 1 mm beads in a bead beater (Biospec). Serial dilutions in CMEM were plated on subconfluent monolayers of NIH 3T12 murine fibroblasts in 6 well plates. Plates were rocked intermittently for 1 h and then overlaid with 3 ml of 8% CMEM + 1.5% methylcellulose. Cells were fixed after 1 week, stained, and counted.

For quantitation of latency, limiting-dilution nested PCR with primers for the MHV68 ORF50 region was used to determine the frequency of virally infected cells. Frozen samples were thawed, resuspended in isotonic buffer, counted, and plated in serial 3-fold dilutions into a 96-well plate in a background of 10^4 NIH 3T12 murine fibroblasts. Six serial dilutions were plated, and 12 wells were plated per dilution. Plates were covered with PCR foil (Eppendorf Scientific), and cells were lysed with proteinase K for 6 h at 56 °C prior to the addition of 10 µl of round 1 PCR mix to each well by foil puncture. Following first-round PCR, 10 µl of round 2 PCR mix was added to each well by foil puncture and samples were subjected to round 2 PCR. Products were resolved on 2% agarose gels and each dilution was scored for positive bands. Control wells containing uninfected cells or 10, 1, and 0.1 plasmid copies of ORF50 were run with each plate to ensure single-copy sensitivity and no false positives. Data is representative of three independent experiments with four to six mice each. Error bars represent standard deviation.

For quantitation of reactivation, a limiting-dilution reactivation assay was performed. Bulk splenocytes in cMEM were plated in serial 2-fold dilutions (starting with 10^5 cells) onto MEF monolayers in each well of a 96-well tissue culture plates. Twelve dilutions were plated per sample, and 24 wells were plated per dilution. Wells were scored for cytopathic effect at 21 d postplating. To detect preformed infectious virus, parallel samples of mechanically disrupted

cells were plated onto MEF monolayers. For T cell co-culture assays, T cells were isolated using positive selection with αCD90.2 microbeads (Miltenyi Biotech) and added at a 5:1 or 25:1 ratio to the splenocytes or peritoneal exudate cells in the explant reactivation plates.

T Cell Transfer. Sts-1/2 dKO and C57BL/6 mice were infected as described above. Spleens were removed 28 dpi, homogenized in CMEM, red blood cell lysed, and filtered through a 100 µM filter. T cells were isolated using positive selection with αCD90.2 microbeads (Miltenyi Biotech). Purity was analyzed by staining cells for CD3 and CD19 with flow cytometry, as described above. WT mice were anesthetized with 5% isofluorane via face mask, and 10^7, 10^6, and 10^5 purified Sts-1/2 dKO or C57BL/6 T cells were injected retroorbitally in 5x C57BL/6 mice. 24 h later mice were infected with 1000 PFU WT MHV68 as described above. After 6 days, lungs were titered as described above. Bar represents geometric mean. *=$p<0.05$.

Statistical Analyses. All data were analyzed by using GraphPad Prism software (GraphPad Software, http://www.graphpad.com). Titer data were analyzed for Gaussian distribution using the D'Agostino and Pearson omnibus normality test followed by an unpaired t-test or a non-parametric Mann-Whitney two-tailed *t*-test. The frequencies of reactivation and viral genome–positive cells were obtained from the nonlinear regression fit of the data where the regression line intersected 63.2% based on the Poisson distribution, and then statistically analyzed by unpaired two-tailed *t*-test.

Results

Sts dKO T cells have heightened responses to MHV68 infected cells in culture. Mice that lack the antiviral cytokine IFNγ or the IFNγ receptor exhibit increased susceptibility to MHV68 infection, characterized by uncontrolled lytic infection, pathology-induced pneumonia, fibrosis of the lungs and spleen, and death (34, 62, 152, 155, 218, 471). A striking phenotype of Sts dKO mice is the production of hyperresponsive T cells that lead to enhanced proliferation and cytokine responses, including IFNγ upon TCR stimulation (468, 469, 472, 473). We reasoned that the T cells from Sts dKO mice might be hyperresponsive to MHV68 infection. To examine the role of Sts-1 and Sts-2 in the T cell response to gammaherpesvirus infection, we infected Sts dKO and WT mice with WT MHV68 by the intranasal route of infection. Splenocytes isolated at 28 days post-infection (dpi) were simulated overnight with 1 μg/ml αCD3 antibody in the presence of monensin to capture intracellular IFNγ production. Unstimulated Sts dKO and WT T cells were largely IFNγ negative **(Fig. 1A, top)**. However, when stimulated with a low level of αCD3, T cells from infected mice were more sensitive to non-specific TCR stimulation than their naïve counterparts **(Fig. 1B, middle)**. The Sts dKO mice had a significant five-fold increase in the production of IFNγ by T cells as compared to WT mice **(Fig. 1A&B, middle)**. Similar to what we observed with non-specific antibody stimulation, there was a significant increase in the levels of IFNγ production in Sts dKO T cells relative to WT T cells following stimulation with gamma-irradiated murine embryonic fibroblasts (MEFs) infected with MHV68 (Figure 1A&B, bottom). This data indicates that Sts dKO T cells from infected mice have a heightened response to viral antigens as compared to T cells from infected WT mice.

We next sought to determine the effector function of both $CD4^+$ and $CD8^+$ T cell subsets by examining IFNγ production and LAMP1 surface staining. LAMP1 marks perforin-containing granules docked at the plasma membrane as an indicator of degranulation. Splenocytes isolated from naïve or infected WT and Sts dKO animals 28 dpi were incubated overnight with 1 μg/ml αCD3 antibody or cocultured with infected MEFs in the presence of monensin. As illustrated in the flow plots of Figure 2A and summarized in the scatter plots of Figure 2B, untreated $CD8^+$ T cells from Sts dKO animals had a slight elevation in LAMP1 production compared to their WT counterparts. αCD3 stimulation induced significantly more IFNγ and LAMP1 in the $CD8^+$ T cells from infected Sts dKO mice **(Fig. 2B, left column)**. Interestingly, Sts dKO $CD8^+$ T cells from infected mice had significantly more IFNγ and surface LAMP1 upon coculture of splenocytes with MHV68-infected MEFs relative to WT $CD8^+$ T cells. We next investigated the responses of $CD8^+$ T cells specific to the viral p79/ORF61 epitope **(Fig. 2B, middle column)**. Without stimulation, the frequency of p79-tetramer+ virus-specific T cells was comparable between infected Sts dKO and WT mice **(Table 1)**, and we observed no difference in IFNγ production or LAMP1. However, TCR stimulation with αCD3 antibody or infected MEFs induced significantly greater IFNγ and LAMP1 in the virus-specific T cells lacking Sts-1 and Sts-2 **(Fig. 2B, middle column)**.

$CD4^+$ T cells play an important role in controlling MHV68 (223). There was a striking increase in IFNγ production and LAMP1 staining in $CD4^+$ T cells from Sts dKO infected mice compared to $CD4^+$ T cells from WT infected mice prior to stimulation **(Fig. 2B, right column)**. TCR complex stimulation with αCD3 antibody induced significantly more IFNγ and LAMP1 in Sts dKO $CD4^+$ T cells than in WT cells, and this heightened response was also observed upon

stimulation by virus-infected MEFs **(Fig. 2B, right column)**. Collectively, the absence of Sts-1 and Sts-2 enhances the T cell response to direct TCR stimulation by CD3 or MHV68-infected cell antigens.

The absence of Sts-1 and Sts-2 does not alter lytic replication *in vivo.* The loss of Sts-1 and Sts-2 leads to greater antiviral cytokine production and degranulation by T cells from infected mice upon stimulation in culture **(Fig. 2)**. However, the role of Sts-1 and Sts-2 in virus replication is unknown. We examined whether loss of Sts-1 and Sts-2 impacted MHV68 replication *in vitro* by examining replication in primary MEFs and primary bone marrow-derived macrophages (BMDMs) prepared from WT and Sts dKO mice. We observed no difference in virus production in either MEFs infected at a low MOI (0.05) or high MOI (5.0) or in BMDMs at a MOI of 10 **(Fig. 3)**. Next, we tested whether heightened effector functions would impact acute MHV68 infection *in vivo* by measuring virus replication in the lungs of infected Sts dKO and WT mice at multiple timepoints. The course of acute replication in Sts dKO and WT mice proceeded with similar kinetics and peaked at similar levels at 9 dpi **(Fig.4)**. Examination of the immune response at 12 dpi revealed no differences in B cell or T cell subsets recruited to the lungs, including no change in the percentage of virus-specific $CD8^+$ T cells as evidenced by staining for the early immunodominant epitope of ORF6 with the p56 tetramer **(Table 1)**. Virus was cleared from the lungs by 16 dpi and remained undetectable at later time points of chronic infection in both genotypes **(Fig. 4)**. We also observed no reduction in MHV68 replication in spleens after intraperitonal infection **(Fig. 5A)**. These findings indicate that Sts-1 and Sts-2 do not influence acute virus replication.

Previous studies have demonstrated that the transfer of virus-specific T cells provides protection against primary MHV68 infection (193, 457) and prevents the expansion of MHV68-immortalized B cells in immunocompromised mice (474, 475). We tested if the transfer of T cells from infected Sts dKO or WT mice could confer protection against primary infection in naïve mice. Donor T cells were enriched from the splenocytes of infected Sts dKO mice and WT mice 28 dpi. Naïve WT mice received 1 X 10^5, 1 X 10^6, or 1 X 10^7 donor T cells by retro-orbital transfer 24 hours prior to MHV68 infection **(Fig. 6A)**. We observed a dose-responsive trend in the reduction of virus replication at 6 dpi with a significant nearly one-log decrease in viral burden upon the transfer of 1 X 10^7 T cells **(Fig. 6B)**. However, both Sts dKO T cells and WT T cells generated similar levels of protection, indicating that in these experimental conditions, WT T cells were as potent as Sts dKO T cells in conferring protection against MHV68 replication *in vivo*.

Immune response is minimally altered in Sts dKO mice. We next monitored if knockout of Sts-1 and Sts-2 alters the T cell profile or the generation of $CD62L^{lo}/CD44^{hi}$ effector T cells over a timecourse of chronic infection. We observed fewer $CD4^+$ T cells at 28 dpi in the infected Sts dKO mice, but the levels increased to that of WT mice by 6 weeks post infection. Sts dKO mice had reduced levels of $CD4^+$ effector T cells at 16 dpi, but had equivalent amounts at 28 dpi, and more at 6 weeks post infection **(Table 2)**. $CD8^+$ T cell populations that were lower at 16 dpi in the absence of Sts-1 and Sts-2 returned to WT levels by 28 dpi. On the other hand, effector $CD8^+$ T cells were significantly increased at 28 dpi in the Sts dKO mice. We observed no difference in virus-specific $p79^+$ $CD8^+$ T cells at early or late stages of latency. The sustained expansion of Vβ4 $CD8^+$ T cells is an immunological hallmark of MHV68 infection of C57Bl/6 mice

(39). The production of IFNγ from Vβ4 CD8+ T cells represses reactivation from peritoneal macrophages (218). Interestingly, there was an enhanced $V\beta4^{+}$ $CD8^{+}$ T cell response at 28 dpi that returned to WT levels by 6 weeks post infection in the absence of Sts-1 and Sts-2.

In addition to being the primary targets for latency establishment in the spleen, B cells also mediate a humoral response to MHV68 infection (169, 214). We examined if the lack of Sts-1 and Sts-2 altered B cell frequency and responses to infection. Levels of B cells in Sts dKO animals were higher at 16 dpi, but not at later time points **(Table 3)**. Activated B cells in Sts dKO animals were higher at 28 dpi but returned to WT levels by 6 weeks post infection. Germinal center B cells typically undergo rapid expansion that peaks approximately two weeks after MHV68 infection (476). This expansion was observed for both WT and Sts dKO infected mice, but the frequency of germinal center B cells was lower in Sts dKO mice compared to WT mice. Taken together, the loss of Sts-1 and Sts-2 led to transient differences in T cell and B cell responses at early and late periods of chronic infection.

Sts-1 and Sts-2 do not impact latency or reactivation from latency. Sts dKO mice did not exhibit sustained alterations in effector T cell responses or the B cell profile between two and six weeks after infection, suggesting a normal course of virus colonization of the spleen. However, Sts-1 and Sts-2 might play intrinsic roles in the latent cell reservoirs that influence establishment of latency and reactivation from latency. Next, we sought to examine whether Sts-1 and Sts-2 influence viral latency in the spleen at early and late time points during chronic infection. To examine changes in latency, we determined the frequency of MHV68-genome positive splenocytes by a limiting dilution nested PCR assay. At 16 dpi we observed no significant difference in the levels of viral latency in the spleen **(Fig. 7A)**. Previous studies found

that mice lacking $CD8^+$ T cells, IFNγ, or perforin exhibit increased reactivation (161, 212, 214). To examine the role of Sts-1 and Sts-2 in reactivation from latency at 16 dpi, we performed a limiting dilution plating assay wherein serial dilutions of intact splenocytes are co-cultured with a monolayer of mouse embryonic fibroblasts that are monitored for cytopathic effect at two and three weeks post infection. There was no change in the frequency of splenocytes undergoing reactivation upon explant from infected mice in the absence of Sts-1 and Sts-2 as compared to WT mice **(Fig. 7B)**. We sought to increase the number of virus-specific T cells present in the explant co-culture reactivation assay by the addition of enriched T cells (70% purity) from previously infected mice at a 5:1 ratio with splenocytes from WT infected mice at 16 dpi. Co-culture with an enriched population of T cells from either Sts dKO or WT infected mice 28 dpi did not alter levels of reactivation **(Fig. 5B)**. Because T cells achieve control of latent gammaherpesviruses in the peritoneal exudate macrophages through the secretion of IFNγ (214), we also enriched T cells from Sts dKO or WT infected mice prior to co-culture at a 5:1 or 25:1 ratio with peritoneal exudate cells (PECs) from infected mice. The hyperresponsive T cells from infected Sts dKO mice did not alter levels of PEC reactivation **(Fig. 5C)**. Lastly, we examined long-term latency and found that levels of latency in Sts dKO mice remained similar to WT levels up to 57 dpi **(Fig. 7C)**. These data indicate that the lack of Sts-1 and Sts-2 does not alter pathogenesis during acute infection or at early or late times during chronic infection.

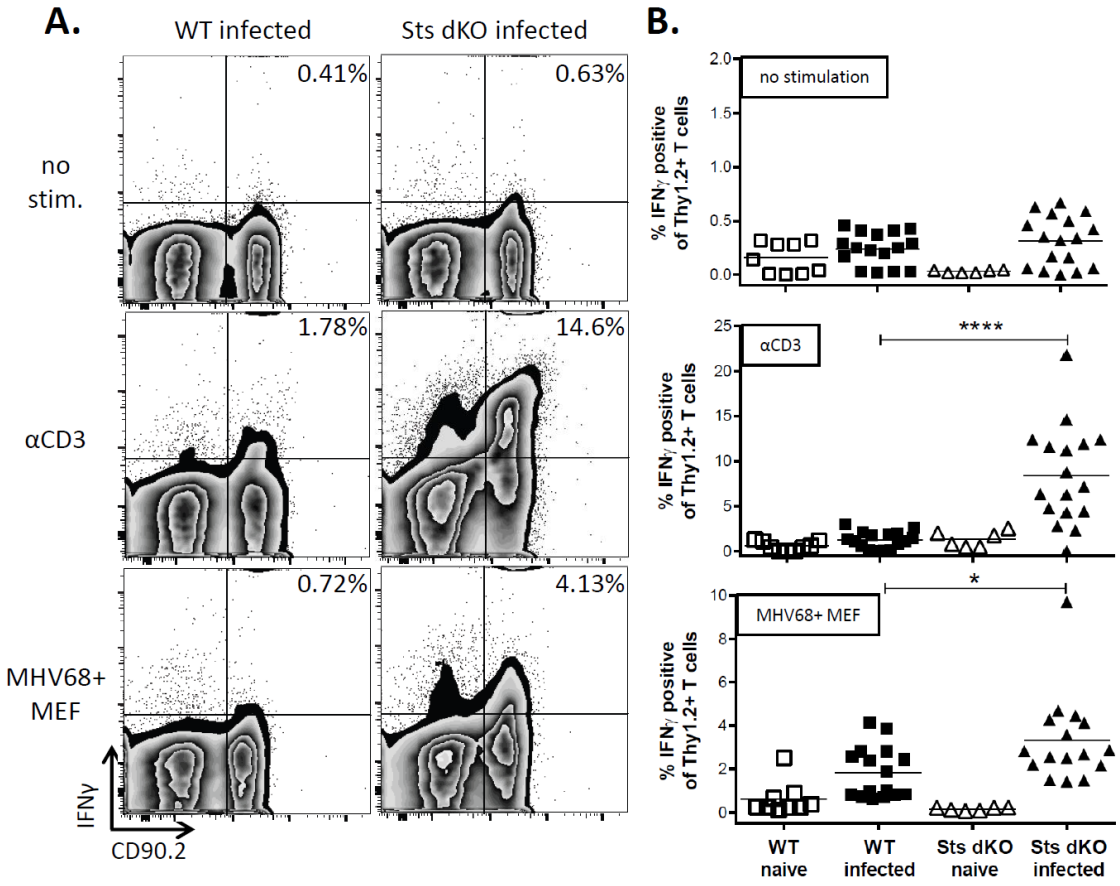

Figure 2.1: Increased IFNγ response to infected cells in the absence of Sts-1 and Sts-2. Sts dKO and C57/BL6 WT mice were infected 1000 PFU of MHV68 by the intranasal route and spleens were harvested 28 dpi. Splenocytes were left untreated or stimulated with 1 ug/ml αCD3 antibody or cocultured with gamma-irradiated MHV68-infected MEFs, both overnight in the presence of monensin. Cells were stained with the T cell marker CD90.2 and the intracellular cytokine IFNγ and analyzed by flow cytometry. (A) Representative flow plots of IFNγ expression in cells stained for CD90.2 are shown for each genotype and culture condition. (B) Scatter plot summary of the percentage of T cells producing IFNγ+ after overnight stimulation. Symbols represent data from individual mice. *= p<.05, **** = p<.0001.

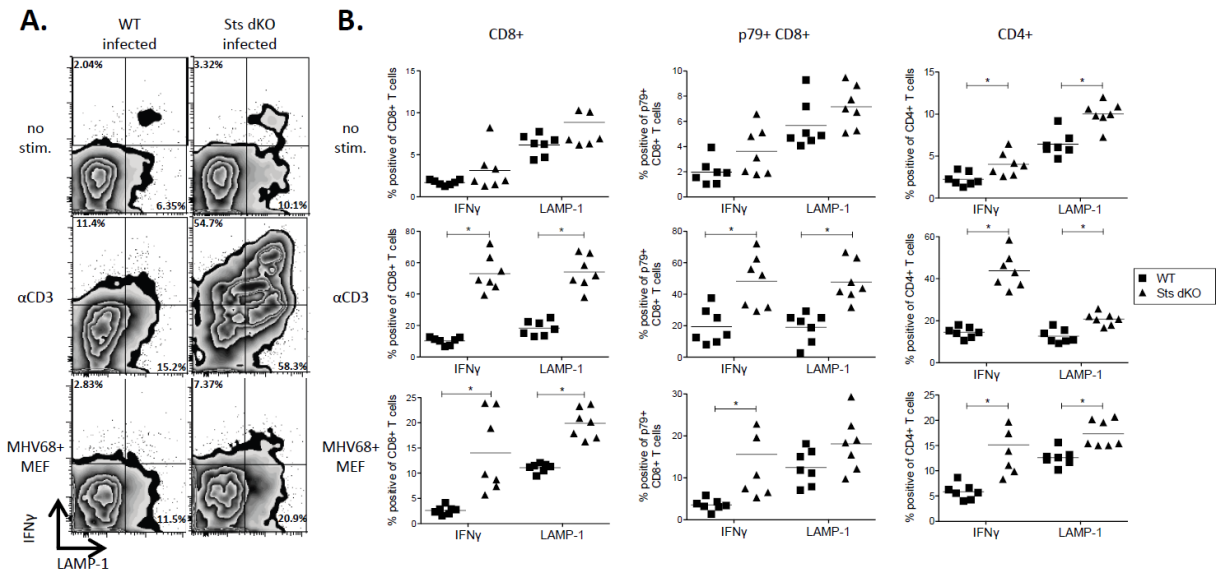

Figure 2.2: Sts dKO CD4+ and CD8+ T cells have increased effector responses to infected cells. Sts dKO and C57/BL6 WT mice were infected 1000 PFU of MHV68 by the intranasal route and spleens were harvested 28 dpi. Stimulations were performed as described in Figure 1 with the addition of αLAMP1, followed by costaining for CD4, CD8, and p79 tetramers. (A) Representative flow plots of LAMP-1 and IFNγ expression on CD8+ T cells are shown for each genotype and culture condition. (B-D) Scatter plot of the percentage of the indicated T cell subsets positive for IFNγ or LAMP-1. Symbols represent data from individual mice. *= p<.05, **= p<.01, ***= p<.001. **** = p<.0001.

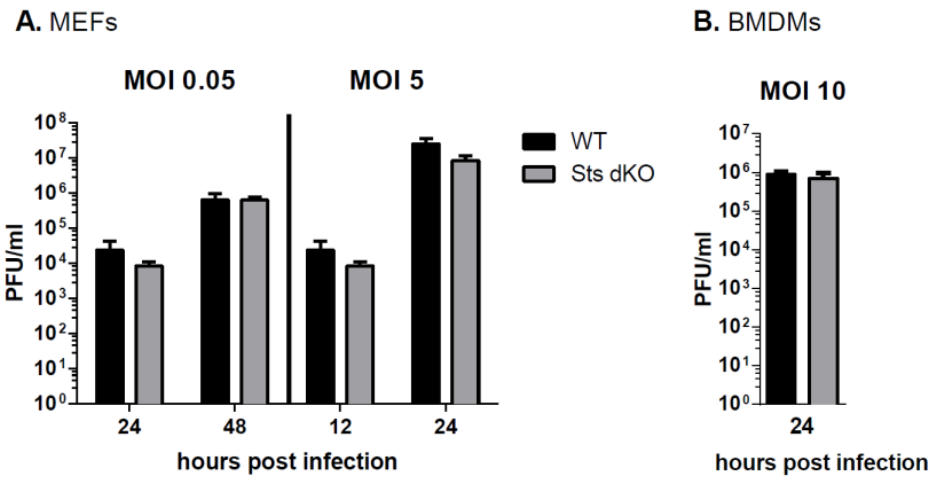

Figure 2.3: MHV68 replication in WT and Sts dKO cells. (A) Murine embryonic fibroblasts cells (MEFs) were infected with MHV68 at a MOI 0.05 or 5. (B) Primary bone marrow-derived macrophages were infected with MHV68 at a MOI 10. At the indicated times post infection, cultures were freeze-thawed and titered on NIH 3T12 fibroblasts.

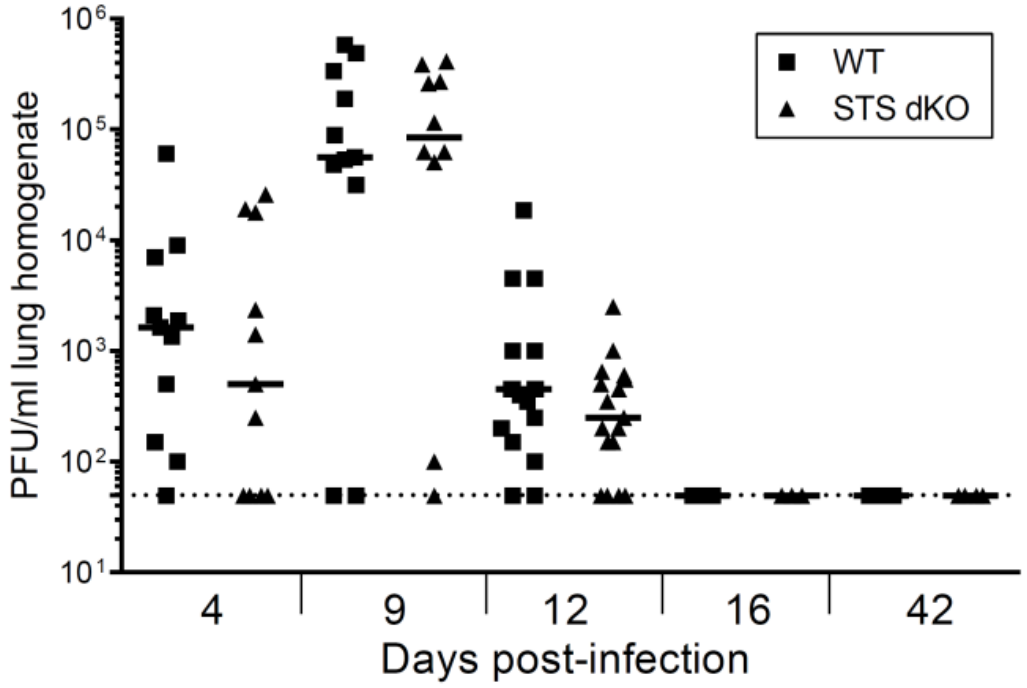

Figure 2.4: Sts dKO T cells do not alter the kinetics or levels of acute replication in the lungs. Sts dKO and C57/BL6 WT mice were infected 1000 PFU of MHV68 by the intranasal route. Lungs were harvested and disrupted at the indicated timepoints to quantitate levels of pre-formed infectious virus. Bar indicates median of $\log_{10}$ transformed data; dashed line indicates limit of detection. 3-8 animals are included per replicate experiment, n=3 for 4 dpi, n=2 for 9 dpi, n=2 for 12 dpi, and n=1 for 16 and 42 dpi; no significant differences were found based on Mann-Whitney non-parametric t-tests.

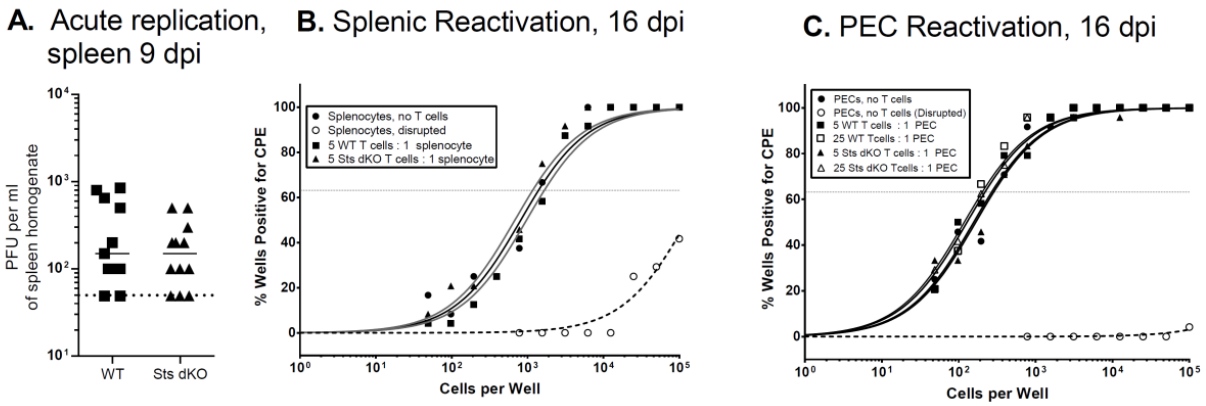

Figure 2.5: Effect of Sts dKO on intraperitoneal infection and reactivation from peritoneal macrophages. Mice were infected intraperitoneally with 1000 PFU of MHV68. (A) Viral titer was measured in the spleen 9 dpi. Bar indicates median of $\log_{10}$ transformed data and the dotted line marks the limit of detection. Data represents two experiments of 4-8 animals; no significant differences were found based on Mann-Whitney non-parametric t-test. (B) Splenocytes and (C) peritoneal exudate cells were harvested from ten WT mice 16 dpi and reactivation was measured by a limiting dilution *ex vivo* reactivation assay without T cells or with enriched T cells from Sts dKO or WT infected mice 28 dpi. The ratios of T cells to target cells are indicated in the legend.

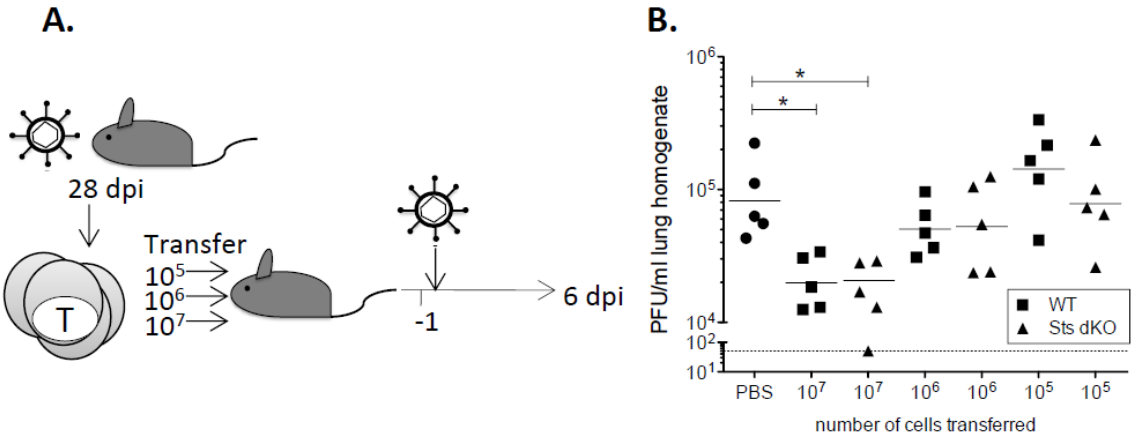

Figure 2.6: T cell transfer prior to infection reduces acute replication. (A) Schematic of T cell transfer experiment. Sts dKO and C57/BL6 WT mice were infected 1000 PFU of MHV68 by the intranasal route and spleens were harvested 28 dpi. Naïve mice received phosphate buffered saline (PBS) or the indicated numbers of enriched T cells by retroorbital transfer one day prior to intranasal infection with 1000 PFU MHV68. (B) Lungs were harvested 6 dpi and pre-formed infectious virus was measured by plaque assays. Symbols represent individual animals; *= p>0.05.

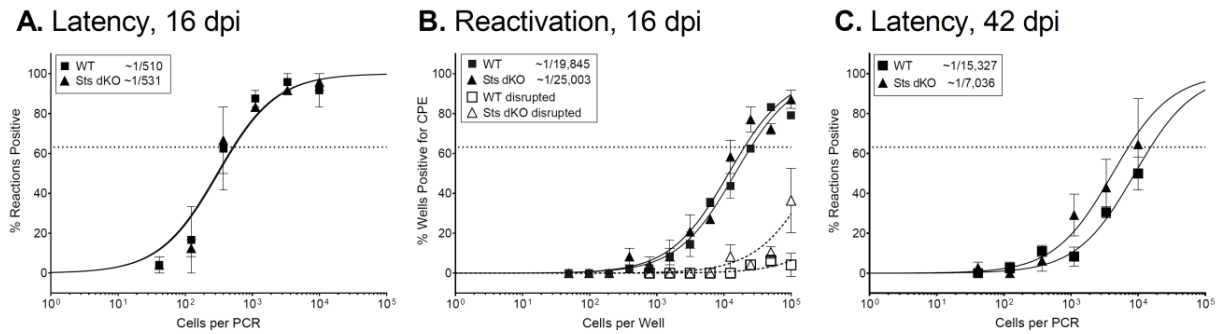

Figure 2.7: Viral latency and reactivation from latency are unchanged in Sts dKO animals. Sts dKO and C57/BL6 WT mice were infected 1000 PFU of MHV68 by the intranasal route and single cell suspensions of the spleens were prepared at 16 dpi. (A) Frequency of splenocytes harboring viral genomes determined by limiting dilution nested PCR assay. (B) Frequency of splenocytes reactivting virus determined by a limiting dilution ex vivo reactivation assay. (C) Frequency of splenocytes harboring viral genomes at 42-60. For all limiting-dilution assays, curve fit lines were derived from nonlinear regression analysis, and symbols represent the mean percentage of wells positive for virus (viral DNA or CPE) ± the standard error of the mean. The dotted line represents 63.2%, from which the frequency of viral genome-positive cells or the frequency of cells reactivating virus was calculated based on the Poisson distribution. Graphs represent 3 independent experiments of 3-4 mice.

Table 2.1. Immune response in the lungs during acute infection.

Cell type	Mean (SD) %[a]	
	WT	Sts dKO
CD4+ T cells	11.68 (0.95)	15.92 (7.53)
Effector CD4+ T cells	28.2 (6.13)	37.04 (16.11)
CD8+ T cells	18.2 (7.19)	15.2 (5.49)
Effector CD8+ T cells	58.79 (17.38)	66.4 (12.34)
p56 tetramer+ T Cells	8.78 (4.96)	9.28 (6.21)
B Cells	13.31 (4.46)	12.31 (3.49)
Activated B Cells	4.21 (1.65)	3.97 (0.79)

[a], The data shown are the percentage +/- standard deviation for each subset derived from FACS analysis of individual infected mice.

[b], CD4+ cells are $CD19^-$ $CD4^+$

[c], CD8+ cells are $CD19^-$ $CD8^+$

[d], Effector cells are $CD62L^-$ $CD44^+$ of the CD4+ or CD8+ gate

[e], p56 tetramer+ cells are $p56^+$ cells of the CD8+ gate

[f], B cells are $CD19^+$

[g], Activated B cells are $CD69^+$ of the B cell gate

Table 2.2. T cell subsets in the spleens of infected mice.

dpi	Mean (SD) %, [a]											
	CD4+ T Cells		Effector CD4+ T Cells		CD8+ T Cells		Effector CD8+ T Cells		p79 tetramer+ T cells		Vβ4+ CD8+ T Cells	
	WT	Sts dKO	WT	Sts dKO	WT	Sts dKO	WT	Sts dKO	WT	Sts dKO	WT	Sts dKO
12	11.81 (2.08)	10.38 (3.19)	6.04 (1.09)	7.82 (0.98)	12.54 (2.34)	9.85 (1.45)	14.08 (6.28)	21.87 (5.84)	1.34 (0.76)	1.75 (0.95)	nd	nd
16	17.8 (3.65)	15.37 (1.3)	**33.67 (1.3)**	**26.8 (2.46)**	**12.6 (1.41)**	**7.16 (1.23)**	25.65 (9.12)	32.57 (8.20)	nd	nd	nd	nd
28	**16.87 (3.93)**	**12.5 (3.44)***	57.07 (3.94)	60.97 (2.66)	17.48 (4.81)	18.53 (4.92)	**45.76 (9.34)**	**58.69 (6.71)***	3.97 (1.84)	4.18 (3.87)	**29.27 (9.96)**	**38.95 (8.51)***
45-57	19.05 (3.83)	16.6 (3.18)	**20.9 (4.81)**	**28.63 (2.24)**	23.89 (2.57)	19.48 (5.61)	33.55 (9.14)	28.84 (9.01)	nd	nd	28.84 (12.97)	28.55 (12.87)

[a], The data shown are the percentage +/- standard deviation for each subset derived from FACS analysis of individual infected mice. *, significant difference ($p<0.05$) between infected WT and STS dKO mice.

[b], CD4+ cells are $CD19^-$ $CD4^+$

[c], CD8+ cells are $CD19^-$ $CD8^+$

[d], Effector cells are $CD62L^-$ $CD44^+$ of the CD4+ or CD8+ gate

[e], p79 tetramer+ cells are $p79^+$ cells of the CD8+ gate

[f], Vβ4+ T cells are Vβ4+ of the CD8+ gate

Table 2.3. B cell subsets in the spleens of infected mice.

dpi	Mean (SD) %, [a]					
	B Cells		Activated B Cells		Germinal Center	
	WT	Sts dKO	WT	Sts dKO	WT	Sts dKO
12	**39.27 (2.55)**	**45.57 (2.38)***	nd	nd	nd	nd
16	55.17 (2.83)	56.3 (6.76)	nd	nd	2.70 (1.14)	2.21 (1.14)
28	43.13 (5.49)	43.26 (5.24)	**3.28 (0.22)**	**3.82 (0.48)***	1.18 (0.45)	1.43 (0.47)
45-57	22.1 (2.33)	24.6 (1.82)	11.38 (0.63)	10.68 (0.38)	**2.38 (0.50)**	**1.70 (0.09)***

[a], The data shown are the percentage +/- standard deviation for each subset derived from FACS analysis of individual infected mice. *, significant difference ($p<0.05$) between infected WT and STS dKO mice.

[b], B cells are $CD19^-$ $CD4^+$

[c], Activated B cells are $CD69^+$ of the $CD19^+$ gate

[d], Germinal center B cells are $CD95^+$ $GL7^+$ of the $CD19^+$ gate

Discussion

In this study we examined how the loss of Sts-1 and Sts-2 influences viral pathogenesis, with a particular interest in whether Sts dKO T cells that exhibit hyper-responsiveness to TCR engagement influence the host immune response and control of gammaherpesvirus pathogenesis. When isolated from MHV68-infected mice, T cells lacking the Sts proteins displayed striking increases (relative to wild-type T cells) in effector responses in response to antibody or viral antigen stimulation. Similarly, *Sts-1*$^{-/-}$*/2*$^{-/-}$ mice had altered splenic T cell responses. However, the absence of Sts-1 and Sts-2 did not impact acute replication, latency establishment or maintenance, and did not reduce reactivation from latency.

Unexpectedly, the enhanced response to infection by the Sts dKO T cells did not impart greater host control. During MHV68 infection of C57BL/6 mice, perforin is critical in maintaining latency in the spleen (214) and controlling viral recrudescence in the lung in the absence of CD4 T cells (152). When stimulated *ex vivo* with virally infected cells, Sts dKO T cells expressed both increased levels of surface LAMP1, indicative of degranulation and perforin secretion, and increased levels of the effector cytokine IFNγ. However, we detected no change in the initial levels of latency established in the spleen or reactivation from splenic latency in Sts dKO mice. Analysis of latency during later time points revealed that Sts dKO mice maintained MHV68 at levels similar to WT. In addition, incubation of explanted splenocytes or peritoneal macrophages with purified T cells from infected mice did not alter levels of reactivation. Taken together, the straightforward interpretation is that T cells enhanced by the removal of these negative TCR regulatory molecules are not functionally significant in the control of gammaherpesvirus latency and reactivation during a normal course of infection in an

immunocompetent animal. However, it must also be noted that $CD4^+$ T cells and $CD8^+$ T cells have antagonistic functions during infection. For instance, upon $CD4^+$ T cell depletion in mice infected with MHV68 and the ensuing viral recrudescence in the lungs, Molloy et al. (193) observed that CD8 T cells secrete the immunosuppressive cytokine IL-10. The administration of antibodies against the IL-10 receptor restored host control of reactivation. Therefore, it is possible that analysis of the individual role of $CD4^+$ T or $CD8^+$ T cell subsets might better reveal specific functions of helper or cytotoxic T cells whose functions have been altered by removal of Sts-1 and Sts-2. In this context, it is also important to note that we have observed recently a significant increase in the frequency of peripheral $CD4^+$ regulatory T cells that are normally thought to act in an immunosuppressive capacity (Carpino, unpublished observations). Thus, the absence of an *in vivo* impact in immunocompetent mice reported here may represent a net neutral outcome from a more complex array of opposing T cell effector functions that will only be revealed by targeted antibody depletions or reconstitution with specific T cell subsets.

Examination of early acute infection by MHV68 revealed that the hypersensitive Sts dKO T cells did not aid in control or clearance of the virus in the lungs of infected mice. There was no difference in the infiltration of B cell or T cells, including p56 tetramer+ virus-specific T cells, into the lungs at 12 dpi. Previous studies demonstrate that the transfer of T cells specific to MHV68 epitopes reduced initial viral replication and reduced latency and long-term maintenance (193, 457). Although we observed a decrease in lytic virus in the lungs after transfer of high numbers of T cells, Sts dKO T cells did not provide increased levels of protection in our adoptive transfer experiments. Based on the fact that $CD8^+$ T cells specific to the immunodominant p79 epitope /ORF61 comprised approximately 4% of the total $CD8^+$ T cell

population **(Table 2)**, we surmise that only a small percentage of the T cells that were transferred were specific to viral epitopes. Further enrichment of virus-specific T cells in combination with an increased dose of infectious particles may reveal a protective effect of hyper-responsive Sts dKO T cells. Sehrawat et al. (457) recently reported the generation of TCR transgenic mice specific for lytic epitopes of MHV68; a source of mono-specific T cells would enhance adoptive transfer studies that seek to enhance T cell effector responses.

Restoration of immune surveillance via the adoptive transfer of T cells specific to both lytic and latent EBV antigens is a highly efficacious therapeutic intervention in the context of immune suppression (477). Gammaherpesvirus-associated lymphoproliferative diseases, such as the EBV-associated nasopharyngeal carcinoma and post-transplant lymphoproliferative disease, express more immunogenic targets than latently infected B cells (232), and adoptive transfer of *in vitro*-expanded virus-specific T cells from an autologous or homologous source can efficiently control transplanted virus-infected tumor lines or PTLD (474, 478, 479). However, adoptive T cell transfer is not always effective in eliminating EBV-associated malignancies (480). T cell therapies primarily focus on the expansion of virus-specific $CD8^+$ T cells, but $CD4^+$ T cells have significant direct effector roles (481, 482) in addition to enhancing $CD8^+$ T cell function (482, 483). An additional challenge is that gammaherpesviruses may induce the T-cell suppressive cytokine IL-10 (129) or encode an IL-10 homologue (vIL-10) (322, 484). In EBV+ PTLD, elevated IL-10 levels have been correlated with an increased failure to respond to adoptive T cell transfer (485). Mechanisms that enhance T cell responses may substitute for $CD4^+$ T cell help or counter inhibitory cytokines produced by the host to improve the efficacy of adoptive T cell transfer.

We conclude that the heightened Sts dKO T cell responses upon stimulation in culture do not confer increased immune control and do not induce pathology in immunocompetent mice infected with MHV68 over an extended timecourse. Interestingly, MHV68 infection and the lack of Sts-1 and Sts-2 have each been reported to promote the development of experimental autoimmune encephalomyelitis (4, 468). Future adoptive T cell transfer studies will examine whether virus-specific Sts dKO $CD8^+$ T cells can augment the control of gammaherpesvirus infections or clearance of lymphomas in immunodeficient hosts. More studies are warranted to ensure that Sts dKO T cells can be therapeutic without leading to autoimmune complications in the context of MHV68 and other infections.

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Chapter 3. Murine gammaherpesvirus 68 pathogenesis is independent of Caspase-1/11 in mice and impairs IL-1β production upon extrinsic stimulation in culture.

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ABSTRACT

Gammaherpesviruses establish life-long infections that are associated with the development of cancer. These viruses subvert many aspects of the innate and adaptive immune response of the host. The inflammasome, a macromolecular protein complex that controls inflammatory responses to intracellular danger signals generated by pathogens, is both activated and subverted during human gammaherpesvirus infection in culture. The impact of the inflammasome response on gammaherpesvirus replication and latency *in vivo* is not known. Caspase-1 is the inflammasome effector protease that cleaves the proinflammatory cytokines IL-1β and IL-18. We infected mice deficient in *caspase-1* with murine gammaherpesvirus 68 (MHV68) and observed no impact on acute replication in the lung or latency and reactivation from latency in the spleen. This led us to examine the effect of viral infection on inflammasome responses in bone marrow-derived macrophages. We determined that infection of macrophages with MHV68 led to a robust interferon response, but failed to activate caspase-1 or induce secretion of IL-1β. In addition, MHV68 infection caused a specific reduction in IL-1β production after extrinsic LPS stimulation or upon co-infection with *Salmonella enterica* Serovar Typhimurium. Interestingly, this impairment occurred at the proIL-1β transcript level and was independent of the lytic viral transcriptional activator RTA. Taken together, MHV68 impairs the inflammasome response by inhibiting IL-1β production during the initial stages of infection.

IMPORTANCE

Gammaherpesviruses persist for the lifetime of the host. To accomplish this, they must evade recognition and clearance by the immune system. The inflammasome consists of proteins that detect foreign molecules in the cell and respond by secreting proinflammatory signaling proteins that recruit immune cells to clear the infection. Unexpectedly, we found that murine gammaherpesvirus pathogenesis was not enhanced in mice lacking caspase-1, a critical inflammasome component. This led us to investigate if the virus actively impairs the inflammasome response. We found that the inflammasome was not activated upon macrophage cell infection with murine gammaherpesvirus 68. Infection also prevented the host cell inflammasome response to other pathogen-associated molecular patterns, indicated by reduced production of the proinflammatory cytokine IL-1β upon bacterial co-infection. Taken together, murine gammaherpesvirus impairment of the inflammatory cytokine IL-1β in macrophages identifies one mechanism by which the virus may inhibit caspase-1-dependent immune responses in the infected animal.

INTRODUCTION

Gammaherpesviruses establish life-long latent infections that are associated with significant morbidity and the development of malignancies (80) (486, 487). Gammaherpesviruses initially infect the mucosal tissues of naïve hosts and undergo productive replication prior to colonization of latency reservoirs, including B lymphocytes and macrophages (21, 476, 488). Murine gammaherpesvirus-68 (MHV68) is a natural pathogen of murid rodents that shares genetic and pathologic similarities to the human gammaherpesviruses (8, 450). The analysis of MHV68 in the context of a mouse infection enables the investigation of the dynamic interplay between the virus and the innate and adaptive immune responses of the host (451, 453).

The inflammasome is a critical mediator of inflammatory responses that restricts the pathogenesis of numerous viruses and bacteria (101). Pathogen-associated molecular patterns (PAMPs) are detected by multiple cellular sensors including Toll-like receptors (TLRs), NOD-like receptors (NLRs), and other RNA and DNA sensors. Triggering of this comprehensive surveillance system leads to upregulation of inflammasome components and induction of the rapid formation of a multi-protein complex containing procaspase-1. Upon procaspase-1 cleavage, the inflammatory cytokines IL-1β and IL-18 are processed and secreted (98, 99, 101). IL-1β is a critical pro-inflammatory cytokine that mediates macrophage and neutrophil recruitment to the site of infection (489, 490). IL-18 induces robust IFN-γ expression from NK and T cells, promoting a Th1 pro-inflammatory response (490).

Inflammasome signaling serves as an important restriction factor against herpesviruses *in vivo*. In the absence of IL-1β, herpes simplex virus type 1 (HSV-1) infection of mice rapidly

advances to lethal encephalitis (491). Similarly, the removal of AIM2, a cytosolic DNA sensor that activates the inflammasome in response to murine cytomegalovirus, leads to an impaired NK cell response and an increase in viral titers during infection (104). The loss of the inflammasome components NLRP3 or ASC, but not the AIM2 sensor, results in an increase of MHV68 genomes in the spleen (105). Thus, the inflammasome response is a critical innate defense with the potential to activate immune cells to control herpesvirus replication.

Recently, herpesviruses including the oncogenic gammaherpesviruses Epstein-Barr Virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV/HHV-8) were found to be sensed by the host DNA sensor IFI16, leading to inflammasome complex formation (106-108). However, like many other viral and bacterial pathogens, herpesviruses employ multiple strategies to inhibit the inflammasome upon infection. HSV-1 rapidly induces IFI16 degradation upon expression of the immediate early gene ICP0 (492). EBV encodes a miRNA that reduces transcription of the NLRP3 sensor protein (109). The KSHV ORF63 tegument protein impairs both the NLRP1 and NLRP3-mediated inflammasomes (17), while the MHV68 ORF64 deubiquinase impairs sensing that reduces type 1 interferon and inflammasome responses upon infection of dendritic cells (105). Gammaherpesviruses activate and inhibit inflammasome responses in culture (17, 106-109), but the influence of caspase 1-dependent inflammatory responses on pathogenesis *in vivo* is unclear.

We assessed the impact of caspase-1-dependent inflammasome activation on murine gammaherpesvirus pathogenesis. The absence of caspase-1 had no effect on viral replication in cell culture or in the lungs of mice after intranasal infection. Establishment of latency and reactivation from latency in the spleen were not altered. The lack of a phenotype *in vivo* led us

to examine whether MHV68 might counteract inflammasome responses. MHV68 did not induce inflammasome activation upon infection of primary bone marrow-derived macrophages (BMDMs). Furthermore, MHV68 inhibited IL-1β production in response to extrinsic LPS stimulation and upon co-infection with *Salmonella enterica* Serovar Typhimurium. We determined that the repression of IL-1β occurred at the transcript level, and was mediated by the tegument or an RTA-independent gene product during the early stages of productive infection. MHV68 subversion of the inflammatory cytokine IL-1β is consistent with the lack of any change in the course of infection in mice lacking *Caspase*-1/11.

MATERIALS AND METHODS

Mice. Mice bearing germline *caspase-1/11* deletions on a C57BL/6 background (99, 493) were bred in our facility. Age and sex matched C57BL/6 mice were purchased from Jackson Labs (Maine, USA) and maintained in our facility. 8-12 week old animals of mixed genders were used in groups of 3-7 for most experiments. Isoflurane was used for anesthesia to conduct infections and terminal harvests**.** The animal experiments described herein were done under a protocol approved by the Stony Brook University Institutional Animal Care and Use Committee. IFNAR1-/- mice (C57BL/6 background) were a kind gift of Dr. Mitchell Grayson and were maintained at the Medical College of Wisconsin (494).

Cell Culture. Low passage murine embryonic fibroblasts were cultured at 37^{o}C with 5% CO_2 in DMEM containing 10% FBS, 2 µM L-glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin (10% cMEM). NIH murine 3T12 fibroblasts were cultured at 37°C with 5% CO_2 in DMEM containing 8% FBS, 2 µM L-glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin (8% cMEM). To generate bone marrow-derived macrophages (BMDMs), bone marrow was flushed from the mouse femur and differentiated for 5 d in DMEM with Glutamax (Life Technologies, Grand Island, NY) containing 20% FBS and 30% L-supplement (BMM-Hi) in low-binding dishes. Cells were then seeded in DMEM with Glutamax containing 10% FBS and 15% L-supplement (BMM-low) in tissue culture treated plates for future experiments.

Virus and infections. For *in vivo* infection experiments, we used the WT MHV68 (WUMS strain, ATCC # VR1465) isolate that was propagated as previously described (64). MHV68-eYFP

expresses YFP driven by a CMV promotor from an intergenic locus (38). MHV68-H2bYFP expresses YFP fused to the H2b protein from an intergenic locus (495). Virus stocks were concentrated to > 1 x 10^8 PFU/ml by centrifugation at 4°C for 120 min at 13,000 x g in a Dupont (Wilmigton, DE) GSA rotor. For intranasal infection, mice were lightly anesthetized using isofluorane and infected with 1,000 plaque forming units (pfu) of virus in a 20 µl bolus of 10% cMEM applied to the nose. For intraperitoneal infections, mice were lightly anesthetized using isofluorane and injected with 1000 PFU of virus in 500 µl of 10% cMEM. Back-titers of inoculate were performed to confirm infectious dose.

MHV68-ORF50stop was produced as previously described (496) with the following modifications. ORF50 cDNA was cloned into pMSCV-puro (Clontech, Mountain View, CA). RTA-encoding retroviruses were packaged by transfecting BOSC23 cells with pMSCV-puro-RTA, and NIH 3T12 fibroblasts were transduced with the resulting virus. Stable cell lines were selected using puromycin in the culture media at a final concentration of 5 µg/ml. RTA-expressing cell lines or empty vector control cell lines were transfected with ORF50stop BAC or WT BAC. Transfected cells were observed for cytopathic effect to ensure that WT contaminant viruses were not present in empty vector control cultures, and viral stocks were generated according to standard MHV68 protocols (64). WT MHV68 and ORF50stop P2 stocks were concentrated to > 1 x 10^8 PFU/ml as described above. Concentrated ORF50stop MHV68 viral stocks were re-evaluated by plaque assay on empty vector control cell lines to confirm that WT contaminants were not present in stocks used for experiments.

Flow Cytometry. For analysis of immune cell responses in mouse tissues, spleen and lungs from

infected mice were homogenized into single cell suspensions. Lungs were digested in 3ml 10% DMEM with 150 U/ml Collagenase and 10 U/ml DNaseI at 37°C. $2x10^6$ cells per sample were resuspended in 200 µl PBS + 2% FBS (FACS). Cells were blocked with TruStain fcX (clone 93, Biolegend, San Diego, CA), washed, and stained to detect the following markers for T cells (CD3 clone 145-2C11; CD4, clone GK1.5; CD8, clone 53-6.7; CD62L clone MEL-14; CD44 clone IM7; Vβ4, clone KT4) and B cells (CD19 clone 6D5; CD69 clone H1.2F3; CD95 clone Jo2; GL7 clone GL7). All conjugated antibodies were purchased from Biolegend except antibodies against CD95 and Vβ4 (BD Pharmingen, San Jose, CA) and anti-GL7 (eBiosciences, San Diego, CA). The p79 tetramer (TSINFVKI/H-$2K^b$) was kindly provided by the NIH Tetramer facility. Cells were analyzed using a FACScalibur (BD Biosciences) or Dxp8 FACScan (BD Biosciences, Cytek Development) and data was analyzed using FlowJo vX (Treestar, Ashland, OR).

Viral pathogenesis assays. For acute titers, mice were sacrificed with isofluorane at the indicated days post infection, and the left lung was removed and frozen at ¯80°C. Lungs were disrupted in 1 ml of 8% cMEM using 1 mm zirconia beads in a bead beater (Biospec, Bartlesville, OK). Serial dilutions in 10% cMEM were plated on subconfluent monolayers of NIH 3T12 murine fibroblasts in 6 well plates. Plates were rocked intermittently for 1 hr and then overlaid with 3 ml of 5% cMEM + 1.5% methylcellulose. Cells were fixed after 1 week, stained with crystal violet, and plaques were counted.

To analyze latently infected cells, mice were sacrificed with isofluorane at 16 or 42 dpi. Spleens were excised, homogenized, and resuspended in 10% cMEM. Peritoneal exudate cells

were isolated by peritoneal injection of 10 ml of 10% cMEM, followed by agitation of the abdomen and withdrawal of the peritoneal wash by syringe.

For quantitation of latency, limiting-dilution nested PCR with primers for the MHV68 ORF50 region was used to determine the frequency of virally infected cells as previously described (166). Briefly, frozen samples were thawed, resuspended in isotonic buffer, counted, and plated in serial 3-fold dilutions in a background of 10^4 NIH 3T12 murine fibroblasts into a 96-well plate. The resultant PCR products were resolved on 2% agarose gels and each dilution was scored for amplimer of the expected sizes. Control wells containing uninfected cells or 10, 1, and 0.1 plasmid copies of ORF50 target sequence were run with each plate to ensure single-copy sensitivity and no false positives.

For quantitation of reactivation, a limiting-dilution reactivation assay was performed as previously described (166). Briefly, bulk splenocytes in 10% cMEM were plated in serial 2-fold dilutions (starting with 10^5 cells) onto MEF monolayers in each well of a 96-well tissue culture plate. Twelve dilutions were plated per sample, and 24 wells were plated per dilution. Wells were scored for cytopathic effect at 14 and 21 d after plating. To detect preformed infectious virus, parallel samples of mechanically disrupted cells were plated onto MEF monolayers.

Bone marrow-derived macrophage cell infection and stimulation. Differentiated BMDMs were seeded onto cell culture-treated dishes. Subconfluent cells were infected with MHV68-H2bYFP, MHV68-eYFP, or MHV68-ORF50stop at 10 MOI for 90 min with intermittent rocking. At 6 or 22 hpi, media was replaced with fresh BMM-low + 100 ng/ml LPS. After 2 hrs, media was replaced with fresh BMM-low + 100 ng/ml LPS + 5 mM ATP and incubated for 1 hr.

S. Typhimurium strain 14028 (ATCC) was used as the wild-type strain. Using standard microbiological techniques, bacteria were grown aerobically overnight at 37°C in LB broth. Bacteria were then subcultured 1:20 in LB Broth for 3 hr to reach late-log phase growth. To infect BMDM with *Salmonella* Typhimurium, WT *Salmonella* was inoculated at an MOI of 1 or 4 CFU/cell. Infected cells were spun at 1000 rpm for 5 min and incubated at 37°C for 2 hrs. Supernatant was removed, and then cells were lysed in 500 µl fresh BMM-low by freezing. Samples were stored at -80^{O}C. Triplicate wells were examined per experiment.

DNA stimulation. Subconfluent BMDMs were incubated in BMM-low media containing 0.5 µM CpG DNA (ODN 1826, Invivogen, San Diego, CA) for 16 h. After incubation, TNF-α secretion was measured by ELISA. For detection of DNA-induced inflammasome activation, subconfluent WT BMDMs were prestimulated for 2 h in BMM-low media containing 100 ng/ml LPS for 4 hr. After prestimulation, 1 µg poly(dA:dT) (Invivogen) was transfected into cells using TransIT LT-1 (Mirus, Madison, WI). After 6 hrs, IL-1β secretion was measured by ELISA.

Bacterial invasion. *Salmonella* Typhimurium ATCC strain 14028 was grown aerobically for overnight at 37°C in LB broth followed by a 3 hr subculture in LB broth prior to BMDM infection. BMDMs were inoculated with *Salmonella* Typhimurium at an MOI of 1 or 4 CFU/cell. Infected cells were spun at 1000 rpm for 5 min and incubated at 37 ^{O}C for 2 hrs. BMDMs were washed using PBS three times in order to remove unattached bacteria, followed by lysing with Triton X-100 (Sigma-Aldrich). Cell lysates were diluted in PBS and plated on LB-agar plates. Bacterial invasion was determined by counting CFUs after overnight incubation at 37 °C.

ELISA and cell death assay. IL-1β and TNF-α were quantified using the Mouse Duoset IL-1β or TNF-α ELISA Kit (R&D Systems, Minneapolis, MN). IL-18 was quantified using Mouse IL-18 ELISA kit (MBL International, Japan). Plates were prepared and assayed according to manufacturer's protocol and signals were read at 450nm with background subtracted. Lactate dehydrogenase (LDH) release into the conditioned media was measured using the CytoTox 96 nonradioactive cytotoxicity assay according to the manufacturer's instruction (Promega, Madison, WI).

Immunoblotting. Infected BMDMs were lysed in RIPA buffer for 20 min. Samples were quantified by Bradford assay (BioRad, Berkeley, CA) and protein was diluted in NuPAGE LDS Sample Buffer (Invitrogen) with Reducing Agent (Invitrogen) before boiling at 95^{o}C for 5 min. Samples were separated by NuPAGE Bis-TRis Mini Gels (Invitrogen), and transferred to Immobilon P membranes (Millipore, Darmstadt, Germany). Primary antibodies against caspase-1 p10 (Santa Cruz Biotechnology, Dallas, TX), IL-1β (R&D systems), IL-18 (BioVision, Milpitas, CA) and GAPDH (Sigma, St. Louis, MO) were used. Rabbit anti-LANA was a kind gift from Dr. Scott Tibbetts. Affinity purified chicken anti-ORF75c antibody (Gallus Immunotech, Cary, NC) was previously described (497). Detection was performed with HRP-conjugated anti-rabbit IgG or anti-mouse IgG (GE Healthcare, Buckinghamshire, UK), HRP-conjugated streptavidin IgG (Rockland, Gilbertsville, PA), or HRP-conjugated goat anti-chicken (Gallus Immunotech, Cary, NC). Data was captured by GE CCD camera and analyzed by ImageQuant software (v7.0, GE Healthcare).

Quantitative RT-PCR. BMDMs were lysed in RLT buffer (Qiagen, Hilden, Germany) supplemented with 1 % BME and stored at -80 °C before RNA extraction. Total RNA was extracted using Qiagen RNEasy Mini Kit according to the manufacturer's instruction. RNA concentration and quality was determined by measuring absorbance at 260 nm and 280 nm. RNA was DNase treated (Ambion Turbo DNA Free kit) and RNA was reverse transcribed into cDNA using SuperScript III (Invitrogen), and the cDNA was input to qPCR using ABsolute Blue QPCR SYBR with Low ROX (Thermo Scientific) in an ABI 7500 real-time PCR system (Applied Biosystems) following the manufacturer's instructions. PCR conditions were 95°C for 15 min followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. Primer sets were purchased from Eurofins genomics (Huntsville, AL) for the following: IL-1β For 5'-TCTTTGAAGTTGACGGACCC-3' Rev 5'-TGAGTGATACTGCCTGCCTG-3' (498); IL-18 For 5'-CAGGCCTGACATCTTCTGCAA-3' Rev 5'-CTGACATGGCAGCCATTGT-3' (499); TNF-α For 5'-GCCTCTTCTCATTCCTGCTTGT-3' Rev 5'-GATGATCTGAGTGTGAGGGTCTG-3' (500), Caspase-1 For 5'-AGATGGCACATTTCCAGGAC-3' Rev 5'-GATCCTCCAGCAGCAACTTC-3' (498); HPRT For 5'-GTAATGATCAGTCAACGGGGGAC-3', Rev 5'-CAGCAAGCTTGCAACCTTAACCA-3' (501). Relative transcript levels were calculated as $E = 2^{-\Delta\Delta CT}$, where E is the gene expression value and $\Delta\Delta CT$ is the difference between HPRT and target genes of the experimental group over the mock treatment group.

Statistical Analyses. All data were analyzed by using GraphPad Prism software (GraphPad Software, http://www.graphpad.com, La Jolla, CA). Titer data were statistically analyzed with Student's two-tailed *t*-test. Based on the Poisson distribution, the frequencies of reactivation and viral genome–positive cells were obtained from the nonlinear regression fit of

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

RESULTS

Lytic replication and early pathogenesis of MHV68 *in vivo* is not affected by the loss of caspase-1/11. To examine the role of inflammatory processes dependent on caspase-1 during gammaherpesvirus pathogenesis, we examined MVH68 replication, latency, and reactivation from latency in the cells and tissues of mice lacking caspase-1 (99). These *Caspase*-1 deficient mice also lack *Caspase*-11 (hereafter referred to as *Caspase*-1/11 -/-) (493). Caspase-1 is the critical effector protease for IL-1β and IL-18 (100). Caspase-11 controls a non-canonical inflammasome pathway that is activated by LPS from Gram negative bacteria (493, 502). Bone marrow-derived macrophages (BMDMs) were prepared from *Caspase*-1/11 -/- and WT mice. Cells were pretreated with LPS for 2h, then stimulated with LPS and ATP for 1 hr to activate the inflammasome. As expected, *Caspase*-1/11 -/- BMDMs failed to secrete IL-1β in response to inflammasome activation **(Fig. 3.1A)**. TNF-α, an inflammasome-independent cytokine, was released from both *Caspase*-1/11 -/- and WT BMDMs **(Fig. 3.1A)**. Caspase-1 has been reported to promote EBV replication (503). We assessed the growth of MHV68 in cells that lacked *Caspase*-1. WT and *Caspase*-1/11 -/- primary murine BMDMs were infected at a low multiplicity of infection (MOI 0.1) and replication was examined over the course of a multi-step growth curve. There was no difference in the kinetics of replication in the *Caspase*-1/11 -/- and WT BMDMs **(Fig. 3.1B)**. Virus replication was also comparable between *Caspase*-1/11 -/- and WT murine embryonic fibroblasts (MEFs) upon infection at an MOI of 0.01 **(Fig. 3.1C)**, indicating that caspase-1 and -11 are not required to support viral replication. Next, *Caspase*-1/11 -/- and WT mice were infected with 1000 PFU of MHV68 via the intranasal route and viral replication in the lungs was measured over a 12 day timecourse. No differences were observed in replication

kinetics or peak viral titers in the lungs of *Caspase*-1/11 -/- mice as compared to WT mice **(Fig. 3.1D)**. In addition, the acute phase of infection was cleared with normal kinetics in *Caspase*-1/11 -/- mice, with viral titers dropping below the limit of detection by 12 dpi.

Long-term latency of MHV68 is unaffected by the absence of caspase-1/11. Following acute lytic replication in the respiratory tract after an intranasal infection, MHV68 establishes latency in B cells and macrophages of secondary lymphoid tissues (26, 64, 476). Spleens from infected mice were harvested 16 dpi and levels of viral latency were determined using a limiting-dilution, nested PCR assay to enumerate the frequency of splenocytes that harbor viral DNA. We found that the absence of caspase-1/11 had no effect on the establishment of latency in the spleen **(Fig. 3.2A)**. Further, caspase-1/11 deficiency did not impact reactivation from latency in intact cells **(Fig. 3.2B, solid lines)** or the low-levels of productive replication detected in disrupted cells **(Fig. 3.2B, dotted lines)** as assessed using a limiting-dilution explant reactivation assay. We evaluated long-term latency at 42 dpi and found no significant change in the maintenance of latency in *Caspase*-1/11 -/- splenocytes **(Fig. 3.2C)**. After intraperitoneal infection, the absence of caspase-1 and -11 did not alter latency **(Fig. 3.2D)** or reactivation **(Fig. 3.2E)** from peritoneal exudate cells, a compartment rich in the macrophage reservoir of latency (64, 476). These data indicate that MHV68 pathogenesis was not enhanced in the absence of caspase-1/11 during the course of lytic and latent infection *in vivo*.

The adaptive immune response to MHV68 infection is not altered by the absence of caspase-1/11. Detection of invading pathogens by cells and the subsequent release of chemokines and cytokines recruits numerous immune cells to the site of infection. IL-1β and IL-18 are critical activators of inflammatory responses by macrophages and NK cells, respectively,

and promote clearance of many bacterial and viral pathogens (504-506). Given that the absence of caspase-1/11 prevents secretion of IL-1β and IL-18, we next examined the immune response in the lungs and spleen at various times post-infection. We measured the level of $CD8^+$ T cells specific to the p79 epitope of ORF61, a dominant antigen during MHV68 infection (206), in the lungs at 10 dpi and found that WT and *Caspase*-1/11 -/- mice had similar frequencies **(Table 3.1)** and similar numbers **(data not shown)** of virus-specific $CD8^+$ T cells.

Examination of B and T cell populations in the spleen at 16 dpi revealed no changes in the total number **(data not shown)** or proportion of B and T cells and their activation **status (Table 3.1)**. $CD4^+$ and $CD8^+$ T cells were recruited and activated to the same extent, and virus-specific p79 $CD8^+$ T cells were efficiently expanded in *Caspase*-1/11 -/- mice. T cells expressing Vβ4 T-cell receptors, a hallmark of MHV68 infection of C57BL/6 mice (39, 218), were enriched in *Caspase*-1/11 -/- mice to levels comparable in WT mice. These data demonstrate that the absence of caspase-1 and 11 does not influence the adaptive immune response against MHV68, which is consistent with our data indicating that deletion of *Caspase*-1/11 does not enhance MHV68 pathogenesis *in vivo*. Given the importance of caspase-1/11 in mediating innate immune responses to other intracellular pathogens (104, 504-506), we hypothesized that WT MHV68 is refractory to caspase-1/11-dependendent inflammasome functions *in vivo* due to a subversion of either the detection or the effector responses of the host.

MHV68 infection does not induce caspase-1 cleavage and IL-1β production. Given our findings that caspase-1/11 do not contribute to the immune response to MHV68 *in vivo,* we next investigated the inflammasome response to MHV68 infection in primary WT BMDMs. Macrophages express a wide range of innate immune sensors, making them ideal for dissecting

viral interactions with intrinsic and innate host defenses. Inflammasome activation upon infection with MHV68-H2b-YFP was assessed by procaspase-1 cleavage and IL-1β release over a 24 hr timecourse of infection (MOI 10). Despite infection levels reaching approximately 40% of cells **(data not shown)**, procaspase-1 cleavage was not observed by western blot at any time point **(Fig. 3.3A)**. The secretion of IL-1β was also not detected in the conditioned media of infected BMDMs, as determined by ELISA **(Fig. 3.3A)**. The lack of response to infection contrasted sharply with robust caspase-1 cleavage and IL-1β release upon stimulation with LPS and ATP, which are classic stimuli of the NLRP3 inflammasome in BMDMs **(Fig. 3.3A)**. We confirmed that BMDMs responded to the foreign DNA ligands CpG and poly(dA:dT) **(Fig. 3.3B)** that bind the TLR9 and AIM2 sensors, respectively, that have been previously shown to detect herpesviruses (104, 507, 508).

Having found that *de novo* infection of BMDMs with MHV68 did not trigger an inflammasome response, we sought to determine whether MHV68 infection hindered inflammasome activation by extrinsic stimuli. Inflammasome function is dependent on the activation of two distinct pathways (509, 510). A first signal is typically TLR activation by PAMPs such as LPS to induce the production of proforms of IL-1β and IL-18. The second signal is the detection of conserved pathogen- or damage-associated molecular patterns, such as ATP, by sensors such as NALP1, NLRP3, AIM2, or IFI16. This drives the assembly of the multi-protein inflammasome complex to mediate the cleavage of procaspase-1 and subsequent processing of pro-inflammatory cytokines IL-1β and IL-18 (511, 512).

To determine if MHV68 infection impaired procaspase-1 or pro-IL-1β cleavage, intracellular levels of IL-1β and caspase-1 were examined in infected BMDMs treated with LPS

alone (signal 1 via TLR4) or primed with LPS (signal 1) followed by ATP (signal 2 via NLRP3) **(Fig. 3.3C)**. While levels of both pro and mature forms of IL-1β were reduced in infected macrophages, levels of cleaved caspase-1 were similar between infected and uninfected cells stimulated with LPS and ATP at 25 hpi **(Fig. 3.3D)**. In agreement with the reduction in intracellular IL-1β protein levels, infection with MHV68 significantly reduced IL-1β secretion upon extrinsic stimulation with LPS and ATP treatment **(Fig. 3.3E, left)**. We next examined IL-18, a cytokine that is also dependent on caspase-1 cleavage for secretion. IL-18 secretion in response to LPS and ATP was also reduced by MHV68 infection **(Fig. 3.3E, middle)**. TNF-α is an NF-κB-responsive cytokine that is not dependent on inflammasome activation for secretion. The levels of TNF-α produced in response to either LPS or LPS and ATP treatment were greatly reduced in the infected BMDMs as compared to uninfected cells **(Fig. 3.3E, right)**. These data indicate that infection of BMDMs reduces cytokine secretion in response to LPS and ATP stimulation, but this inhibition is not due to interference with caspase-1 activation.

Viral particle release initiates around 24 hpi **(Fig. 3.1C)**. We found that infection did not induce cell death, as indicated by no increase in lactate dehydrogenase release compared to uninfected cells **(Fig. 3.3F, left)**. However, upon treatment with either LPS alone or LPS and ATP there was significantly more cell death in the infected BMDMs than in the uninfected cells **(Fig. 3.3E, middle, right)**. Due to the increase in cell death observed at 25 hpi, we next examined if MHV68 exerted a repression on cytokine secretion at an earlier stage of infection.

Next, BMDMs were stimulated at 6 hpi and both secreted and intracellular levels of IL-1β, IL-18, and TNF-α were measured by ELISA at 9 hpi **(Fig. 3.4A)**. When MHV68-infected macrophages were examined, we observed a dramatic reduction in intracellular IL-1β levels in

LPS or LPS and ATP treated cells **(Fig. 3.4B, top left)**, and a reduction in secreted IL-1β in response to LPS and ATP **(Fig. 3.4B, bottom left)**. We observed a slight but significant reduction in intracellular IL-18 levels from infected cells that were LPS and ATP treated **(Fig. 3.4B, top middle)**, concomitant with the increase in the secretion of IL-18 **(Fig. 3.4B, bottom middle)**. At this earlier timepoint, infection did not alter intracellular or secreted TNF-α in response to either LPS or LPS and ATP **(Fig. 3.4B, right)**. While LPS and ATP treatment resulted in a significant increase in cell death, infection did not significantly affect cell death upon LPS treatment alone **(Fig. 3.4C)**. Taken together, MHV68 infection alone does not trigger inflammatory cytokine production. In addition, infection potently impairs IL-1β production by extrinsic LPS stimulation at 9 hpi. This inhibition is not attributed to an increase in cell death.

Inflammasome components and inflammasome activation can be reduced by type 1 interferon (IFN) signaling (513). We observed a potent interferon response based on upregulation of interferon-stimulated genes **(data not shown)**, consistent with a previous report of macrophage infection with MHV68 (140). To assess the role of paracrine IFN-β signaling in the repression of IL-1β, we infected IFNαβ receptor-/- BMDMs with MHV68 and evaluated IL-1β production and secretion after LPS stimulation. MHV68 infection reduced IL-1β levels upon extrinsic stimulation even in the absence of the IFNαβ receptor **(Fig. 3.4D)**. This indicates that MHV68 infection inhibits IL-1β production independent of type 1 interferon signaling.

MHV68 impairs IL-1β production induced by *Salmonella* Typhimurium. Having found that MHV68 inhibits IL-1β production induced by LPS and ATP treatment **(Fig. 3.4)**, we next examined whether MHV68 infection could impair inflammasome induction through multiple

sensors. The bacterial pathogen *Salmonella enterica* Serovar Typhimurium triggers inflammasome responses via flagellin sensing by NLRC4 and LPS sensing by NLRP3 (101, 514). To determine the effect of MHV68 infection on the inflammasome response to *S.* Typhimurium, BMDMs were infected with MHV68 at an MOI of 10 for 7 hr prior to infection with *S.* Typhimurium at MOI of 1 or 4. Cytokine secretion was measured after 2 hrs of coinfection **(Fig. 3.5A)**. In the absence of MHV68, we observed a dose-dependent increase in the inflammasome response upon infection with *S.* Typhimurium, as indicated by elevated levels of both the intracellular and secreted IL-1β **(Fig. 3.5B)**. MHV68 infection significantly reduced both intracellular production and secretion of IL-1β induced by secondary *S.* Typhimurium infection **(Fig. 3.5B)**. TNF-α secretion in response to *S.* Typhimurium was not changed by MHV68 infection **(data not shown)**, suggesting this phenomenon is not common to all NF-κB-driven genes. Under the growth conditions used to prepare the bacteria, *S.* Typhimurium infection of BMDMs is known to trigger inflammasome activation and a type of caspase-1-dependent cell death termed pyroptosis (515, 516). Cell viability was slightly reduced upon co-infection at an MOI of 1, but no difference was detected between cells infected with the higher MOI of 4.0 **(Fig. 3.5C)**. We examined if MHV68 reduced the infectivity and survival of *S.* Typhimurium that might, in turn, reduce inflammasome activation. MHV68 infection increased invasion at 1 MOI, but did not impair *S*. Typhimurium invasion at 4 MOI **(Fig. 3.5D)**. Thus, the block in IL-1β production by MHV68 extends beyond extrinsic stimulation with LPS to a secondary bacterial infection.

MHV68 infection reduces IL-1β transcripts. Having observed the specific decrease in IL-1β production and secretion in MHV68 infected BMDMs after extrinsic stimulation, we examined

levels of the pro- and mature forms of inflammasome components by immunoblot. Levels of procaspase-1 and proIL-18 proteins did not change upon infection at 9 hpi **(Fig 3.6A)**. In addition, there was no difference in cleavage of the proforms of these inflammasome components between infected and uninfected cells upon LPS and ATP treatment. However, as seen for 25 hpi, infection reduced the levels of both pro and mature IL-1β compared to uninfected cells treated with LPS and ATP **(Fig. 3.6A)**. The approximate 2.5-fold reduction in proIL-1β occurred even upon stimulation with only the first priming signal, LPS. Thus, the virus does not impair NLRP3 inflammasome activation, but instead reduces the production of proIL-1β. We assessed the changes in transcript levels of *Il1b, caspase1*, *il18*, or *tnfa* in the infected cells upon LPS stimulation. MHV68 infection led to an approximate 3.5-fold reduction of *Il1b* transcripts in response to LPS stimulation, while the transcript levels of caspase1 and the other NF-kB responsive cytokines *il18* and *tnfa* were not changed by MHV68 infection **(Fig. 3.6B)**. Thus, MHV68 infection interferes with inflammasome responses in part by impairing the induction of *Il1b* transcripts.

MHV68 repression of IL-1β upon extrinsic stimulation is independent of the viral transactivator RTA. Having observed that MHV68 infection impairs IL-1β transcript levels, we sought to identify what aspect of viral infection mediated this reduction. To determine whether tegument protein was responsible, we prevented *de novo* gene expression by UV inactivating MHV68 prior to infection. MHV68 that was UV inactivated by six orders of magnitude did not trigger caspase-1 cleavage and failed to inhibit LPS-induced IL-1β production **(data not shown)**. This data is consistent with a role for some component of the virion tegument in this blockade. However, the levels of tegument protein ORF75C delivered to the cell were also reduced upon

UV treatment **(data not shown)**, leaving open the possibility that the virions may have been damaged such that less virus entered the cell. Thus, we used a genetic approach to identify the class of viral genes that might contribute to this blockade of intrinsic activation. MHV68 gene *Orf50* encodes RTA, a conserved immediate-early viral transactivator that is necessary and sufficient for early viral gene expression (496, 517-520). Mutations in MHV68 *orf50* severely limit viral gene expression and block virus replication (496, 521). RTA has additional functions in host signaling pathways, including the degradation of NF-κB signaling proteins, impairment of cytokine production, and interference with TLR signaling (408, 409, 522). To examine if RTA or RTA-dependent gene expression drives suppression of IL-1β, we infected BMDMs with a recombinant MHV68-ORF50stop virus (496) prior to LPS and ATP treatment and evaluated the intracellular and secreted levels of IL-1β at 9 hpi. BMDMs infected with MHV68-ORF50stop had a reduction in intracellular and secreted IL-1β upon stimulation with LPS alone or in combination with ATP, respectively, as observed with WT infection **(Fig. 3.7A)**. TNF-α secretion was not reduced in cultures infected with either WT or MHV68-ORF50stop virus **(data not shown)**. Levels of the tegument protein ORF75C delivered to infected BMDMs were similar between WT and ORF50stop virus at 4 hpi, prior to immediate-early gene expression by MHV68 **(Fig. 3.7B, left)** (523). By 9 hpi, there were increased levels of ORF75C in WT infected BMDMs as compared to ORF50stop infected cells **(Fig. 3.7B, right)**, consistent with *de novo* gene expression driven by RTA transactivation. Expression of the immediate early gene LANA was ORF50 independent since it was observed in both WT and ORF50stop virus infected cells. These data indicate that RTA does not directly block IL-1β expression, nor is the expression of an RTA-dependent factor responsible. This function is likely mediated by a tegument factor or a newly

expressed viral or virus-induced host factor that is independent of the major lytic gene transactivator RTA.

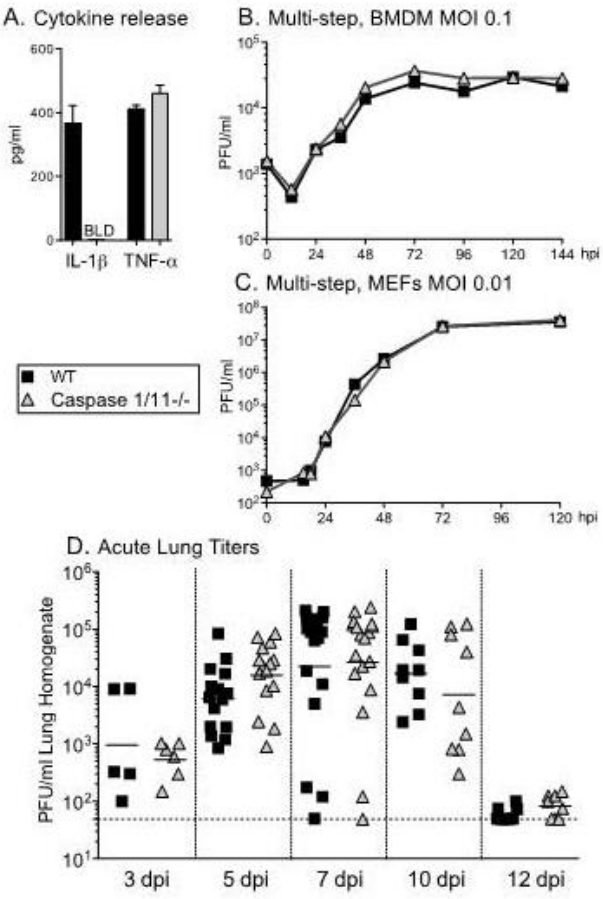

Figure 3.1: Loss of caspase-1/11 does not impact acute replication of MHV68 in mice. (A) WT and *Caspase*-1/11-/- BMDMs were treated for 2 hrs with LPS followed by 1 hr with both LPS and ATP. Cytokine release was measured by ELISA. (B) Multi-step growth curve in WT and *Caspase*-1/11-/- BMDMs upon infection with MHV68-eYFP at a low MOI (0.1 PFU/cell). (C) Multi-step growth curve in WT and *Caspase*-1/11-/- MEFs upon infection with MHV68-eYFP at a low MOI (0.1 PFU/cell). At the indicated times post infection, viral titer was assayed by plaque assay. Symbols represent mean +/- SD of triplicate samples. (D) C57BL/6 WT or *Caspase*-1/11-/- mice were infected with 1000 PFU of WT MHV68 via the intranasal route. At the indicated days post infection, lungs were removed, homogenized, and titered by plaque assay.

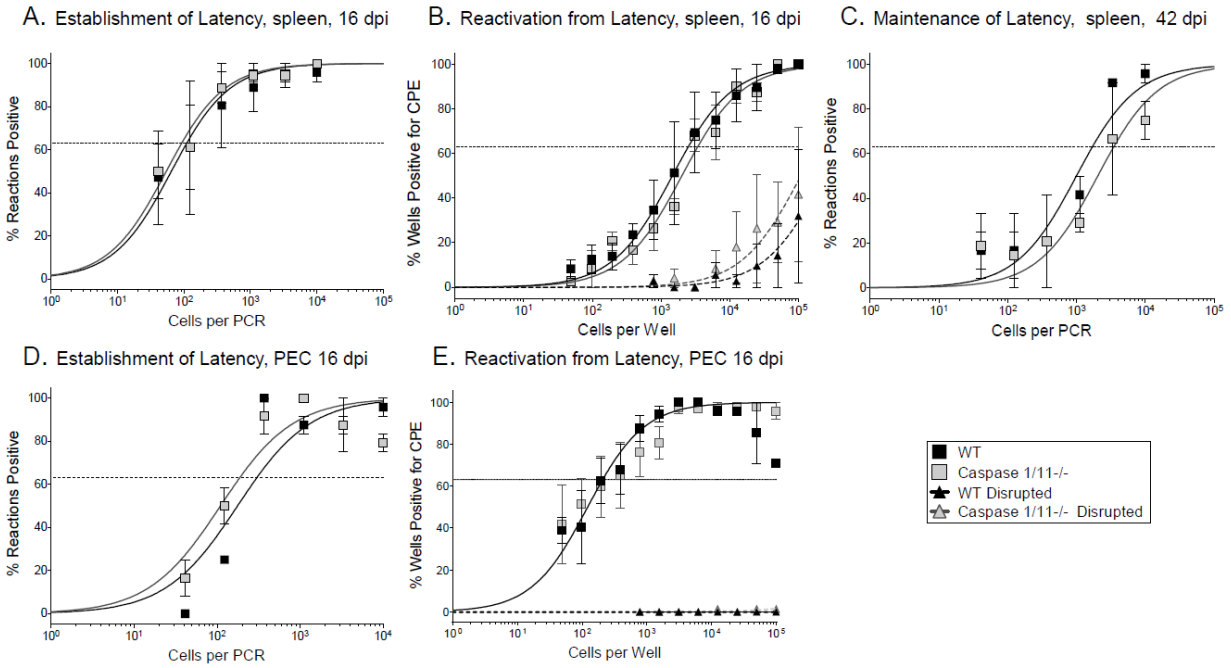

Figure 3.2: Loss of caspase-1/11 does not impact latency and reactivation of MHV68 in mice. C57BL/6 WT or *Caspase*-1/11-/- mice were infected with 1000 PFU of WT MHV68 via the intranasal route. (A) Spleens were harvested at 16 dpi. Latency was analyzed using a limiting dilution nested PCR assay. (B) Reactivation from latency was analyzed using a limiting dilution *ex vivo* coculture assay. Dotted lines represent disrupted splenocytes used to measure preformed infectious virus. (C) Spleens were harvested at 42 dpi. Latency was analyzed using a limiting dilution nested PCR assay. (D) WT or *Caspase*-1/11-/- mice were infected with 1000 PFU of WT MHV68 via the intraperitoneal route. At 16 dpi, the peritoneum was flushed with DMEM the frequency of genome-positive cells was analyzed using a limiting dilution nested PCR assay. (E) Reactivation from latency was analyzed using a limiting dilution *ex vivo* coculture assay. Dotted lines represent disrupted splenocytes used to measure preformed infectious virus. Graphs represent two to three independent experiments of 3–5 mice.

Table 3.1: Immune Response to MHV68 infection of *Caspase*-1/11$^{-/-}$ and WT mice.

	Mean (SD) %[a]			
	Caspase-1/11 -/- Naïve	*Caspase*-1/11 -/- Infected	WT Naïve	WT Infected
Lung, 10 dpi				
p79+ T cell[b]	1.1 (0.2)	4.1 (2.0)	1.2 (0.9)	3.5 (1.1)
Spleen, 16 dpi				
B cell[c]	40.5 (10.5)	43.8 (7.2)	46.7 (8.6)	46.3 (9.8)
Activated B cell[c]	1.7 (0.3)	4.5 (1.8)	1.6 (0.2)	6.1 (1.9)
CD4+ T cell[d]	18.6 (1.1)	11.9 (1.6)	15.9 (3.5)	11.0 (1.6)
Effector CD4+ T[d]	14.5 (0.6)	23.0 (3.3)	16.5 (1.7)	26.3 (5.8)
CD8+ T cell[d]	13.6 (0.3)	14.7 (2.0)	12.5 (1.2)	13.4 (3.1)
Effector CD8+ T[d]	8.0 (1.0)	33.3 (6.7)	7.5 (1.3)	30.9 (8.2)
p79+ CD8+ T cell[b]	0.38 (0.1)	8.4 (3.4)	0.74 (0.3)	3.9 (1.9)
Spleen, 42 dpi				
B cell[c]	41.5 (0.6)	38.7 (5.2)	42.6 (3.3)	38.2 (1.7)
Activated B cell[c]	1.8 (0.5)	1.0 (0.2)	1.3 (0.1)	0.91 (0.1)
CD4+ T cell[d]	17.5 (1.1)	17.8 (2.4)	18.5 (1.4)	16.4 (2.3)
Effector CD4+ T[d]	17.8 (2.4)	17.2 (2.4)	15.4 (1.7)	16.4 (6.4)
CD8+ T cell[d]	13.9 (1.8)	22.4 (2.8)	15.2 (1.9)	19.3 (5.6)
Effector CD8+ T[d]	7.1 (0.5)	43.2 (6.3)	9.0 (1.3)	41 (5.8)
p79+ CD8+ T cell[b]	0.28 (0.1)	2.7 (0.7)	0.36 (0.1)	2.3 (1.1)
Vβ4+ CD8+ T cell[e]	7.7 (2.5)	33.8 (9.4)	6.0 (0.4)	30.5 (10.2)

[a], The data shown are the percentage +/- the standard deviation of values obtained by FACS analysis of individual infected mice. 3-5 mice in 1-2 experiments were analyzed per time point. No significant differences were observed for any population measured.

[b], p79 tetramer CD8$^+$ T cell surface markers: CD4, CD8, CD62L, p79 tetramer

[c], Activated B cell surface markers: CD19, CD69

[d], Effector T cell surface markers: CD4, CD8, CD62L, CD44

[e], Vβ4 T cell surface markers: CD3, CD8, CD62L, Vβ4

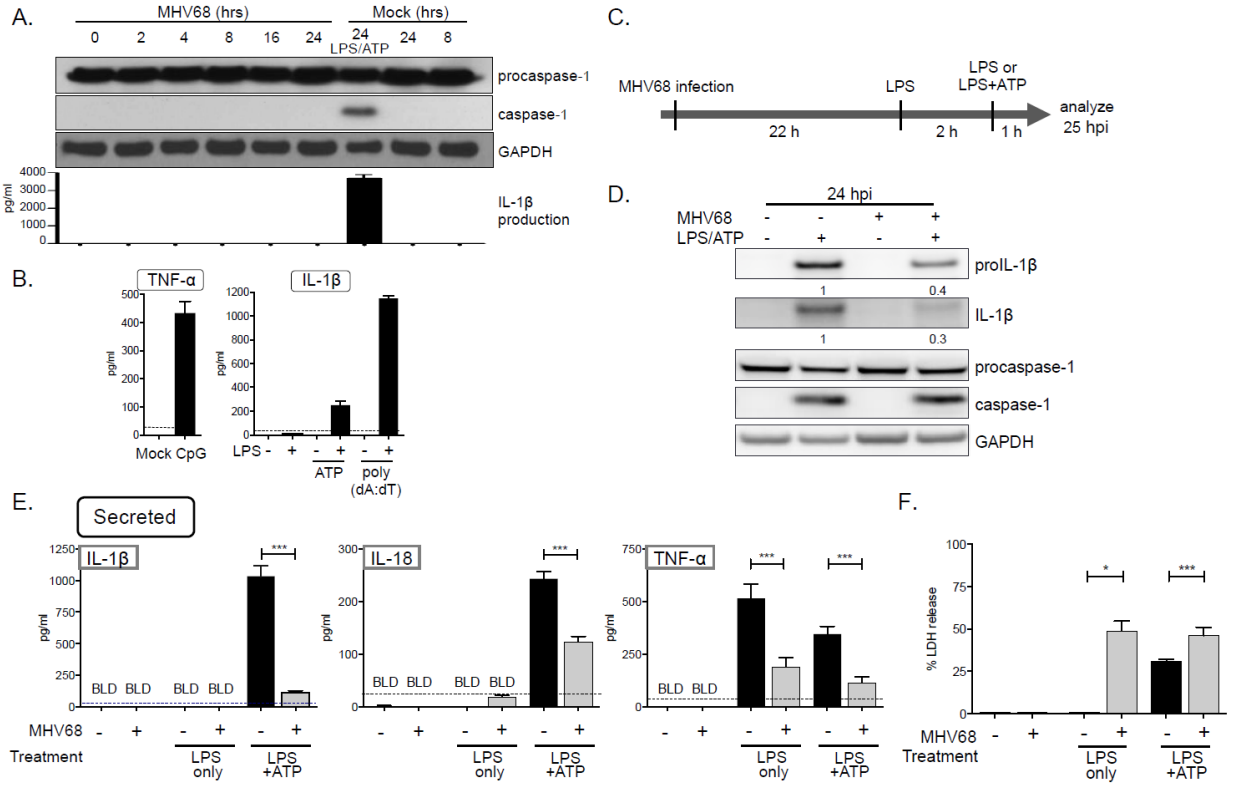

Figure 3.3: MHV68 impairs inflammatory response in BMDMs. (A) WT C57BL/6 BMDMs were infected with WT MHV68 at an MOI of 10. For each panel, procaspase-1 and caspase-1 levels were detected by immunoblot and secreted IL-1β was measured by ELISA. BMDMs treated for 2 hrs with LPS followed by 1 hr with both LPS and ATP served as a positive control for caspase-1 cleavage and IL-1β secretion ($p<0.0001$ for LPS and ATP compared to untreated mock control). GAPDH was used as a loading control. (B) WT BMDMs were treated with CpG DNA for 16 h and TNF-α secretion was measured by ELISA. For detection of DNA-induced inflammasome activation, WT BMDMs were treated for 4 h with LPS and then transfected with poly(dA:dT) for 6 hrs and IL-1β secretion was measured by ELISA. (C) Infection and stimulation schematic describing infection of WT C57BL/6 BMDMs at an MOI of 10 for 22 hrs, followed by 2 hrs LPS and an additional 1 hr with LPS alone or LPS and ATP. (D) Proform and mature cleaved forms of the indicated proteins at 25 hpi were detected by immunoblot and normalized to the GAPDH loading control. Values are the protein levels relative to the infected cultures treated with LPS and ATP. (E) Secreted cytokine levels were determined by ELISA. (F) Cell death was monitored by LDH release into conditioned medium relative to the freeze-thawed samples. Bars represent mean +/- SD of triplicate samples. Dashed line represents the limit of detection. BLD, below limit of detection. * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$.
Data in this figure was generated by Qiwen Dong and included with permission.

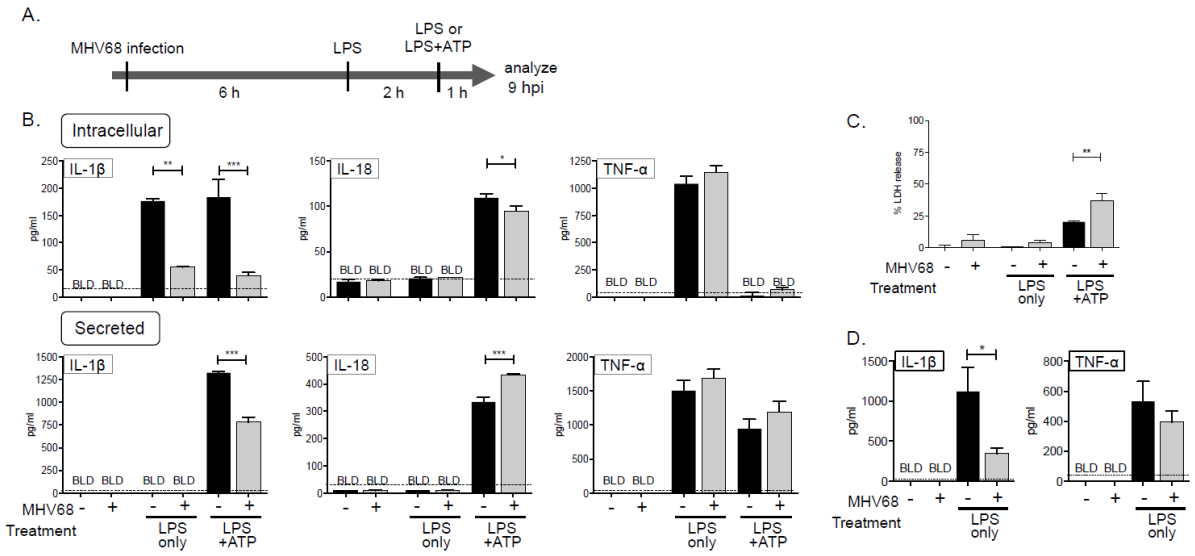

Figure 3.4: MHV68 infection specifically impairs IL-1β production upon LPS and ATP stimulation and is independent of the type I IFN response. (A) Infection and stimulation schematic describing infection of WT C57BL/6 BMDMs at an MOI of 10 for 6 hrs, followed by treatment with LPS for 2 hrs and LPS alone or LPS and ATP for an additional 1 hr. (B) Levels of intracellular (top panels) or secreted (bottom panels) cytokines were measured by ELISA. (C) Cell death at 9 hpi was monitored by LDH release into conditioned medium relative to freeze-thawed samples. (D) IFNαβR-/- BMDMs were infected with MHV68 at an MOI of 10, followed by the treatment as described above. Intracellular levels of the indicated cytokines were measured by ELISA. Bars represent mean +/- SD of triplicate samples. Dashed line represents the limit of detection. BLD, below limit of detection. * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$.
Data in this figure was generated by Qiwen Dong and included with permission.

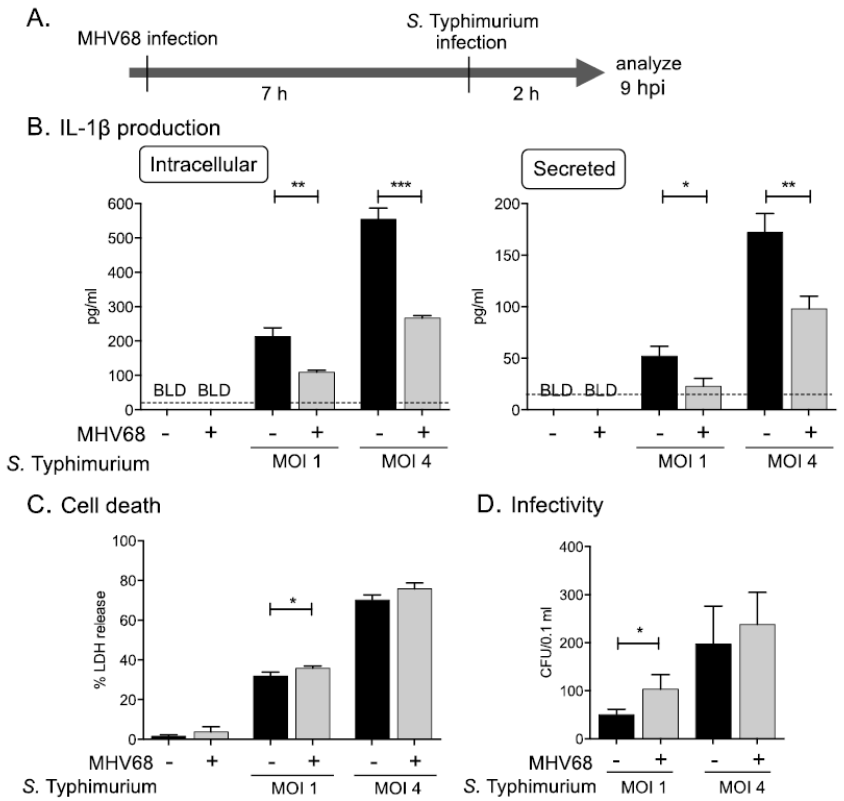

Figure 3.5: MHV68 impairs IL-1β production induced by *S.* Typhimurium co-infection. (A) Infection and stimulation schematic describing infection of WT C57BL/6 BMDMs at an MOI of 10 for 7 hrs, followed by *S.* Typhimurium infection at MOI of 1 or 4 CFU/cell for 2 hrs. (B) Intracellular levels (left panels) or secreted levels (right panels) of IL-1β were analyzed by ELISA. (C) Cell death was monitored by LDH release into conditioned medium relative to the freeze-thawed samples. (D) Bacterial invasion was determined by plating then counting CFUs. Bars represent mean +/- SD of triplicate samples. Dashed line represents the limit of detection. BLD, below limit of detection. * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$.
Data in this figure was generated by Qiwen Dong and included with permission.

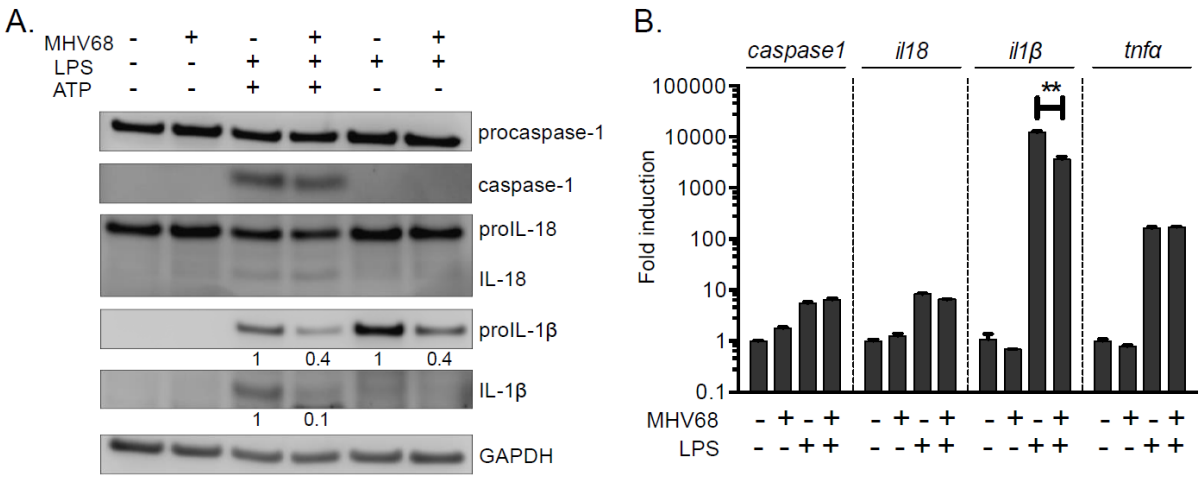

Figure 3.6: MHV68 infection impairs IL-1β at the transcript level. WT C57BL/6 BMDMs were infected with MHV68 at an MOI of 10 for 6 hrs, followed by treatment with LPS for 2 hrs and LPS alone or LPS and ATP for an additional 1 hr. (A) Proform and mature cleaved forms of the indicated proteins at 9 hpi were detected by immunoblot and normalized by the GAPDH loading control. Values are the protein levels relative to either the uninfected cultures treated with LPS and ATP or LPS alone. (B) Transcripts of inflammasome components were analyzed by RT-qPCR and ΔΔ*CT*. HPRT served as the housekeeping gene for normalization. ** = $p<0.01$; *** = $p<0.001$.
Data in this figure was generated by Qiwen Dong and included with permission.

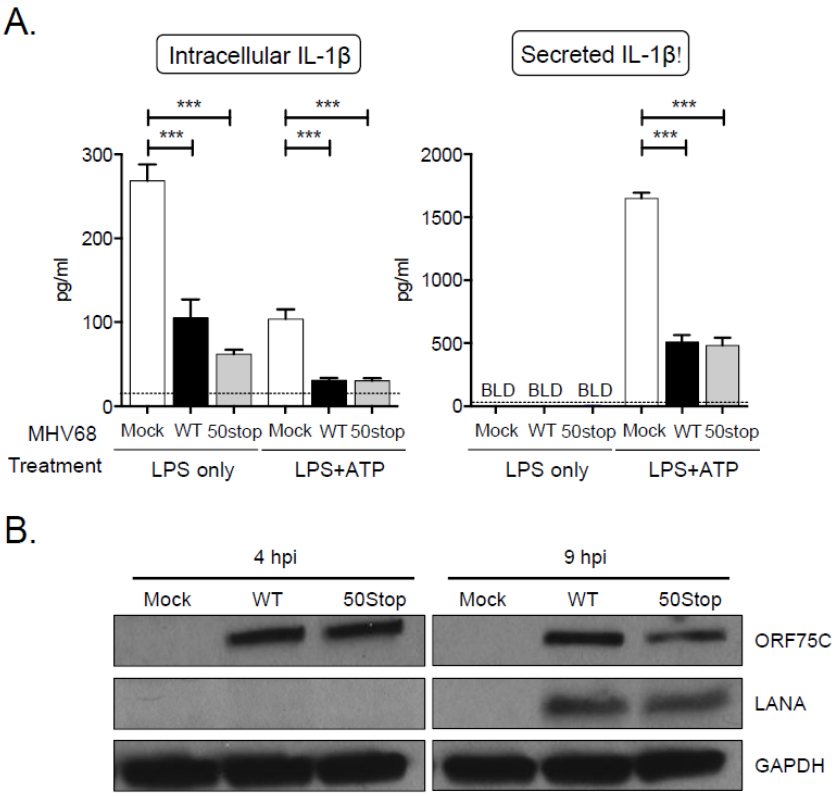

Figure 3.7: MHV68 impairment of IL-1β is independent of ORF50 function. (A) WT C57BL/6 BMDMs were infected with WT MHV68 or MHV68-ORF50stop at an MOI of 10 for 6 h, followed by treatment with LPS for 2 hrs and LPS alone or LPS and ATP for an additional 1 hr. Intracellular levels (left panels) or secreted levels (right panels) of IL-1β were analyzed by ELISA. Bars represent mean +/- SD of triplicate samples. Dashed line represents the limit of detection. BLD, below limit of detection. ** = $p<0.01$; *** = $p<0.001$. (B) BMDMs were infected with WT MHV68 or MHV68-ORF50stop at an MOI of 10 and expression of the indicated viral proteins at 4 hpi and 9 hpi were detected by immunoblot. GAPDH used as a loading control.
Data in this figure was generated by Qiwen Dong and included with permission.

DISCUSSION

In this study we examined how impairment of the inflammasome impacts gammaherpesvirus pathogenesis and, in turn, how a gammaherpesvirus impacts the inflammatory response of the host. We determined that the absence of caspase-1/11 does not impact virus replication or pathogenesis of WT MHV68 in mice. Consistent with this observation, the inflammasome was not activated upon *de novo* infection of BMDMs. MHV68 infection of BMDMs repressed IL-1β production upon extrinsic activation by LPS and ATP or co-infection with *Salmonella* typhimurium. The inhibition of IL-1β occurred at the transcript level early during infection and was independent of the viral immediate-early transactivator protein RTA. Our findings indicate that MHV68 evades inflammasome activation in BMDMs and impairs inflammatory cytokine responses to extrinsic stimuli, likely contributing to immune evasion during the initial stages of gammaherpesvirus infection.

During EBV infection, activated caspase-1 cleaves BPLF1, a viral ubiquitin and NEDD8-specific deconjugase, to promote BPLF1 nuclear localization and facilitate DNA replication (503). However, MHV68 replication in primary fibroblast or BMDM cells isolated from *Caspase*-1/11 -/- mice was not impaired by the absence of caspase-1 **(Fig. 3.1)**. Lung tissue is more restrictive for the replication of mutant viruses that are only slightly attenuated for replication in culture (524, 525). However, the kinetics and peak level of MHV68 replication were not changed in the lungs of mice lacking caspase-1/11 **(Fig. 3.1)**. While these data do not rule out a possible decrease in replication that is counter-balanced by a loss of immune control, the absence of any change in replication in primary fibroblasts or BMDMs suggests that caspase-1 does not influence virus replication at the cellular level.

Acute MHV68 replication in the lungs of mice is enhanced in mice lacking either NF-κB signaling components or interferon responses (155, 334, 409), including the cGAS cytoplasmic DNA sensor, which typically activates IRF3 signaling (526). The inflammasome is a powerful proinflammatory signaling pathway that restricts multiple pathogens (104, 504-506). During the preparation of this manuscript, it was reported that mice lacking the inflammasome components NLRP3 or ASC maintain higher levels of MHV68 viral DNA in the spleen during late infection (105). However our studies found that mice lacking the inflammasome effector caspase-1/11 maintained levels of MHV68 similar to WT **(Fig. 3.2)**. This discrepancy may stem from inflammasome-independent functions for the upstream molecule NLRP3 and ASC. Activation of ASC, also known as TMS1, has been implicated in the induction of cell death (527). The loss of ASC could dysregulate apoptosis in response to infection and lead to an increase in viral load. A distinction between the pathogenesis studies in the *NLRP3* -/- and *ASC* -/- mice reported by Sun et al. (105) compared to the *Caspase*-1/11 -/- pathogenesis data reported here is the methodology for measuring levels of virus infection. Sun et al. (105) quantified total viral DNA in disrupted spleen tissues wherein an increase in viral load might involve an increase in viral episome copy number per cell or a slight increase in lytic replication. Here, the *Caspase*-1/11 -/- studies measured the frequency of intact splenocyte and peritoneal exudate cells that harbor the viral genome and reactivate virus. Changes in the copy number of viral genomes per cell will not skew the frequency determination, and preformed infectious virus generated by lytic productive infection is monitored in the associated LDA assay and, in these experiments, was not a contributing factor. Regardless, these phenotypic differences point to possibly subtly

different roles for upstream inflammasome components, the sensor NLRP3 and the adaptor ASC, compared to the executioner caspase-1 and 11 that require further investigation.

Having observed no significant change in pathogenesis in mice lacking caspase-1/11, we examined MHV68 inhibition of inflammasome responses *in vitro.* MHV68 infection did not trigger inflammasome activation as evidenced by lack of caspase-1 cleavage upon infection of BMDMs at 10 MOI **(Fig. 3.3)**. This data agrees with a recent report that an extremely high MOI of 100-1000 is required to see levels of inflammatory cytokine production by MHV68 that still do not approximate the robust induction by HSV-1 and MCMV at much lower MOI of 3 and 1, respectively (105). It is possible that the herpesvirus capsid shields the viral DNA from detection by intracellular DNA sensors in the cytoplasm that are present in specific cell-types. We have previously found that APOBEC3s, which are intrinsic antiviral host factors that deaminate foreign DNA to mediate viral restriction, only impair MHV68 replication initiated from naked DNA, not an intact virion, suggesting that the viral DNA remains protected by the capsid during MHV68 infection (528). Indeed, the HSV-1 capsid has been reported to be ubiquitinated and degraded en route to the nucleus, exposing the viral DNA for recognition by IFI16 (529). Interestingly, mutation of the MHV68 tegument protein ORF64 deubiquinase leads to the mislocalization of viral DNA and augments type 1 interferon and IL-1β secretion in dendritic cells (105), and this phenotype is lost in cells lacking the cytosolic DNA sensor STING (105). Gammaherpesviruses can also directly inhibit inflammasome activation. KSHV encodes ORF63, a tegument protein that impairs NLRP1 and NLRP3 inflammasome activation (15, 17). Further investigation of the roles of tegument proteins in counteracting inflammasome activation

during gammaherpesvirus infection is critical to fully understand how gammaherpesviruses evade and subvert the innate immune system.

The down-regulation of genes for costimulatory signaling molecules and inflammatory cytokines was reported for the THP-1 macrophage cell line latently infected with KSHV, but the mechanism remains undefined (530). In this study, we uncovered a novel outcome of MHV68 infection in LPS-stimulated primary macrophages; the reduction of *il1b* transcript levels **(Fig. 3.6)**. The lytic transactivator RTA has been reported to inhibit multiple cell signaling pathways, including the TLR, MAVS, and NF-κB signaling pathways (408, 409, 522). However, the reduction in IL-1β upon infection did not require RTA or RTA-dependent early and late viral genes. Interestingly, LPS-induction of *il18* and *tnfa* mRNA levels was not impacted by infection.Taken together, MHV68 does not globally repress the TLR4/MyD88/NF-κB axis early during BMDM infection, but instead impairs IL-1β mRNA levels via a different mechanism driven by a virion component or RTA-independent immediate early gene

While *il1b* and *il18* transcripts are both induced and stabilized by LPS treatment (531-533), *il1b* mRNA, but not *il18* mRNA, contains an AU-rich destabilization element in the 3' UTR (534). Like KSHV, MHV68 encodes an alkaline nuclease (ORF37/vSox) that mediates host shutoff and influences virion protein composition (141, 535, 536). vSox may play a role in the general repression of cytokines observed late during infection (Fig. 3), but a mechanism by which vSox would selectively target *IL*-1β mRNA, but not *IL-18* and *TNF-α* mRNA, early after infection is unclear. Loss-of-function screens coupled with transcriptome profiles will likely reveal several layers of viral interference with host sensing and innate immune effector responses such as inflammatory cytokine production.

Gammaherpesvirus infection must avoid clearance by both the adaptive and innate immune systems. To reach the lymphoid reservoir of latency, gammaherpesviruses infect multiple cell types, including dendritic cells and macrophages (58, 66). Some of the mechanisms by which the gammaherpesviruses impair and subvert the inflammatory response have only recently come to light, and the specific inhibition of *Il1b* mRNA in response to extrinsic LPS stimulation and bacterial co-infection reveals a novel mechanism of viral interference with inflammatory signaling in a reservoir of infection in the host. Gaining a better understanding of the interplay of gammaherpesviruses with the innate immune system will help identify molecules critical for viral evasion mechanisms that could be targeted to reduce the burden of gammaherpesvirus infection.

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Chapter 4. Evaluation of the role of IKKα-mediated non-canonical NF-κB signaling in gammaherpesvirus infection.

Abstract

During the course of infection, the gammaherpesviruses infect numerous cell types before establishing latency in germinal center B cells. However, the signaling events that determine the lytic or latent course of infection, as well as maintain latency or initiate reactivation, are not well understood. The NF-κB signaling pathway is highly activated during B cell maturation and in B cells undergoing the germinal center reaction. NF-κB signaling occurs via two distinct pathways; the canonical and non-canonical NF-κB signaling pathways. Canonical NF-κB signaling is activated in numerous cell types through cytokine receptors, toll-like receptors, and antigen receptors specifically in lymphocytes. Non-canonical NF-κB signaling is activated in a more restricted set of cells through cytokine receptor ligation. Activation of both of these pathways leads to cell survival and proliferation. Infection of B cells by the human gammaherpesviruses leads to constitutive NF-κB activation through the expression of viral proteins and miRNAs that mimic endogenous receptor signaling. While these molecules are important for infected cell survival *in vitro,* the role of NF-κB signaling *in vivo* is not understood. Previous work by our laboratory examined the role of the IKKβ-dependent canonical NF-κB signaling pathway in latency establishment and found that deletion of a canonical transcription factor p50 or virus-mediated expression of a dominant negative inhibitor of canonical signaling reduced viral latency and reactivation in B cells. Here, we sought to examine the role of IKKα-mediated non-canonical NF-κB signaling in latency establishment. In order to examine the role of non-canonical signaling in latency establishment, we generated a virus that expresses a dominant negative IKKα molecule that blocks signaling. We characterized our mutant viruses

and found they overexpressed both the IKKα transgene and YFP. We also found that MHV68 infection could impair non-canonical pathway activation independently of transgene expression. To examine the role of IKKα in latency maintenance, we generated mixed bone marrow chimeras with WT bone marrow and bone marrow that can be induced to delete IKKα after latency is established. While latency was established normally in our mixed bone marrow chimeras, a low level deletion of IKKα independent of tamoxifen treatment was associated with a loss of latency at later times post infection. Together these data indicate that IKKα is targeted by MHV68 during lytic infection and likely contributes to latent infection, but additional experiments are necessary to further understand the interactions between MHV68 and non-canonical NF-κB signaling.

Introduction

Gammaherpesviruses infect numerous cell types including epithelial and endothelial cells, macrophages, and B cells (21-23, 25, 26, 234, 476). Infections may result in lytic infection and production of infectious virus or quiescent latency. B cells are the predominant latency reservoir. The ability of the gammaherpesviruses to maintain a non-integrated viral genome (18) with highly circumscribed viral gene expression during latency (31, 232, 235, 243) allows them to evade detection by the immune system and persist for the life of the host. In the context of host immunosuppression, the control of viral latency and productive infection is impaired (197, 455), predisposing the host to the development of malignancies. Despite the importance of latency in the viral life cycle, the mechanisms that drive latency establishment in some cells types but not others, and the cellular signals that maintain latency or initiate reactivation are poorly understood. Better understanding of the host signaling pathways that contribute to the establishment and maintenance of a latent infection is critical for the development of therapeutics against infection and virus-associated malignancies.

The strict host tropism of the human gammaherpesviruses makes the study of the host determinants of EBV and KSHV latency difficult. Murine gammaherpesvirus (MHV68) infection of mice is used to test the contributions of viral and host factors in the establishment of latency. MHV68 is a natural pathogen of murid rodents that has genomic, biologic, and pathogenic parallels to the human gammaherpesviruses (8, 26, 451, 452). The MHV68 model pathogen system allows for the manipulation of both viral and host genes to examine how they contribute to latency establishment.

The canonical pathway and non-canonical pathway of host NF-κB signaling each play a critical role in the survival of B cells in the secondary lymphoid organs, especially during the germinal center reaction (279, 283, 400, 437, 442, 537). Inhibiting canonical NF-κB signaling in the infected cell through overexpression of the repressor IκBα does not impair lytic replication in the lung, but significantly reduces the levels of latency established in the spleen (333). In mixed bone marrow chimeras bearing both WT B cells and B cells lacking the canonical transcription factor p50, latency establishment is impaired in the $p50^{-/-}$ B cells (334). However, after the impairment of canonical signaling, latency was established, albeit at lower levels, suggesting other signals promote latency establishment. Non-canonical NF-κB signaling is highly active at multiple stages of B cell development, including entry into the germinal center (278, 279, 400, 434, 437, 441, 442). The non-canonical pathway is activated by a set of receptors that are expressed primarily on B cells (279), whereas the canonical receptors are expressed on most cell types (538). Mice lacking the BAFF receptor have severely reduced mature B cell populations and form transient germinal centers (278, 279). BAFFR-KO mice have a defect in supporting normal levels of MHV68 latency in the context of reduced B cell reservoirs (539). The importance of non-canonical signaling in the B cell reservoir suggested that it may represent a critical determinant for the establishment and maintenance of a latent infection. Thus, we sought to examine the role IKKα-mediated non-canonical NF-κB signaling on gammaherpesvirus pathogenesis.

We utilized multiple strategies to impair IKKα-dependent signaling during infection. First, we generated an MHV68-IKKαSA virus that expressed a dominant negative form of IKKα. Transgene expression did not affect lytic replication. In addition, genetic ablation of IKKα in

MEFs did not impact WT virus replication. Unexpectedly, non-canonical signaling was repressed independent of the IKKαSA transgene during lytic infection. Next, we generated mixed bone marrow (BM) chimeras with a B cell deficient bone marrow and a conditional deletion of IKKα in the hematopoietic lineage. Upon deletion of the floxed IKKα gene in B cells by a tamoxifen-inducible Cre recombinase, most mature populations of B cells survived at levels comparable to undeleted chimeras for up to 2 weeks. Next, we generated a second set of mixed bone marrow chimeras with both WT and conditional IKKα deletion in the B cell reservoir. Latency was established at normal levels, but we observed a significant loss of viral latency in both the WT and IKKα-deleted B cell subsets. These results suggest that MHV68 suppresses non-canonical NF-κB signaling during lytic infection. A determination of the role for non-canonical signaling in the establishment and maintenance of a latent infection *in vivo* will require additional experiments.

Materials and methods

Mice. Ly5.1+ C57BL/6 mice were purchased from Jackson Labs (Maine, USA) and bred at the Stony Brook Division of Lab Animal Resources. Mice bearing germline *CreER^{T2}/IKKα$^{fl/fl}$* on a C57BL/6 background were bred in our facility (540). μMT mice on a C57BL/6 background were purchased from Jackson Labs (Maine, USA) and maintained in our facility. 8-12 week old animals of mixed genders were used in groups of 3-7 for most experiments. Isoflurane was used for anesthesia to conduct infections, bone marrow transfer, and terminal harvests**.** Cheek vein punctures were used to acquire peripheral blood. The animal experiments described herein were approved by the Stony Brook University Institutional Animal Care and Use Committee.

Cell Culture. Low passage murine embryonic fibroblasts were cultured at 37^{o}C with 5% CO_2 in DMEM containing 10% FBS, 2 μM L-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin (10% cMEM). NIH murine 3T12 fibroblasts were cultured at 37°C with 5% CO_2 in DMEM containing 8% FBS, 2 μM L-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin (8% cMEM). Non-canonical pathway activation in MEFs was stimulated with 0.3μg/ml or 1 μg/ml αLTβR clone 5G11 (Abcam, Cambridge, MA, USA) for 5-18 h.

Virus and infections. For *in vivo* infection experiments, we used the recombinant reporter virus MHV68-H2bYFP that expresses YFP fused to the H2B protein from an intergenic locus (495). Virus stocks were concentrated to > 1 x 10^8 PFU/ml by centrifugation at 4°C for 120 min at 13,000 x g in a Dupont GSA rotor (Wilmigton, DE). For intranasal infection, mice were lightly anesthetized using isofluorane and infected with 1,000 plaque forming units (pfu) of virus in a

20 µl bolus of 10% cMEM applied to the nose. For viral growth curves, virus was diluted and cells were infected with a low volume of inoculate for 1 h. Cells were rocked every 15 min. After 1 h of infection, inoculate was removed, cells were washed once in PBS, and overlaid with 2ml of 8% CMEM. For other infections the inoculate was not removed.

MHV68-IKKαSA and MHV68-IKKαSTOP were generated using BAC-mediated recombination onto the WT MHV68 BAC (46, 47). First, the ECMV IRES was cloned out of pIRES (Clontech, Mountain View, CA, USA) into pEYFP-N1 30bp upstream of the eYFP. The pCMVIE-IRES-eYFP-SV40polyA sequence was excised using restriction digestion, blunted using the Klenow fragment of DNA polypmerase (NEB, Ipswitch, MA, USA), and blunt-end ligated into the TOPO-TA blunt vector (Thermo-Fisher Scientific, Waltham, MA, USA). Human IKKαSA was amplified from pBIP-IKKαSA (541) and ligated into the TOPO-TA vector upstream of the IRES. The complete pCMVIE-IKKαSA-IRES-YFP-SV40polyA was excised using NsiI PstI double restriction digestion, gel purified, and ligated into NsiI and PstI sites in pUC19 (NEB, Ipswitch, MA, USA). A kanamycin resistance cassette and I-Sce cut site was cloned into the IRES of our construct. The entire construct was amplified by PCR, transformed into *E. coli* containing the WT BAC, and carbenicillin/kanamycin resistant clones were selected. I-Sce expression was induced at 37^{o}C. Carbenicillin-resistant and kanamycin sensitive colonies were selected. BAC clones were sequenced using Illumina miSeq whole genome sequencing and assembled to the predicted genome sequence.

Next, this BAC was further modified to repair mutations in the IKKα coding sequence and modify the IRES and YFP reporter gene to improve poor YFP expression. First, a gBlock (Integrated DNA Technologies, Coralville, IA, USA) was synthesized with an extended IRES, a

sequence deletion between the IRES and eYFP, and an H2B coding sequence fused to YFP in addition to a kanamycin-resistance cassette and an I-Sce cut site. Mutants were generated by *en passant* recombination onto the IKKαSA BAC to generate the IKKαSA-IRES-H2BYFP BAC (referred to as IKKαSA). Next IKKα mutations were repaired by amplification of the IKKα gene from the BAC, site directed mutagenesis, followed by *en passant* recombination as described above. Next, to generate an IKKαSTOP virus, an all-frames STOP cassettes was inserted at bases 111 and 1310 of the IKKα gene using sequential site-directed mutagenesis of PCR products from the IKKαSA BAC followed by *en passant* recombination as described above. Repair of IKKα, insertion of STOP cassettes, extension of the IRES, and addition of H2BYFP were confirmed by Sanger sequencing. For restriction fragment length polymorphism (RFLP) analysis, BAC DNA was digested using HindIII, EcoRI, or BamHI and resolved on a 2% agarose gel at low voltage for 8 hours.

IKKα deletion in MEFs. MEFs generated from *CreER^{T2}/IKKα$^{fl/fl}$* mice were treated daily with 100 μM tamoxifen (Sigma-Aldrich, St. Louis, MO, USA) + 10% CMEM for 8 days.

Virus growth curve. To measure virus replication, subconfluent WT MEFs were infected with recombinant MHV68 viruses at an MOI of 0.05. At the indicated times post infection, triplicate wells of cells and conditioned medium were frozen at -80^{o}C. Cell homogenate was serially diluted and used for infection of NIH 3T12 murine fibroblasts for 1 hour with rocking every 15 min. Infected cells were overlaid with 1.5% methylcellulose in 5% CMEM. After 8 days the

methylcellulose was removed, cells were wasted once with PBS, fixed in methanol, and stained with 0.1% crystal violet stain in 10% methanol.

Immunoblotting. Stimulated or infected cells were lysed in RIPA buffer and quantified by Bradford assay (BioRad, Berkeley, CA) and protein was diluted RIPA Buffer (150 mM sodium chloride, 1.0% IGEPAL CA-630, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 50 mM Tris pH 8.0) supplemented with a protease inhibitor cocktail (Sigma, St. Louis MO) and PMSF) before boiling at 95^{o} C for 5 min. Proteins were separated on 10% SDS-PAGE and transferred to polyvinylidene fluoride membrane. Primary antibodies against IKKα (Cell Signaling Technology, Rabbit polyclonal, Danvers, MA, USA), p65 (Cell Signaling Technology, Clone C22B4), p100 (Cell Signaling Technology, Rabbit polyclonal), GAPDH (Sigma-Aldrich, Rabbit Polyclonal), and Lamin A/C (Cell Signaling Technology, Clone 4C11) were used. Detection was performed with HRP-conjugated anti-rabbit IgG or anti-mouse IgG (GE Healthcare, Buckinghamshire, UK), Data was captured by GE CCD camera and analyzed by ImageQuant software (v7.0, GE Healthcare).

Assessment of YFP expression. MEFs were infected with recombinant MHV68 viruses expressing H2BYFP at an MOI of 10. After 24 h, cells were washed with PBS and trypsinized for 10 min. Cells were resuspended in 500 µl of FACs Buffer (2% FBS in PBS) and analyzed by flow cytometry. Cells were gated on YFP+ signal and the MFI of YFP+ cells were measured.

Nuclear fractionation. Stimulated MEFs were washed in cold PBS and scraped into 1 ml cold PBS. Cells were pelleted by centrifugation at 600x g and resuspended in 300-500 µl of hypotonic

lysis buffer. Cells were incubated for 15 min on ice, then 5% volume of 10% NP-40 was added to cells. Cells were vortexed for 15 s then centrifuged at 10,000 xg for 10 min. Supernatant was stored as the cytoplasmic fraction. The remaining pellet was washed twice in 500 µl of hypotonic lysis buffer (10 mM HEPES pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, protease inhibitor cocktail (Sigma, St. Louis MO)), then resuspended in 50 µl of complete lysis buffer (Active Motif, Carlsbad, CA, USA). Pellets were shaken at 4^{o}C for 2 h, then centrifuged at 10,000x g for 10 min. The supernatant was saved as the nuclear fraction.

p52 ELISA. The Active Motif TransAM p52 ELISA kit was used to quantify p52 activation. 20 µg of nuclear extract was used for assays, which were carried out according to manufacturer's instructions.

Generation of bone marrow chimeras. Bone marrow recipients were given 2 doses of 475 rads 8 hours apart using a gamma-irradiator (GammaCell). The second dose of irradiation was given immediately before bone marrow transfer. Bone marrow was harvested from the tibias and femurs of donor mice. Pooled bone marrow from each genotype was resuspended in ACK lysis buffer for 5 minutes to remove red blood cells. Marrow was resuspended in 10% CMEM and counted. For µMT/AFTC chimeras, a preparation of 1.4 x 10^8 µMT and 0.6 x 10^8 AFTC bone marrow cells were prepared per ml. Recipient mice were lightly anesthetized with isofluorane and injected retroorbitally with 100 µl of bone marrow slurry (1.4 x 10^7 µMT and 0.6 x 10^7 AFTC cells) using a 28 gauge needle. For WT/AFTC chimeras, WT Ly5.1+ recipients were given 1.4 x 10^7 Ly5.1+ WT and 0.6 x 10^7 AFTC bone marrow cells under identical conditions. For 2 weeks

post transfer, chimeras were given fresh water with 12.5 μg/ml Polymixin B (Sigma-Aldrich) and 1mg/ml Neomycin (Sigma-Aldrich) every 3 days. Mice were allowed to drink *ad libitum.* From 2-8 weeks after infection, fresh water with 12.5 μg/ml Polymixin B and 12.5 μg/ml Polymixin B was given weekly. After 8 weeks, mice were no longer given antibiotics in their water.

Assessment of immune reconstitution. At 8 weeks post infection immune reconstitution was measured using peripheral blood. Mice were lightly anesthetized using isofluorane and 4-5 drops of blood were collected by cheek puncture with a small lancet. Bleeding was stopped with gauze and gentle pressure. Cells from μMT/AFTC mice were washed in PBS then Fc blocked with Trustain FcX (Clone 93, Biolegend, San Diego, CA) in FACs buffer for 20 min. Cells were pelleted by centrifugation and incubated with anti-CD19-PE (Biolegend) in FACs buffer for 20 min. Cells were washed twice in FACs buffer, then analyzed for CD19+ cells by flow cytometry. Cells from WT/AFTC chimeras were processed in a similar fashion and stained for anti-CD19-PE (Biolegend), anti-Ly5.2-AlexaFluor700 (Thermo-Fisher Scientific, Waltham, MA, USA), and anti-Ly5.1-Pacific Blue (Biolegend) in FACs buffer for 20min. Cells were washed twice in FACs buffer, then CD19+ cells were analyzed for Ly5.1+ and Ly5.2+.

B cell enrichment. Spleens were harvested from chimeras at the indicated days and homogenized into a single cell suspension. Splenocytes were ACK treated for 5 min to remove red blood cells, then resuspended in 10% CMEM and pelleted. Cells were resuspended in 90 μl of MACS buffer (PBS + 2% FBS + 5 mM EDTA) per 1 x 10^7 cells. 10 μl of anti-CD90.2 T cell positive selection beads (Miltenyi Biotec, Bergisch Gladbach, Germany) were added per 1 x 10^7

cells. Cells were incubated on ice for 15 min and shaken every 3 min prior to wash and preparation of a LS MACs column (Miltenyi Biotec, Bergisch Gladbach, Germany) in a magnet (Miltenyi Biotec, Bergisch Gladbach, Germany) per manufacturer's recommendations. B cells were collected in the run-through as the T-cell depleted fraction.

FACS sorting of B cells. Enriched B cells from WT/AFTC chimeras were counted and blocked with Trustain FcX (Biolegend) for 20min. Cells were washed in FACS buffer, pelleted, and stained with anti-CD19-PE (Biolegend), anti-ly5.2-Brilliant Violet 510 (Biolegend), and anti-Ly5.1-Alexa Fluor 700 (Thermo-Fisher Scientific) in sterile FACS buffer for 20min. Cells were washed twice in sterile FACS buffer. CD19+ B cells were FACS sorted into Ly5.1+ or Ly5.2+ populations using a FACS AriaIII (BD Biosciences).

RT-PCR to determine IKKα deletion. RNA from 2 x 10^6 enriched µMT/AFTC splenocytes or purified Ly5.1+ and Ly5.2+ B cells was column purified, DNAseI treated, then input into a Superscript III reverse transcription reaction (Thermo-Fisher Scientific). Products were treated with RNaseH, then 100 ng of RT product was used as a template for PCR with IKKα primers F-GAGTGACATAGGATCTGGGATC and R-CTCCAGTCATCTCTTCACATGC spanning the deleted exons 6 and 7, or HPRT (F-GTAATGATCAGTCAACGGGGGAC and R-CAGCAAGCTTGCAACCTTAACCA).

Flow Cytometry. For the analysis of B cells, 2x10^6 splenocytes were resuspended in 200 µl of FACS buffer (PBS with 2% Fetal Bovine Serum) and blocked with TruStain fcX (Biolegend). The cells were washed and stained to identify B cell subsets with fluorophore-conjugated antibodies

against CD19 (clone 6D5), B220 (clone RA3-682) CD138 (clone 281-2), CD95 (clone 15A7), CD21 (Clone 7E9), CD23 (Clone B3B4), AA4.1 (Clone AA4.1), or biotinylated antibodies against GL7 (clone GL-7) that were detected by secondary streptavidin-conjugated allophycocyanin. All antibodies were purchased from Biolegend or BD Biosciences (San Jose, CA). The data were collected using an LSRII (BD Biosciences) and analyzed using FlowJoX v10.0.7 (Treestar Inc, Ashland, OR).

Viral pathogenesis assays. For acute titers, mice were sacrificed with isofluorane at the indicated days post infection, and the left lung was removed and frozen at ¯80°C. Lungs were disrupted in 1 ml of 8% cMEM using 1 mm zirconia beads in a bead beater (Biospec, Bartlesville, OK). Serial dilutions in 10% cMEM were plated on subconfluent monolayers of NIH 3T12 murine fibroblasts in 6 well plates. Plates were rocked intermittently for 1 hr and then overlaid with 3 ml of 5% cMEM + 1.5% methylcellulose. Cells were fixed after 1 week, stained with crystal violet, and plaques were counted.

To analyze latently infected cells, mice were sacrificed with isofluorane at 16 or 42 dpi. Spleens were excised, homogenized, and resuspended in 10% cMEM. Peritoneal exudate cells were isolated by peritoneal injection of 10 ml of 10% cMEM, followed by agitation of the abdomen and withdrawal of the peritoneal wash by syringe. For quantitation of latency, limiting-dilution nested PCR with primers for the MHV68 ORF50 region was used to determine the frequency of virally infected cells as previously described (166). Briefly, frozen samples were thawed, resuspended in isotonic buffer, counted, and plated in serial 3-fold dilutions in a background of 10^4 NIH 3T12 murine fibroblasts into a 96-well plate. The resultant PCR products

were resolved on 2% agarose gels and each dilution was scored for amplimer of the expected sizes. Control wells containing uninfected cells or 10, 1, and 0.1 plasmid copies of ORF50 target sequence were run with each plate to ensure single-copy sensitivity and no false positives.

For quantitation of reactivation, a limiting-dilution reactivation assay was performed as previously described (166). Briefly, bulk splenocytes in 10% cMEM were plated in serial 2-fold dilutions (starting with 10^5 cells) onto MEF monolayers in each well of a 96-well tissue culture plate. Twelve dilutions were plated per sample, and 24 wells were plated per dilution. Wells were scored for cytopathic effect at 14 and 21 d after plating. To detect preformed infectious virus, parallel samples of mechanically disrupted cells were plated onto MEF monolayers.

Statistical Analyses. All data were analyzed by using GraphPad Prism software (GraphPad Software, http://www.graphpad.com, La Jolla, CA). Titer and flow data were analyzed with Student's two-tailed *t*-test. Growth curve data were analyzed with two-tailed ANOVA with multiple comparisons. Based on the Poisson distribution, the frequencies of reactivation and viral genome–positive cells were obtained from the nonlinear regression fit of the data where the regression line intersected 63.2%. The frequencies of reactivation and genome-positive cells were analyzed by unpaired two-tailed *t*-test.

Results

IKKα is not required for lytic infection. The role of the non-canonical pathway in lytic virus infection has not been reported. We prepared murine embryonic fibroblasts (MEFs) from mice bearing both a tamoxifen-inducible Cre and IKKα alleles with critical exons flanked by LoxP elements (AFTC, *CreER*^{T2}*/IKKα*$^{fl/fl}$) (540). MEFs were tamoxifen treated for 8 days to induce Cre translocation into the nucleus and drive complete IKKα deletion **(Fig. 4.1A)**. Untreated and tamoxifen-treated AFTC MEFs were infected with WT MHV68 at an MOI of 5. Deletion of IKKα did not impair virus replication at 24 or 48 hpi **(Fig. 4.1B)**. These results indicate that lytic viral replication occurs independently of IKKα signaling.

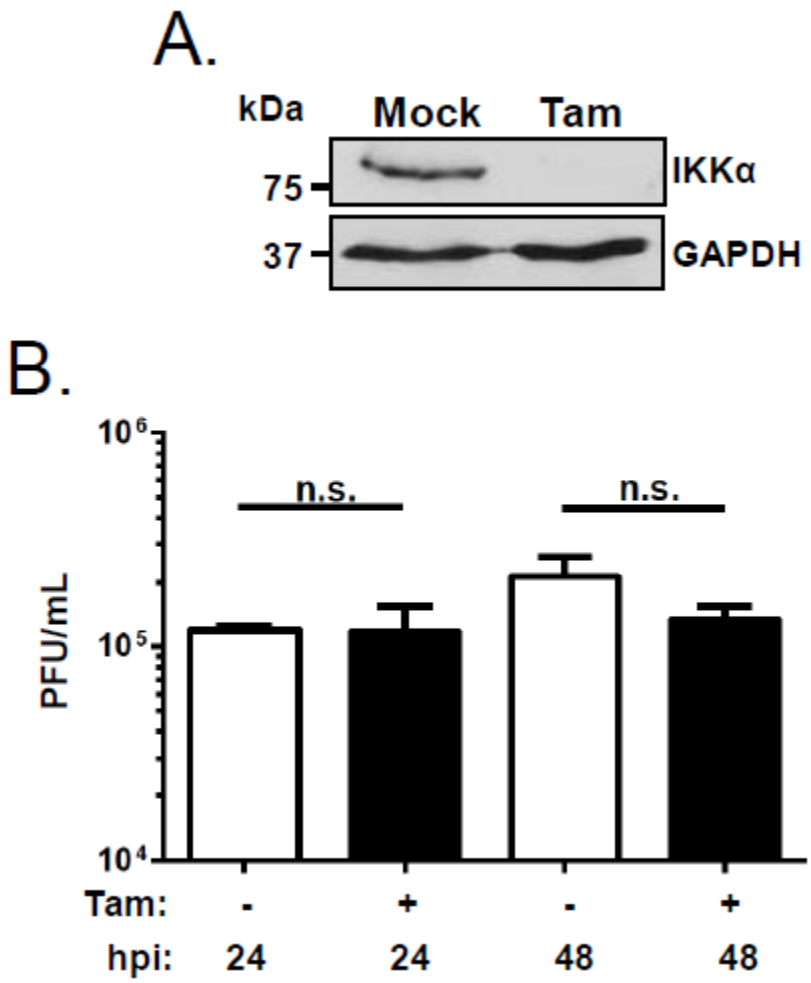

Figure 4.1 IKKα signaling is not necessary for MHV68 lytic infection. MEFs were generated from AFTC (*CreER^{T2}/IKKα$^{fl/fl}$*) mice and treated with tamoxifen daily for 8 days to drive IKKα deletion. (A) Cells were lysed and probed for IKKα protein to verify loss. GAPDH was used as a loading control. (B) IKKα$^{-/-}$ and WT MEFs were infected with MHV68-WT at an MOI of 5. At 24 and 48 hpi cells were harvested and viral replication was measured by plaque assay. Timepoints were measured in triplicate. Data was analyzed using Student's T test, n.s.=p>0.05.

Generation of recombinant viruses expressing dominant-negative IKKα. Transdominant transgene expression has been successfully employed to suppress NF-κB signaling (333). To specifically impair non-canonical NF-κB signaling in the infected cell, we engineered a gammaherpesvirus expressing a dominant negative form of IKKα, IKKα (S176A,S180A) (IKKαSA) (542), from a neutral intergenic locus between ORF57 and ORF58 in the MHV68 genome (523). The IKKαSA bears two serine to alanine mutations in the T loop that prevents phosphorylation by NIK and downstream signaling (399, 400, 442). In order to track changes in infected cells mediated by IKKα inhibition, we also designed an internal ribosome entry sequence (IRES) driven histone 2B-YFP fusion gene (H2BYFP) (495) **(Fig. 4.2A)**. A bicistronic transcript expressing IKKαSA and H2BYFP would ensure that YFP-expressing cells also express IKKαSA. This recombinant virus was termed IKKαSA. Two independent clones of IKKαSA were generated.

To control for changes in viral fitness due to gene insertion into the ORF57-58 locus, we also generated a virus bearing two all-frames stop codons prior to the IKKα gene region encoding the T-loop containing the NIK-phosphorylated serines (399). This recombinant virus was termed IKKαSTOP. Two independent clones of IKKαSTOP were generated using site-directed mutagenesis of the IKKαSA BAC. The mutant viruses were generated using BAC-mediated *en passant* recombination into a previous sequence-verified version of the IKKαSA BAC by our collaborator Dr. Darby Oldenberg in the White Lab (Gundersen Health System). The IKKαSA, IRES, and H2bYFP coding sequences were then confirmed by Sanger sequencing. Purified viral BACs were analyzed using restriction fragment length polymorphism (RFLP). As expected, the IKKαSA and the IKKαSTOP viruses had no visible changes following the Bam*HI* digest, a size increase from a 5042 bp band to a 5437 bp band following the Eco*RI* digest, and a

size increase from a 6174 bp to a 6569 bp band indicating there were no large deletions or insertions into the viral genome **(Fig. 4.2B, arrows)**.

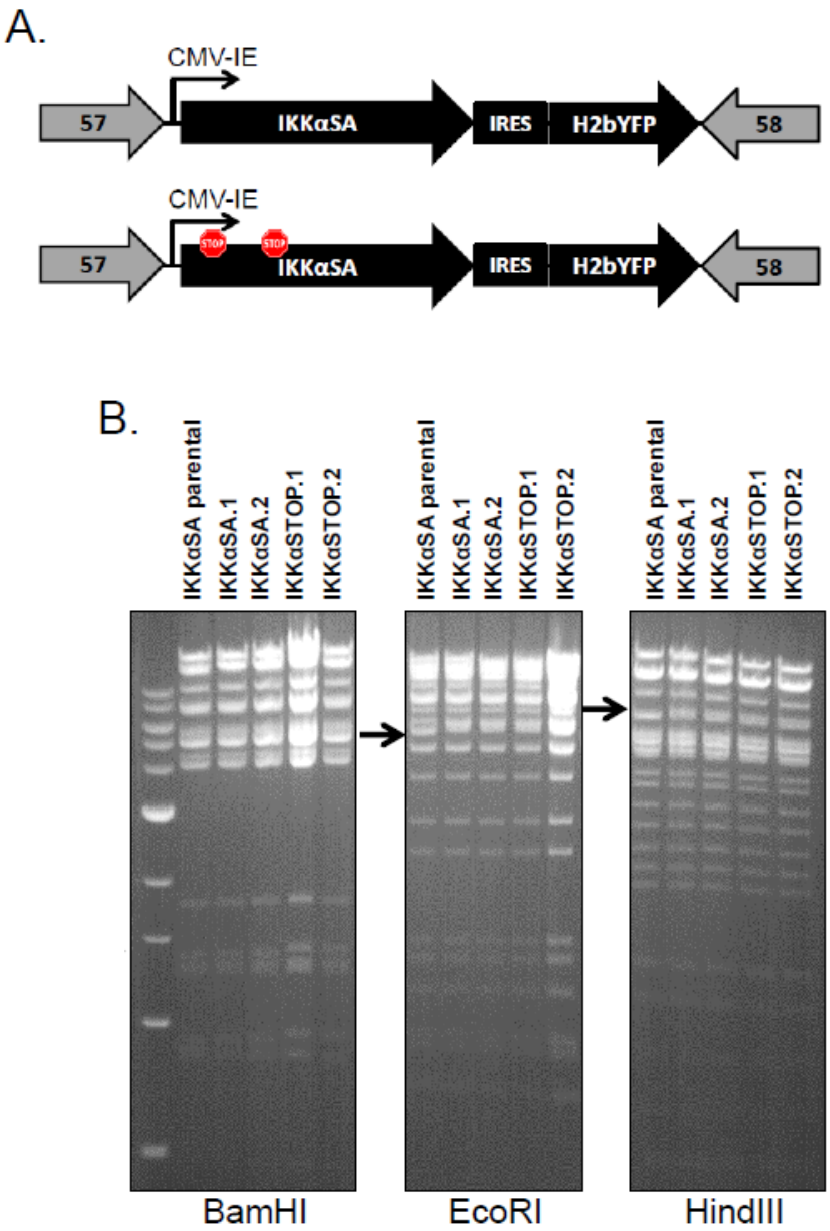

Figure 4.2 Generation of mutant MHV68 that impairs IKKα signaling. (A) A cassette encoding a CMV-IE protomor driving IKKαSA or IKKαSTOP, an IRES, and an H2BYFP was inserted into the neutral locus between ORF57 and ORF58 using BAC-mediated recombination. (B) BACs of the MHV68-IKKαSA or IKKαSTOP clones were digested using Bam*HI*, Eco*RI*, or Hind*III* for RFLP analysis. Restriction fragments were run on a gel and compared to the parental BAC. Arrows indicate larger bands due to STOP cassette insertion.

To confirm transgene expression from the mutant viruses, murine embryonic fibroblasts (MEFs) were infected with the IKKαSA and IKKαSTOP viruses at an MOI of 5 and lysates were collected 24 hpi. IKKα was expressed at much high levels in cells infected with the IKKαSA virus. The IKKαSTOP infected cells had low levels of IKKα similar to uninfected cells **(Fig. 4.3A)**. Using flow cytometry, we compared the mean fluorescence intensity (MFI) of YFP in the mutant viruses compared to the benchmark MHV68-H2BYFP virus (495) and found the IKKαSA and IKKαSTOP viruses expressed YFP at comparable levels to H2BYFP **(Fig. 4.3B)**. Next, we assessed if transgene overexpression impaired viral replication in MEFs. MEFs were infected with IKKαSA, IKKαSTOP, or H2BYFP at a low multiplicity of infection (MOI 0.05) to allow for multiple rounds of replication. The IKKαSA and IKKαSTOP viruses replicated to similar titers at levels 5-fold lower titers than H2B virus **(Fig. 4.3C)**, indicating transgene insertion had minimal effect on lytic infection. Together these data indicate the IKKαSA virus expresses the IKKαSA transgene during infection while the IKKαSTOP does not, and that both viruses express YFP at levels comparable to the H2BYFP virus.

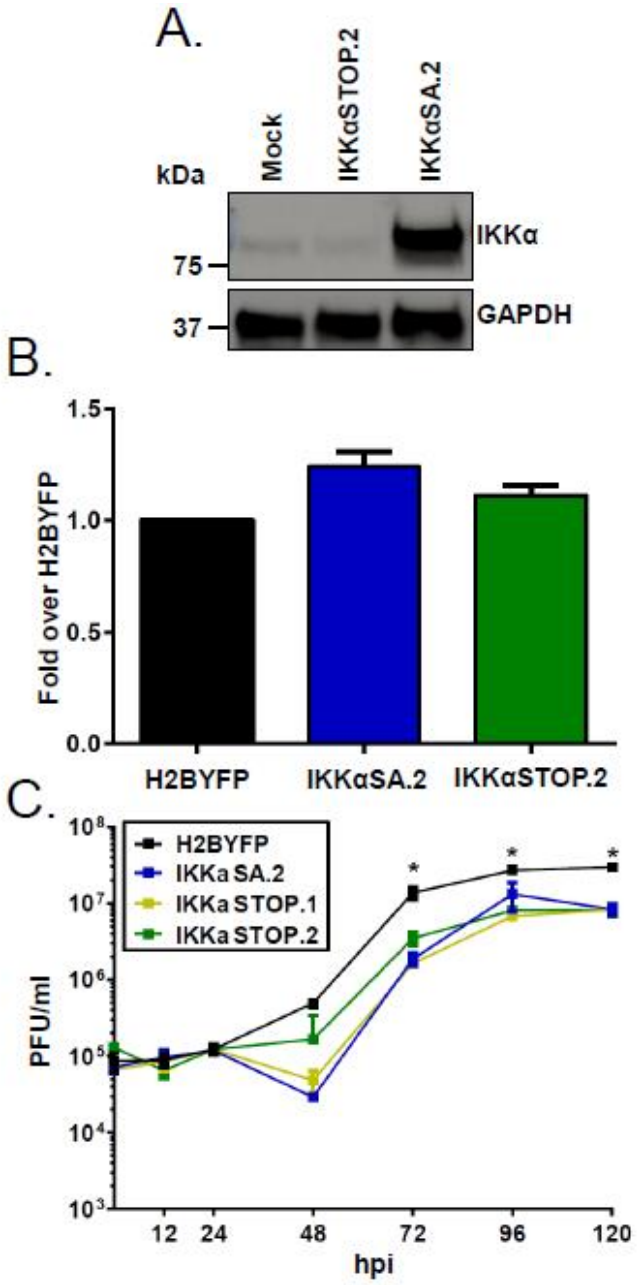

Figure 4.3 Function and growth of MHV68-IKKαSA and IKKαSTOP. WT MEFs were infected with IKKαSA or IKKαSTOP at an MOI of 10. (A) At 24 hpi cells were harvested and lysates were probed for IKKα. GAPDH was used as a loading control. (B) At 18 hpi cells were trypsinized and YFP fluorescence was measured by flow cytotmetry. MFI of mutant viruses was compared to the MFI of MHV68-H2BYFP. YFP expression from each virus was measured in triplicate. (C) Murine 3T12 fibroblasts were infected at an MOI of 0.05. Cell lysate was harvested at the indicated times post infection and viral growth was measured by plaque assay. Timepoints were measured in triplicate. Results were analyzed using 2-way ANOVA with multiple comparison post-test. *=$p<0.05$.

Viral infection impairs alternative pathway signaling during lytic infection. We next tested whether we could detect impairment of alternative pathway signaling by infection with MHV68-IKKαSA. Activating the lymphotoxin beta receptor (LTβR) in MEFs by an antibody against the LTβR induces non-canonical signaling (404). NIK accumulates in the cytoplasm of the cell over time, leading to the phosphorylation and activation of IKKα. IKKα then phosphorylates p100, inducing its degradation into p52 and its translocation to the nucleus. MEFs were stimulated for 16 hr with 10 μg/ml LTβR antibody and tested for p100 cleavage in the whole cell, cytoplasmic, and nuclear fractions. We also stimulated MEFs with the canonical pathway activator TNFα for 15 min. p100 cleavage and p52 translocation was observed only after LTβR stimulation **(Fig. 4.4A lane 3, 6, 9)**. TNFα stimulation but not LTβR antibody treatment led to p65 depletion from the cytoplasm, confirming the specificity of these stimuli for the canonical and non-canonical pathways. To quantitate alterations in alternative pathway signaling after infection, MEFs were stimulated for five or 16 hours with either a high or low dose of αLTβR. The levels of activated p52 in nuclear fractions that recognized immobilized oligonucleotides with p52 consensus binding sites were measured in a p52 NF-κB enzyme-linked immunosorbent assay (ELISA). I observed a time-dependent induction of p52 nuclear translocation in cells stimulated with either dose of αLTβR **(Fig. 4.4B)**.

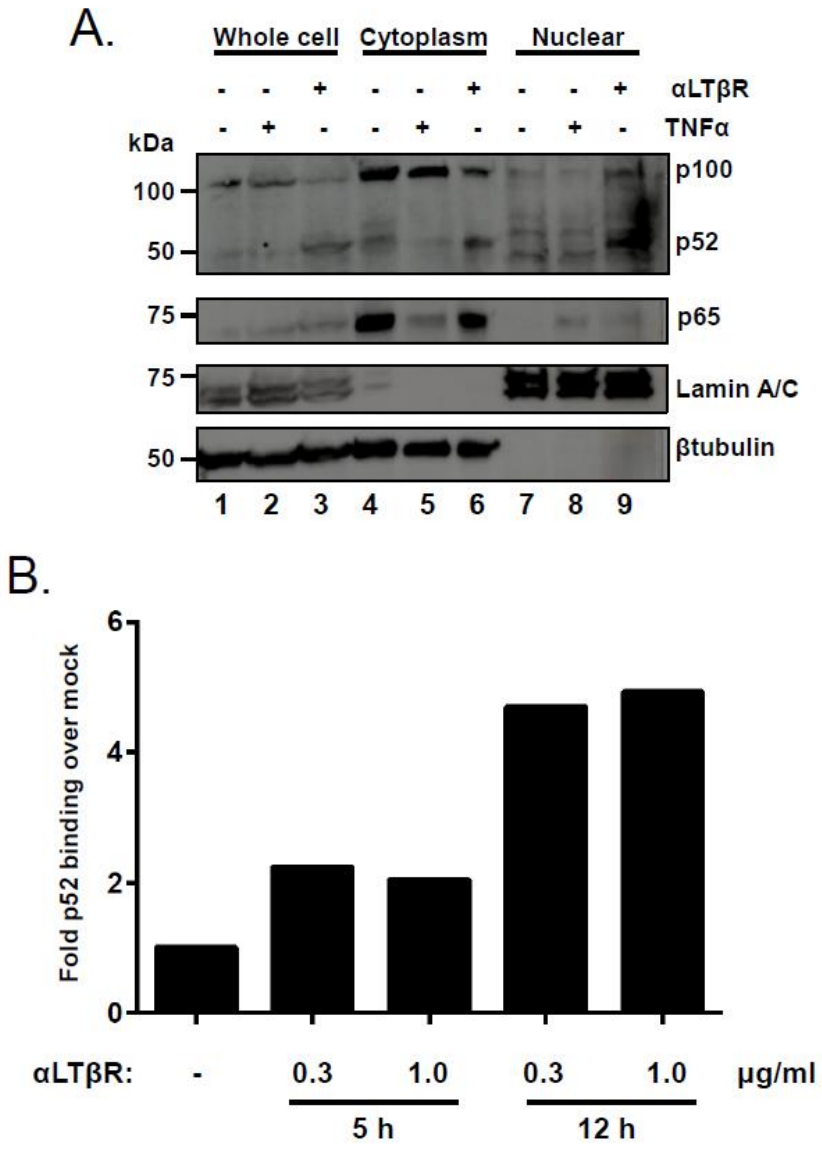

Figure 4.4 αLTβR stimulation induces p100 degradation in MEFs. (A) Primary WT MEFs were stimulated with 1 μg/ml αLTβR for 18 h or 50 ng/ml TNF-α for 15 min. Cell lysates were fractionated to collect cytoplasmic and nuclear fractions. p100 cleavage and p65 were detected by western blotting. Lamin A/C was used as a control nuclear protein and GAPDH was used as a control cytoplasmic protein. (B) MEFs were stimulated with various concentrations of αLTβR for 5 or 12 h. Nuclear fractions were tested for p52 activation by p52 ELISA. Data normalized to mock stimulated MEFs.

Next, MEFs were infected with IKKαSA and IKKαSTOP virus at an MOI of 5 for four hours prior to stimulation of cells with αLTβR for five hours. Nuclear fractions were harvested at nine hpi. NF-κB p52 binding activity was induced upon stimulation with αLTβR antibody in the uninfected cultures **(Fig. 4.5 top)**. Infection with the IKKαSA reduced p52 binding activity approximately 2 fold, but this reduction was also observed in the IKKαSTOP, which does not produce the inhibitory IKKα **(Fig. 4.5 bottom)**. There was a slight but insignificant increase in p52 binding activity in the unstimulated IKKαSTOP infected cells compared to the IKKαSA infected cells. The reduction in nuclear p52 in infected, stimulated cells indicates that MHV68 impairs non-canonical NF-κB signaling during lytic infection. Due to this unexpected yet novel finding, functionality of the IKKαSA transgene was not validated.

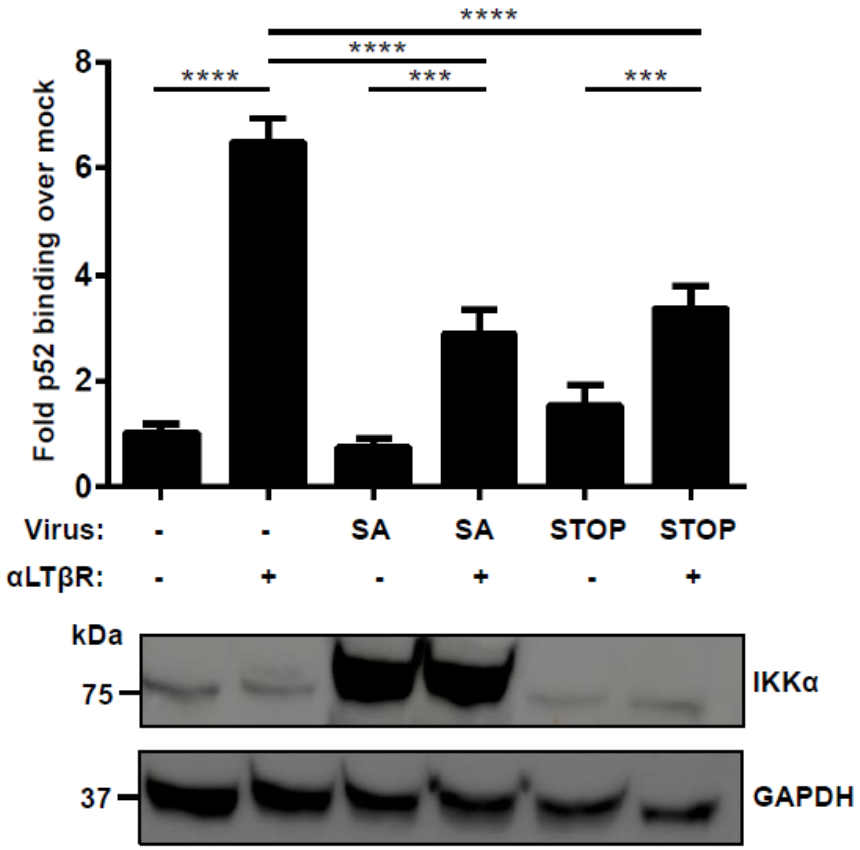

Figure 4.5 MHV68 lytic infection impairs non-canonical NF-κB signaling. Primary MEFs were infected with MHV68-IKKαSA or IKKαSTOP at an MOI of 10. At 4 hpi, cells were stimulated with 0.3 μg/ml of αLTβR antibody for 5 h. At 9 hpi, cells were harvested. Nuclear fractions were probed for p52 activation using a p52 ELISA. Results were normalized to uninfected, unstimulated cells. IKKαSA overexpression was measured in the cytoplasm by western blotting. GAPDH was used as a loading control. p52 entry into the nucleus was measured in triplicate, N=1. Results were analyzed using 2-way ANOVA with multiple comparison post-test. ***=p<0.001, ****=p<0.0001

IKKα signaling is not required for mature B cell survival. The role of the non-canonical signaling pathway in gammaherpesvirus latency has not been thoroughly investigated. Apoptosis of the B cell subset at the T1 to T2 transition stage is observed in mice bearing a germline kinase-dead IKKα (543). Thus the role of IKKα in the survival of mature B cell subsets was not known. Given the defects in early maturation and subsequent survival in the germinal center of IKKα$^{-/-}$ B cells (400, 442), we first examined if IKKα signaling is critical for survival of mature B cells.

To determine if ablation of IKKα affected mature B cell subsets, we generated mixed BM chimeras with BM from IKKα inducible knockout mice (AFTC, *CreER^{T2}/IKKα$^{fl/fl}$*) and B-cell deficient μMT mice. Upon transfer to a gamma-irradiated μMT recipient, the AFTC bone marrow would generate cells of the hematopoietic lineage that are inducible for IKKα deletion. Every cell type except for B cells would have a WT counterpart derived from the μMT lineage to ensure that secondary effects from IKKα loss in other immune cell subsets would be minimized **(Fig. 4.6A)**. Immune reconstitution and successful engraftment of AFTC bone marrow were assessed at eight weeks post transfer. The μMT recipients had CD19+ B cells in their periphery, indicating successful engraftment of the AFTC donor BM **(Fig. 4.6B)**. Chimeras were then administered tamoxifen or vehicle by intraperitoneal injection daily for 4 days to induce deletion of IKKα. Loss of IKKα in B cells was confirmed by RT-PCR to detect the Cre-mediated deletion of the IKKα exons **(Fig. 4.6C)**. Two weeks after the last tamoxifen treatment a majority of B cell subsets, including marginal zone and naïve mature follicular B cells, were maintained at levels comparable to untreated mice even in the absence of IKKα **(Fig. 4.7A)**. We observed a two-fold reduction in newly formed B cells, which includes the T1 and T2 B cells, in tamoxifen-treated chimeras, consistent with previous reports of the requirement for IKKα signaling in

early maturation (400, 437). Entry into the germinal center was also completely ablated in IKKα-deficient cells **(Fig. 4.7B)**, in agreement with Senftleben et al. (400). Examination of plasma cells in IKKα-deficient mice revealed a two-fold decrease in frequency, but not a total loss **(Fig. 4.7C)**. Taken together, these data confirm that IKKα is important for early B cell maturation and participation in the germinal center, but is not required for the maintenance of the majority of mature B cells for at least 2 weeks. This suggests that induction of IKKα deletion in the context of latent infection could reveal *bona fide* roles for IKKα in maintenance of viral latency.

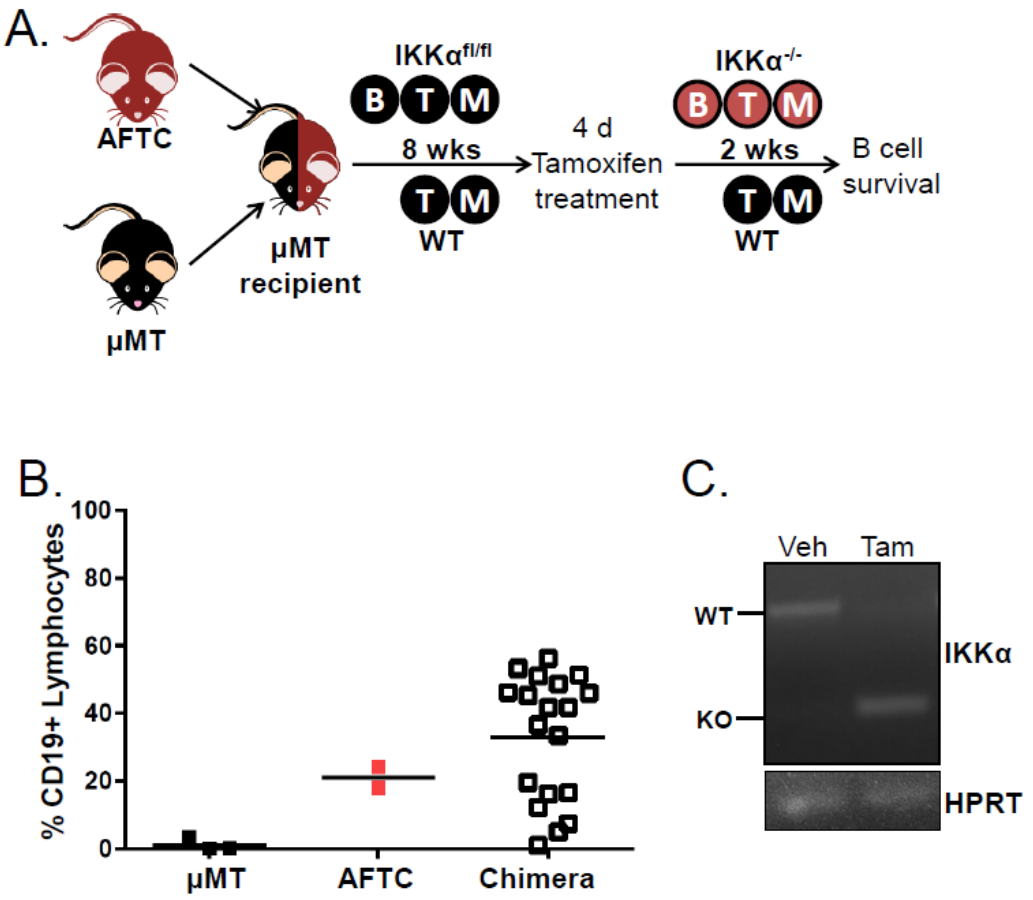

Figure 4.6 Generation of mixed bone marrow chimeras from µMT and AFTC donors. (A) Mixed bone marrow chimeras were generated in a gamma-irradiated µMT recipient using AFTC (*CreER*^{T2}*/IKKα*$^{fl/fl}$) and µMT donors. After 8 weeks of immune reconstitution, chimeras were tested for establishment of chimerism. Chimeras were treated with tamoxifen by intraperitoneal injection daily for 4 days, then B cell survival was examined after 2 weeks. (B) Establishment of chimerism was measured by flow cytometric analysis of CD19+ cells in peripheral blood. Cells were gated on live lymphocytes. (C) IKKα deletion was measured by RT-PCR of column-purified B cells from vehicle or tamoxifen-treated chimeras. IKKα deletion results in a smaller band than WT. HPRT was used as a loading control.

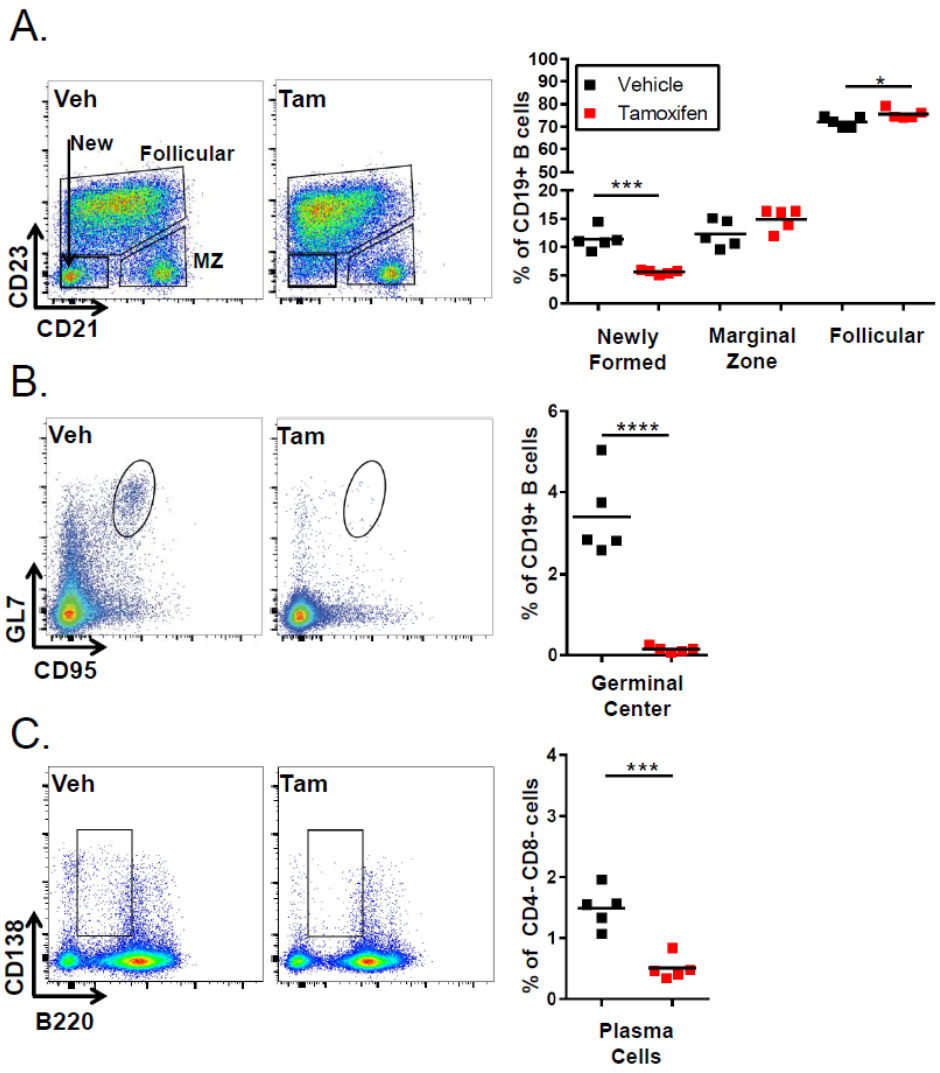

Figure 4.7 IKKα is not necessary for survival of mature B cells. B cell subsets from vehicle and tamoxifen-treated μMT/AFTC chimeras were analyzed by flow cytometry. (A) Splenic subsets were analyzed using CD21 and CD23 staining of CD19+ B cells. Subsets were defined as newly formed = $CD21^{lo}$ $CD23^{lo}$; marginal zone = $CD21^{hi}$ $CD23^{lo}$; follicular = $CD21^{+}$ $CD23^{hi}$. (B) Germinal center B cells were analyzed using CD95 and GL7 staining of $CD19^{+}$ B cells. Germinal centers were characterized as $CD95^{hi}$ $GL7^{hi}$. (C) Plasma cells were analyzed using B220 and CD138 staining of CD4/CD8- cells. Plasma cells were characterized as $B220^{lo}$ $CD138^{hi}$. 5 mice were used per experiment, N=1. Results were analyzed using T test. *= $p<0.05$, *** = $p<0.005$, ****= $p<0.0005$.

Induction of IKKα loss after latency is established leads to reduced levels of latency in both IKKα$^{-/-}$ and WT B cells. Next, we examined the contribution of IKKα signaling to the maintenance of MHV68 latency. In the mixed BM chimera studies described above, we sought to maintain functional T cells and DCs in the μMT BM to provide WT support to B cells after IKKα loss. To examine the role of IKKα in gammaherpesvirus latency, we used a competitive *in vivo* approach with bone marrow chimeras of mixed WT and AFTC bone marrow. WT B and T cells would provide normal immune support and WT latency reservoirs. We generated mixed BM chimeras with a mix 70% WT and 30% AFTC BM on a WT Ly5.1 background. The AFTC hematopoietic linages express the Ly5.2 cell surface marker and WT linages express Ly5.1 to allow for differentiation of the cell subsets by flow cytometry **(Fig. 4.8A)**. At 8 weeks post-transplant all chimeras established a mixed population of Ly5.1+ and Ly5.2+ B cells, indicating successful engraftment and reconstitution **(Fig. 4.8B)**.

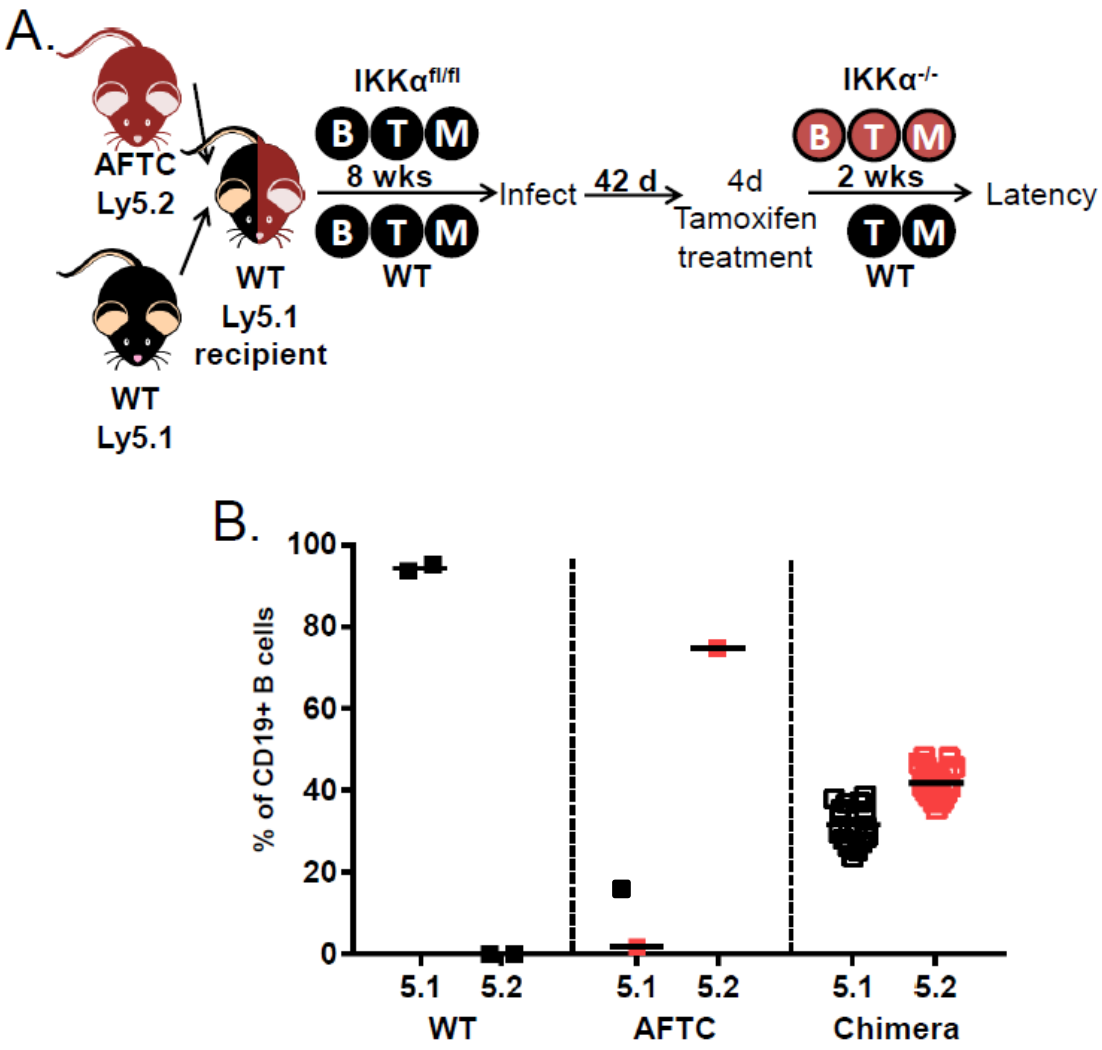

Figure 4.8 Generation of mixed bone marrow chimeras from WT and AFTC donors. (A) Mixed bone marrow chimeras were generated in a gamma-irradiated Ly5.1+ WT recipient using Ly5.2+ AFTC (*CreER*^{T2}*/IKKα*$^{fl/fl}$) and Ly5.1+ WT donors. After 8 weeks of immune reconstitution, chimeras were tested for establishment of chimerism. Chimeras were infected with 1000 PFU of MHV68-H2BYFP via the intranasal route. At 16 dpi latency establishment was measured. At 42 dpi chimeras were treated with tamoxifen by intraperitoneal injection daily for 4 days, then latency was examined after 2 weeks. (B) Establishment of chimerism was measured by flow cytometric analysis of Ly5.1+ and Ly5.2+ CD19+ cells in peripheral blood. Cells were backgated on live lymphocytes.

Next, mixed WT/AFTC BM chimeras were infected via the intranasal route with 1000 PFU of MHV68-H2bYFP and latency establishment was examined at 16 dpi. The frequency of genome-positive B cells was measured using limiting dilution PCR (LDPCR). Frequency analyses of bulk samples were consistent with frequencies typically observed for WT mice. Sorted CD19+ Ly5.2+ AFTC and Ly5.1+ WT subsets revealed both subsets supported comparable levels of latent viral infection **(Fig. 4.9A)**. The frequency of reactivating cells was measured using a limiting dilution coculture assay (LDA). Reactivation from bulk splenocytes occurred at frequencies typical for WT infection **(Fig. 4.9B)**. Sorted CD19+ Ly5.1+ or Ly5.2+ populations had comparable levels of reactivation.

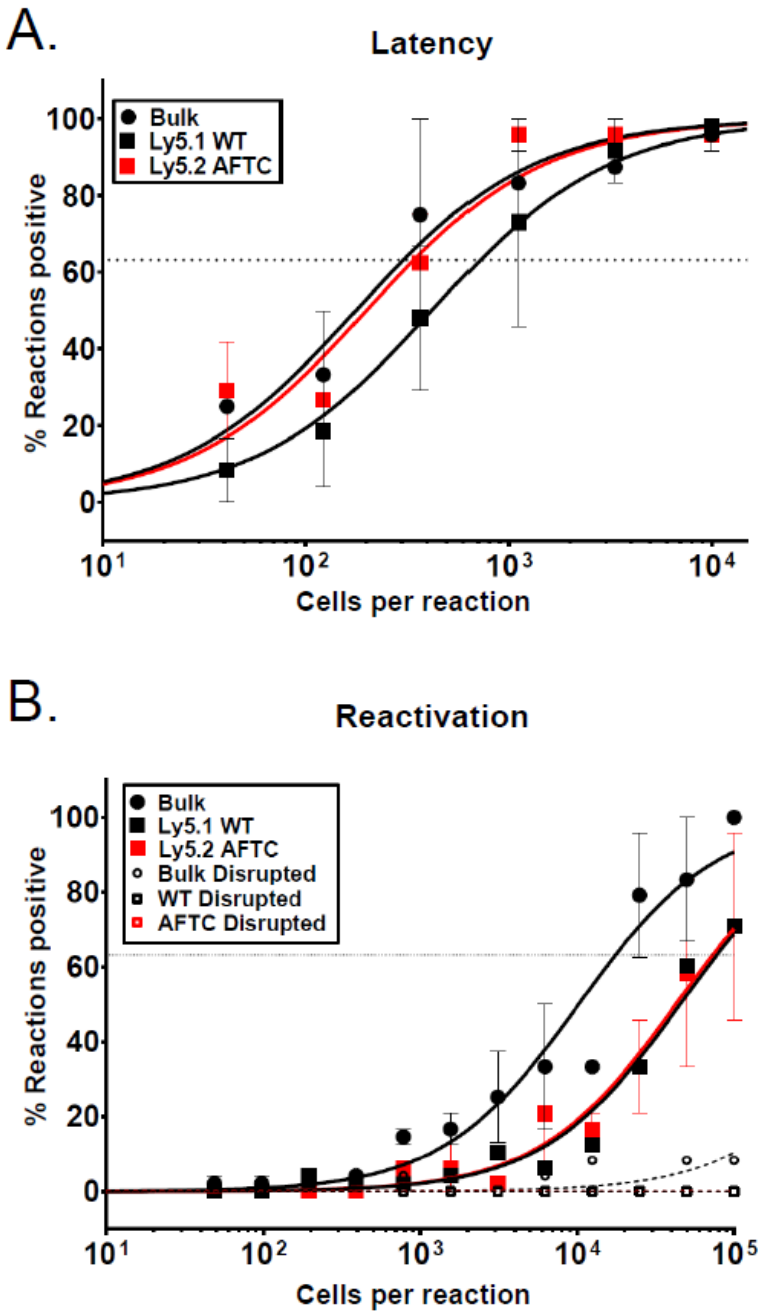

Figure 4.9 MHV68 establishes normal levels of latency in AFTC + WT mixed bone marrow chimeras. At 16 dpi spleens from infected chimeras were harvested. Ly5.1+ and Ly5.2+ CD19+ B cells were flow sorted and assessed for latency and reactivation. (A) Latency was measured in Ly5.1+ B cells, Ly5.2+ B cells, and bulk splenocytes by LD-PCR. Briefly, intact splenocytes were serially diluted and subjected to nested PCR reactions to detect viral ORF50. (B) Reactivation was measured by coculturing serial dilutions of intact splenocytes onto a monolayer of MEFs. 2-3 weeks post-coculture reactivation events were measured by the presence of CPE in the MEF monolayer. 4-5 mice were used per experiment, N=2. Differences in frequencies of latency or reactivation were analyzed by Students T test.

Having determined that latency was established normally in AFTC/WT bone marrow chimeras, we administered tamoxifen or vehicle intraperitoneally daily for 4 days to induce deletion of IKKα in the AFTC compartment. Two weeks after the last tamoxifen treatment CD19+ Ly5.1+ WT and CD19+ Ly5.2+ AFTC cells were isolated by flow sorting and the levels of latency in these two populations was analyzed by LD-PCR. We found that in vehicle-treated chimeras, levels of latency in Ly5.1+ WT B cells had contracted approximately 1 log from their d16 levels, which is typical for 60 dpi **(Fig. 4.10A)**. However, latency in Ly5.2+ AFTC B cells had contracted much more, and levels of latency were below the limit of detection of the assay. It is unclear what caused this loss of latency. A nonspecific response to corn oil, the vehicle, was monitored upon intraperitoneal injection of purified sterilized corn oil. B or T cell activation was not detected by CD69 upregulation **(data not shown)**. Examination of the rearrangement status of IKKα rearrangement status by RT-PCR of sorted B cells revealed a low level of IKKα rearrangement in AFTC cells the absence of tamoxifen treatment **(Fig 4.10C)**.

Additionally, we found that both the Ly5.1+ WT and Ly5.2+ AFTC B cell populations of chimeras treated with tamoxifen suffered significant reductions in latency **(Fig. 4.10B)**. IKKα was almost entirely rearranged in AFTC B cells, while WT B cells only had full-length transcript **(Fig. 4.10B)**. The cause of the unexpected loss of latency in the control WT, tamoxifen-insensitive cells is not clear. Tamoxifen treatment caused a significant reduction in the T1, T2, and germinal center populations of AFTC B cells, which was expected **(Fig. 4.11A, D)**. However, we noted a number of differences in the B cell subset frequencies in the corn oil vehicle treated animals **(Fig. 4.11A)**. Marginal zone, germinal center, and plasma B cells were underrepresented in the AFTC Ly5.2+ subsets, while the T2 B cell subset was present at higher

frequencies in the AFTC compartment, suggesting the AFTC genetic background is not completely identical to C57Bl/6. Taken together, the RT-PCR and flow data indicate that tamoxifen treatment induced deletion of IKKα. The unexplained depletion of latency from AFTC cells in the absence of tamoxifen treatment could stem from differences in the genetic background of the AFTC mice or leaky IKKα deletion that disrupts latency in the infected B cells.

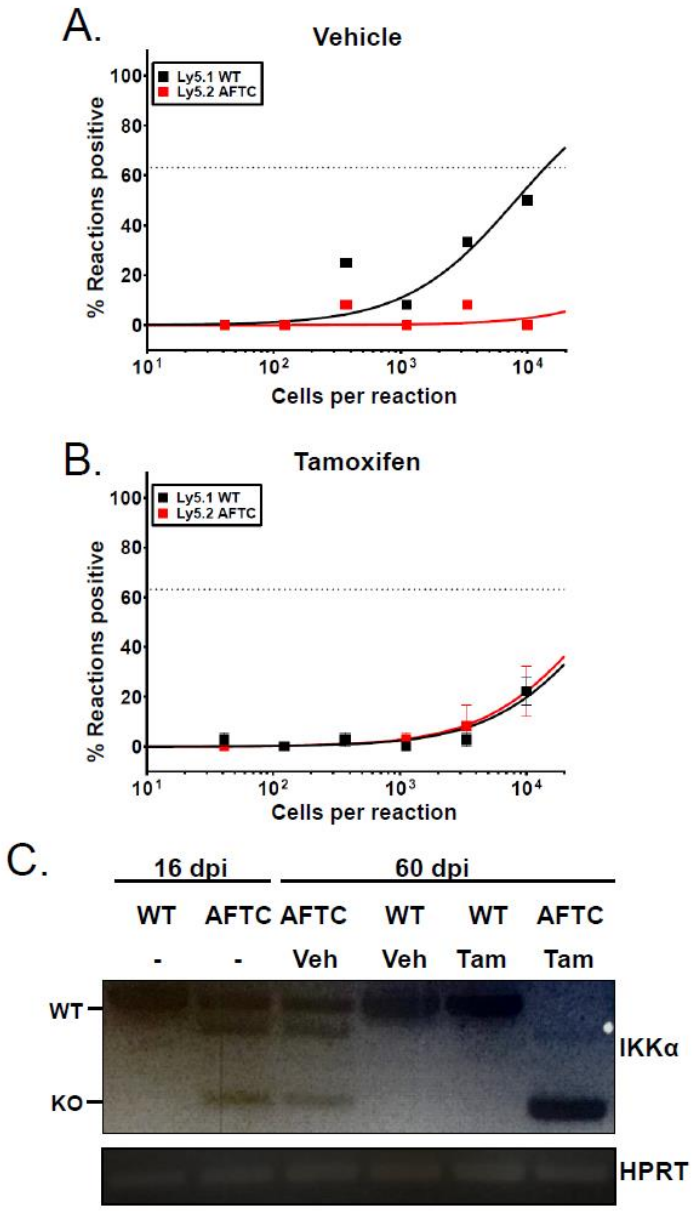

Figure 4.10 Tamoxifen treatment causes loss of latency from WT and AFTC B cells. MHV68-H2BYFP infected chimeras were treated with tamoxifen or vehicle intraperitoneally for 4 days, and spleens were harvested after 2 weeks. Ly5.1+ and Ly5.2+ CD19+ B cells were flow sorted and tested for latency and IKKα deletion. (A) Latency was measured in vehicle-treated chimeras using LD-PCR. N=1. (B) Latency was measured in tamoxifen-treated chimeras using LD-PCR. N=2. (C) IKKα deletion was detected in purified Ly5.1+ or Ly5.2+ CD19+ B cells by RT-PCR. IKKα deletion results in a smaller band. HPRT was used as a loading control. 6-10 mice were used per experiment, N=2 for tamoxifen treatment, N=1 for vehicle treatment. Differences in frequencies of latency or reactivation were analyzed by Students T test.

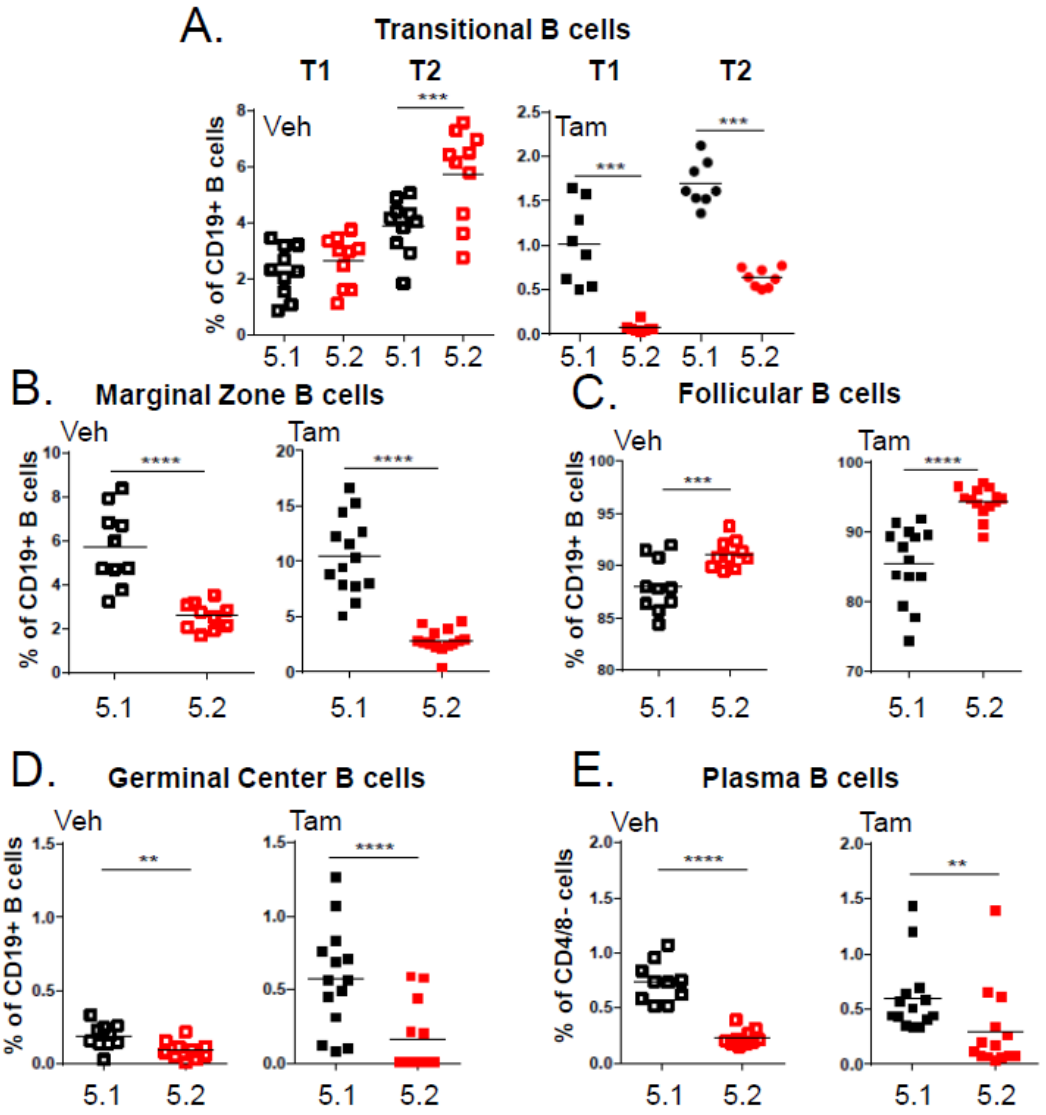

Figure 4.11 Tamoxifen treatment causes rearrangement of IKKα and loss of newly formed and germinal center B cells in AFTC B cells. B cell subsets from vehicle and tamoxifen-treated WT/AFTC chimeras were analyzed by flow cytometry. (A) Transitional B cells were measured using AA4.1 on $CD19^{+}$ $CD23^{-}$ B cells. T1 B cells were defined as $AA4.1^{+}$ $CD23^{-}$; T2 B cells were defined as $AA4.1^{-}$ $CD23^{-}$. (B,C) Marginal zone and follicular B cells were analyzed using CD21 and CD23 staining of $CD19^{+}$ B cells. Subsets were defined as (B) marginal zone = $CD21^{hi}$ $CD23^{lo}$; (C) follicular = $CD21^{+}$ $CD23^{hi}$. (D) Germinal center B cells were analyzed using CD95 and GL7 staining of $CD19^{+}$ B cells. Germinal centers were characterized as $CD95^{hi}$ $GL7^{hi}$. (E) Plasma cells were analyzed using B220 and CD138 staining of $CD4^{-}/CD8^{-}$ cells. Plasma cells were characterized as $B220^{lo}$ $CD138^{hi}$. 10 mice were used per experiment. Data is representative of N=1 vehicle treated chimeras and N=2 tamoxifen treated chimeras. Results were analyzed using Student's T test. *= $p<0.05$, **= $p>0.01$, *** = $p<0.005$, ****= $p<0.0005$.

Discussion

I examined the role of IKKα, an important mediator of B cell signaling, in the productive replication of MHV68 *in vitro* and the establishment and maintenance of latency *in vivo*. The *in vitro* experiments revealed that non-canonical NF-κB signaling is not required for lytic infection. Infection with the virus did not induce non-canonical signaling in primary MEFs. Extrinsic stimulation of non-canonical signaling was impaired during infection, revealing a novel effect of viral infection **(Fig. 4.5)**. With regard to *in vivo* analysis of the non-canonical signaling pathway, I generated mixed bone marrow chimeras that harbor inducible IKKα knockout hematopoietic lineages on a μMT recipient that lacks B cells. IKKα deletion in donor-derived mature AFTC B cells did not lead to death of marginal zone or follicular B cells, and only reduced plasma cell survival by two-fold **(Fig. 4.7)**. However, upon using this strategy to induce IKKα deletion in the context of mixed WT/AFTC BM chimeras infected with MHV68, I observed significant loss of latency from $IKK\alpha^{fl/fl}$ cells in the absence of tamoxifen-induced gene deletion. Additionally, we observed significant loss of viral latency from both $IKK\alpha^{-/-}$ and WT cells upon tamoxifen-induced gene deletion **(Fig. 4.10)**. Together these results suggest IKKα impacts the lytic phase of MHV68 infection, but we could not determine the intrinsic role for IKKα in the infected B cell population.

MHV68 interacts with many cell signaling pathways to promote lytic or latent infection. Canonical NF-κB is activated in response to inflammatory signals such as IL-1β and TNF-α (124, 490). These potent cytokines stimulate receptors on nearly every cell type to activate the canonical NF-κB pathway. Canonical NF-κB activation plays a deleterious role in lytic replication (411), and consequently the gammaherpesviruses have strategies to repress canonical NF-κB

activation by targeting the NF-κB subunit p65 for degradation (302, 408, 409). The receptors that mediate non-canonical NF-κB signaling have restricted expression patterns, are more commonly associated with organogenesis or immune cell maturation (278, 400, 437, 544-546). However, the non-canonical NF-κB pathway can be involved in immune responses such as chemotaxis (540, 547, 548).

We examined the effect of the loss of IKKα, the critical non-canonical kinase, on lytic infection and found that MHV68 did not require IKKα for replication **(Fig. 4.1)**. We observed a slight but statistically insignificant increase in p52 activity upon infection with the control virus MHV68-IKKαSTOP in the absence of stimulation. It is unclear what would trigger non-canonical signaling in the context of acute infection, and how the virus mediates this inhibition. It is possible that non-canonical signaling is activated slightly in response to infection, but is in turn inhibited by viral infection. Additionally, we observed a two-fold reduction in αLTβR-stimulated p52 activation in cells infected with either MHV68-IKKαSA or the control virus. Because we observe inhibition of p52 translocation, which constitutes the very last step of non-canonical NF-κB signaling, the upstream steps must be further investigated.

The downregulation of non-canonical signaling occurred as soon as nine hpi, suggesting that early genes or tegument proteins mediate this effect. Several MHV68 ORFs have functions that might contribute to inhibition of the non-canonical pathway. MHV68 RTA plays a critical role in promoting lytic gene transcription (145) and targets p65 for degradation (408). Effects on non-canonical subunits were not reported. The vSOX protein mediates global mRNA turnover which could affect NIK mRNA and subsequent translation (549). The importance of ubiquitin moieties in changing TRAF2, TRAF3, and cIAP1/2 activity (391-393, 396) and mediating

NIK and p100 degradation makes them a potential target for viral modulation. The tegument protein ORF64 functions as a viral deubiquitinase (550) and plays an important role in innate immune evasion that is dependent on its ubiquitinase activity (105). The ubiquitin modification states of non-canonical pathway signaling intermediates were not examined.

Previous studies examining the effect of the loss of cellular genes on latency have depended on constitutive germline deletion of the gene (334) or Cre-mediated excision of the gene in the infected cell (86). While these methods are effective and have uncovered roles for host genes in the viral life cycle, they abrogate the host gene very early after infection, impacting events during acute infection and the establishment of latency. I sought to delete IKKα only after latency had been established using a tamoxifen-inducible cre system. To avoid global immune dysregulation, I generated mixed bone marrow chimeras bearing both WT and IKKα-inducible knockout immune cells with the expectation that the WT cells would provide functional T and B cell control after tamoxifen treatment. While latency establishment in these chimeras appeared normal prior to tamoxifen treatment, I observed an unexpected reduction in viral latency in the IKKα$^{fl/fl}$ cells at 60 dpi, two weeks after vehicle treatment **(Fig. 4.10A)**. The reduction in viral latency in the vehicle treated animals occurred in the absence of the loss of germinal centers or newly formed B cells **(Fig. 4.11),** suggesting that widespread spurious deletion of IKKα does not underlie this phenomenon. However, I observed low levels of IKKα recombination in AFTC B cells **(Fig. 4.10C)**. How this could cause such a drastic loss in the infected B cell reservoir is not clear. One explanation is that a low level of leaky Cre recombination of IKKα occurs in cells harboring the virus. This could prove detrimental to the cell, removing it from the pool of latency and leading to a drop in viral load over time. The

continual replenishment of B cells from the AFTC bone marrow could be masking the loss of cells in the B cell subsets we examined. It is possible the leakiness of the CreER^{T2} system is specific to B cells. Examining IKKα rearrangement in other cell types could indicate if it occurred in other populations, and examining uninfected age-matched chimeras could indicate if rearrangement is tied to rapid proliferation by immune activation. Bone marrow from donor mice was mixed prior to transfer, resulting in a chimera with multiple donors from each background. It is possible that leakiness varies on an individual basis. Testing IKKα rearrangement status in the peripheral lymphocytes of individual AFTC mice could reveal changes within the population, and could allow us to select donors with tight regulation, which could minimize IKKα rearrangement in the absence of tamoxifen treatment.

Upon tamoxifen treatment of WT/AFTC mixed BM chimeras, there was a reproducible loss of latency in both the WT and AFTC B cells **(Fig. 4.10B)**. Tamoxifen treatment led to IKKα deletion and loss of IKKα cells, as expected. We suspect that the simultaneous loss of IKKα in almost 50% of B cells could induce a global stress to the immune system. Reactivation of MHV68 can be triggered upon activation of immune signaling receptors such as the TLRs or by coinfection with helminths (81, 88). Additional experiments examining IKKα deletion, immune activation, latency, and reactivation in a time course after tamoxifen treatment would be necessary to examine what occurred in these chimeras. Unfortunately unexpected results preclude us from determining if IKKα-mediated signaling intrinsic to the infected cell contributes to latency maintenance.

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Chapter 5: Discussion

I. Overview. The gammaherpesviruses represent an ancient and highly successful family of viruses that co-evolved with their hosts over the course of 60-80 million years (551). Infection by the gammaherpesviruses leads to the establishment of a life-long infection in the lymphocyte reservoir (26, 232, 552), where the virus maintains the ability to reactivate to infect new cellular reservoirs and new hosts. During this long co-evolution the gammaherpesviruses have developed strategies to evade, control, and hijack multiple aspects of the immune system such that even infection with a single infectious unit can establish long-term latency (553).

I have undertaken three studies examining the interactions that occur between the host and the virus during infection. In the study of T cell control of gammaherpesvirus pathogenesis, I demonstrated that T cells lacking the Sts proteins were hyperresponsive to MHV68-infected cells, yet they did not reduce viral pathogenesis better than WT T cells. Another study investigated the course of viral pathogenesis in mice lacking *caspase-1*, the primary mediator of the inflammasome. C*aspase-1* deletion did not affect viral pathogenesis. My laboratory made two additional observations *in vitro* that may explain my *in vivo* findings; MHV68 establishes lytic infection in macrophages without activating the inflammasome, and MHV68 infection impaired the generation of IL-1β mRNA upon extrinsic stimulation. Lastly, I found that non-canonical signaling was not necessary for MHV68 lytic infection and I also observed inhibition of the non-canonical signaling pathway upon *de novo* infection. Overall these studies uncover novel interactions between the virus and the host immune system whereby the virus impairs cell signaling pathways critical to innate and adaptive immune responses.

II. *Sts-1* and *-2* dKO improves the cellular response against gammaherpesvirus-infected cells.

Arguably the most important aspect of host control of lytic and latent gammaherpesvirus infection is the T cell response. A robust virus-specific T cell response is generated rapidly upon infection (200, 210, 213, 554), and in the mouse, CD4 and CD8 T cells collaborate to clear virus from the lung by 12 dpi (34, 192, 205, 221). T cells then mediate long term control through cell-cell interactions and release of anti-viral and cytotoxic molecules (152, 155, 162, 214, 474). Gammaherpesviruses effectively evade clearance despite the highly active anti-viral immunity that persists for the lifetime of the host. We sought to determine if Sts-1/2 double knockout T cells that have a lower threshold for activation and increased effector responses could overcome gammaherpesvirus immune evasion and clear a latent infection. While we confirmed that the Sts dKO T cells could react more strongly to virally infected cells in culture (**Fig. 2.2)**, this did not lead to clearance of long term latency *in vivo* **(Fig. 2.4)**.

This study suggests targeting the Sts proteins to drive immune clearance of latency in the immunocompetent host would not be effective. However, blocking these negative regulators of T cell signaling could improve the effectiveness of adoptive cytotoxic T cell (CTL) therapy used against gammaherpesvirus-driven malignancies. Transfer of gammaherpesvirus-specific T cells prior to hematopoietic stem cell transplant has excellent efficacy in preventing virus-driven PTLD, an EBV-driven malignancy that expresses the viral type III latency proteins (477). However, CTL therapy against virus-associated malignancies that have more restricted gene expression as well as highly immunosuppressive microenvironments has a higher failure rate (478, 480). Improving the ability of transferred T cells to detect and destroy infected cells could improve the efficacy of virus-specific T cell therapy. The closely related STS molecules

represent potential targets for inhibitory drugs that would prevent their function and increase T cell sensitivity. In the era of targeted genome editing, it is likely possible to specifically delete the *sts* genes in virus-specific CTLs cultured *in vitro* (555, 556). Only the virus-specific T cells would be hypersensitive upon reinjection into the patient, which may improve treatment efficacy while preventing off-target effects. In addition, limiting gene deletion to one subset of the T cell population avoids global effects on T cell function that inhibitory drugs could cause. Further investigation of these cells could reveal a novel cellular target to improve therapies against virus-driven malignancies.

In order to determine if Sts dKO T cells could improve the treatment of virus-driven malignancies, we would need to test the functionality of Sts dKO T cells in the control of malignancies in SCID mice. Murine fetal liver B cells can be latent infected and immortalized with MHV68 (45). Upon injection into SCID mice, these cells form tumors. If adoptive transfer of Sts dKO T cells into these mice could impair MHV68+ tumor growth better than WT T cells, we could next examine their efficacy against EBV-infected cells. We could expand EBV-specific T cells isolated from PBMCs of infected individuals and delete *Sts-1* and *Sts-2* using CRISPR-based gene deletion. These experiments could determine if targeting the *sts* genes could be used to enhance EBV-specific T cell responses for CTL therapy.

III. Innate immunity and gammaherpesvirus infection. The innate immune system is comprised of cells and molecules that react to moieties common to pathogens. This allows the innate immune system to mount a rapid response to control and eliminate the pathogen while also mobilizing the adaptive immune response. One of the primary mediators of intracellular surveillance against pathogens is the inflammasome. The inflammasome is activated after *in*

vitro infection of macrophages by HSV-1 or CMV, members of the alpha and betaherpesvirus families (104), and this activation is critical in preventing lethal encephalitis after intranasal infection of HSV-1 (491).

We sought to determine if the inflammasome also played a role during gammaherpesvirus infection. We found that infection of mice lacking *caspase-1* and *caspase-11* did not lead to exacerbated lytic infection, nor did it alter latency **(Fig. 3.2)**. Further examination revealed that MHV68 infection of BMDMs at an MOI of 10 occurred without inflammasome activation **(Fig. 3.3)**, and that infection reduces the inflammasome response to extrinsic stimulation by reducing IL-1β at the mRNA level **(Fig. 3.6)**. Many pathogens have evolved methods to subvert or avoid detection and clearance by the innate immune system. While we did not see IL-1β release in response to MHV68 infection at an MOI of 10 **(Fig. 3.3),** a concurrent study by Sun et al. found that MHV68 infection at an MOI of 100 could induce IL-1β transcription (105). The sensor responsible for this activation was not identified. Unlike the murine gammaherpesvirus, the human gammaherpesviruses EBV and KSHV appear to induce inflammatory signaling in cell lines and patient samples (106-108) in spite of encoding molecules that impair the inflammasome (17, 109). Both EBV and KSHV are sensed in the nucleus by the dsDNA sensor IFI16 (106, 108). The putative mouse homolog of IFI16, p204, has primarily been implicated in control of cell differentiation and proliferation (557). It is possible that in the absence of a nuclear DNA sensor, MHV68 did not evolve to persist in the context of inflammasome activation, and instead evades inflamasome signaling all together.

Our studies determined that IL-1β repression occurred within eight hours post infection and in the absence of the viral transactivator RTA (ORF50), however additional work is

necessary to determine the mechanism of inhibition and the viral molecules responsible for this effect. It is possible that the inhibitor of IL-1β transcript is a *de novo* expressed gene independent of RTA. Future studies should characterize the gene expression profile of the ORF50 stop virus by microarray analysis of infected BMDMs. These data would reveal the extent of viral gene transcription that occurs in the absence of RTA and the extent of changes in host gene expression that are dependent on RTA function. Viral genes expressed in the absence of RTA could be potential mediators of IL-1β expression inhibition or transcript degradation. We could then proceed to examine if lentiviral transduction of these RTA-independent genes into BMDMs prior to stimulation could impair IL-1β mRNA levels.

Alternatively, MHV68 tegument proteins could mediate the reduction of IL-1β transcript. Many tegument proteins for MHV68 have been identified by mass spectrometry of purified virions (14, 558), and constructs encoding these proteins can be transduced into BMDMs to test for impairment of IL-1β upon stimulation. We would confirm the role of any protein found to downregulate IL-1β by generation of a mutant virus with a stop codon insertion into the viral gene. Infection of BMDMs with the mutant virus should lead to an increase in IL-1β upon extrinsic stimulation. The MHV68 ORF64 tegument protein mediates inflammasome evasion upon infection of BMDMs (105). This inhibition was dependent on the deubiquitinase function of ORF64, which was critical for localizing the viral genome to the nucleus upon infection. However, it was not determined if ORF64 affected IL-1β mRNA levels. It is possible these additional experiments could reveal an additional role for ORF64 or, alternatively, a novel protein that cooperates with ORF64 to impair inflammasome signaling.

To test if MHV68 infection impairs transcription of IL-1β, we would clone the IL-1β promotor upstream of a luciferase reporter gene. Lentiviral transfection of this reporter construct into BMDMs and subsequent stimulation or infection and stimulation would allow us to measure changes in IL-1β transcription caused by MHV68 infection. IL-1β mRNA can also be directly regulated through the action of the human miRNA miR146a (559). Mice and other vertebrates encode miR146a as well, but its function is not well understood. The gammaherpesvirus utilize mimicry of cellular miRNAs to regulate cellular gene transcription (109, 260, 269, 272), and can influence the transcription of cellular miRNAs (239). We might also examine whether the 14 miRNAs expressed by MHV68 mediate IL-1β inhibition by lentiviral transfection of miRNA-expressing constructs into BMDMs prior to stimulation. Changes in the levels of cellular miRNAs that target IL-1β, such as miR146a (559), could also be examined upon MHV68 infection.

The IL-1β transcript contains mRNA instability elements that can promote its rapid degradation (533). Infection with mutant MHV68 that lacks the ability to induce global mRNA instability via the vSox protein (141) would indicate if the effect we observed was due to MHV68 vSox. Determining how MHV68 mediates the specific repression of IL-1β would reveal a novel level of regulation of the infected cell. Additionally, gammaherpesvirus-mediated changes in inflammatory signaling are associated with oncogenesis (560), and further understanding how gammaherpesviruses alter innate immune signaling could help us understand and better combat virus-driven malignancies.

IV. Gammaherpesvirus infection impairs non-canonical NF-κB signaling during lytic infection.

Gammaherpesviruses are capable of infecting a wide range of cell types (21, 23, 24, 26, 33,

561), however they primarily establish latency in the B cell compartment (25, 26, 552). NF-κB signaling through the canonical and alternative arms is highly activated during B cell maturation in the spleen (400, 437, 442, 537) and engagement in the germinal center reaction (279, 438). Our lab determined that the canonical pathway of NF-κB signaling contributed to latency establishment in the spleen (333, 334), but the non-canonical pathway had not yet been examined. I examined if the non-canonical NF-κB pathway contributed to infection by targeting IKKα. I found that MHV68 infection did not require IKKα, the central mediator of non-canonical signaling, for lytic infection **(Fig. 4.1)**. Additionally I observed an impairment of non-canonical signaling upon extrinsic activation during lytic infection **(Fig. 4.5)**. I also found that deletion of IKKα from mature B cells did not impair the survival of most mature B cells subsets. Recent studies have confirmed that induced IKKα deletion does not affect maintenance of mature B cell populations (562).

While I did not determine if IKKα signaling is required for latency establishment and maintenance, I did find that MHV68 impaired non-canonical signaling during lytic infection of MEFs. MHV68 impairs canonical signaling during lytic infection (408, 409) to counteract repressive functions of activated canonical transcription factors on lytic gene expression (411) and impair the production of inflammatory cytokines *in vivo* (409). While canonical NF-κB signaling is activated by numerous inflammatory signals, it is unclear to what extent alternative pathway signaling is activated during infection *in vivo*. Non-canonical signaling can be activated by the chemokine HMGB1, inducing chemotaxis towards a site of injury (540, 547, 548). Future studies should measure the induction of chemokines during MHV68 infection in the lung using a multiplex cytokine assay of bronchoalveolar lavage and bulk lung tissue. It may be necessary to

compare the cytokine profile of infection by an MHV68 deleted for the secreted chemokine binding protein M3 to observe the profile of the uninhibited host cytokine response to infection. Induced cytokines that are known to require non-canonical signaling, such as CXCL12 (547) orB6 (563) would indicate a function for blocking signaling in the infected cell. If we identify the viral mediator of inhibition, we would then examine how non-canonical pathway-dependent cytokine induction affects infection.

The mechanism of non-canonical signaling inhibition by the virus is also an important line of investigation for future studies. Virus infection could mediate inhibition at multiple steps. Examining the accumulation of NIK as well as the phosphorylation state of IKKα after αLTβR stimulation during infection would identify the step at which MHV68 is acting. Infection may alter TRAF3 binding to cIAP1/2, a necessary step for NIK degradation. This would be examined by coIP pulldown and probing for their binding partners. *De novo* translation of NIK is required for non-canonical signaling, such that repression of NIK transcription or degradation of NIK mRNA could be methods to block non-canonical signaling downregulation. MHV68 infection may impair the accumulation of NIK after receptor stimulation. If NIK levels are low, we could block proteasome function with MG132 upon infection and stimulation to detect virus-mediated NIK degradation. MHV68 vSox degrades cellular RNA, and a virus deficient in vSox could be deficient in non-canonical pathway inhibition. Investigation into the mechanisms of the inhibition we observed could reveal a novel aspect of gammaherpesvirus pathogenesis that might inform additional strategies by which the gammaherpesviruses subvert the host.

V. Conclusion. The long coevolution of the gammaherpesviruses and their hosts have resulted in the gammaherpesviruses subverting many aspects of host immunity, culminating in the

establishment of a life-long infection that causes little deleterious effect to the host. However, in the context of immunosuppression, these latent infections predispose their host to the development of malignancies. Only by careful investigation of the many strategies the gammaherpesviruses use to evade the host immune system and establish and maintain a long term infection can we develop better therapeutics against virus-driven malignancies and extirpate latent infection. Murine gammaherpesvirus infection of mice serves as a powerful system to test the complex interactions of viral and host factors that contribute to pathogenesis in a natural host. We can test the contributions of both innate and adaptive immune cells in control of the virus, and we can determine the limit of their natural functionality. Manipulating the activation thresholds of immune cells, increasing their specific responses, and extending the duration of their activation beyond their normal parameters could give the host immune system the upper hand in the 80 million year evolutionary tug-of-war, aiding clearance of virus-driven malignancies and possibly impairing and clearing viral latency. The increased responsiveness of the Sts dKO T cells suggests that targeting these host proteins, or other negative regulators, could augment cytotoxic T cell therapy against virus-driven malignancies. Examining the *in vivo* response to viral infection also clues us in to the Achilles' heels of the immune system, as well as how the virus may exploit them. The absence of an expected response in the *caspase-1/11* knockout mice led us to uncover novel strategies of by which the virus impairs a central host signaling pathway. Examination of non-canonical NF-κB signaling during lytic infection revealed an unexpected repression of this pathway, suggesting a negative impact on productive infection that the virus has evolved to avoid. These may reveal new avenues for examining the host response to infection and the generation of cytokine-mediated

antiviral therapy. The mouse model also allows us to unravel how viral manipulation of the host cell and hijacking of normal immune responses culminates in life long latency. Viral latency involves both maintenance of the viral genome as well as reactivation to seed new cells and new hosts. We can examine the contribution of cellular factors such as IKKα in control of viral gene expression and observe how their disruption disturbs the establishment and maintenance of latency within multiple B cell subtypes. Together these studies revealed new modes of immune evasion by the gammaherpesviruses and set the foundations for additional investigation into viral inhibition of innate immune signaling, T cell control of infected cells, and into the effect of the non-canonical signaling blockade on gammaherpesvirus latency.

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