

# Unraveling immunity to $\gamma$ -herpesviruses: a new model for understanding the role of immunity in chronic virus infection

Herbert W Virgin IV\* and Samuel H Speck†

Murine  $\gamma$ -herpesvirus 68 ( $\gamma$ HV68) infection is a new model for understanding how immunity and chronic  $\gamma$ -herpesvirus infection inter-relate.  $\gamma$ HV68 is closely related to the human Epstein–Barr virus and Kaposi's sarcoma herpesvirus and is associated with tumors, vasculitis of the great elastic arteries and splenic fibrosis. Advances in the past year have provided an even stronger foundation for believing that  $\gamma$ HV68 infection of normal and mutant mice will become the pre-eminent animal model for understanding  $\gamma$ -herpesvirus pathogenesis and immunity.  $\gamma$ HV68 latency has been characterized employing new assays for quantitating cells carrying the  $\gamma$ HV68 genome and cells that reactivate  $\gamma$ HV68 and for detecting the presence of preformed infectious virus in tissues. These advances have fostered the first steps towards a molecular definition of  $\gamma$ HV68 latency. It appears that  $\gamma$ HV68 shares latency programs with human  $\gamma$ -herpesviruses – including the loci for gene 73, v-bcl-2 and the viral homolog of the G-protein coupled receptor. This provides candidate antigens for analysis of the role of T and B cells in regulating latency. Multiple cellular reservoirs for  $\gamma$ HV68 latency were uncovered with the demonstration that  $\gamma$ HV68 latently infects macrophages in addition to B cells. A critical role for B cells in regulating the nature of  $\gamma$ HV68 latency was discovered and the mechanism was shown to be via alteration of the efficiency of reactivation. Studies of the response of CD4<sup>+</sup> and CD8<sup>+</sup> cells during acute and chronic  $\gamma$ HV68 were performed. These new studies provide key building blocks for further development of this novel and interesting model system.

## Addresses

Department of Pathology, Washington University School of Medicine, Box 8118, 660 South Euclid Avenue, St Louis, MO 63110, USA

\*e-mail: virgin@immunology.wustl.edu

†e-mail: speck@pathology.wustl.edu

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## Abbreviations

<b><math>\beta</math>2m</b>	$\beta$ 2-microglobulin
<b>EBV</b>	Epstein–Barr virus
<b><math>\gamma</math>HV68</b>	$\gamma$ -herpesvirus 68
<b>HVS</b>	herpesvirus saimiri
<b>IFN-<math>\gamma</math></b>	interferon $\gamma$
<b>KSHV</b>	Kaposi's sarcoma herpesvirus
<b>MCMV</b>	murine cytomegalovirus
<b>PCR</b>	polymerase chain reaction
<b>PEC</b>	peritoneal exudate cell
<b>RT-PCR</b>	reverse-transcriptase PCR
<b>v-GCR</b>	viral G-protein coupled receptor

## Introduction

Herpesvirus infection of normal and mutant mice presents an outstanding opportunity for understanding host

responses, and viral genes that counter host responses, during chronic persistent infection and latent infection. Immune responses during chronic herpesvirus infection may differ from such responses to other viruses for several reasons. First, the antigens expressed during latent herpesvirus infection differ significantly from those expressed during lytic infection. Second, the presence of immune-avoidance genes in herpesviruses probably influences the nature and efficacy of the immune response. Last, latent infection of hematopoietic cells may fundamentally alter the efficacy of immune responses to latent antigens.

There are three classes of herpesviruses:  $\alpha$ ,  $\beta$  and  $\gamma$ . Medically important  $\gamma$ -herpesviruses include Epstein–Barr virus (EBV) and Kaposi's sarcoma herpesvirus (KSHV or human herpesvirus 8). Such  $\gamma$ -herpesviruses are characterized biologically by their association with tumors in immunosuppressed hosts. EBV is associated with B cell lymphomas and nasopharyngeal carcinoma in humans [1,2]. KSHV is associated with Kaposi's sarcoma, body cavity based B cell lymphomas and Castleman's disease in humans [3–6]. Herpesvirus saimiri (HVS) causes lymphomas in primates and rabbits and can transform T lymphocytes [7–10]. Until recently, mechanistic studies of the role of immunity in  $\gamma$ -herpesvirus infection have been hampered by the species specificity of EBV and KSHV and the difficulties and expense inherent in pathogenetic studies in primate models.

Infection of mice with murine  $\gamma$ -herpesvirus 68 ( $\gamma$ HV68, also called murine herpesvirus 68 [MHV-68]) provides a promising new system for addressing fundamental issues in herpesvirus pathogenesis and immunity. In this review we will focus on recent advances that fundamentally alter our understanding of  $\gamma$ HV68 latency and how the immune system regulates both persistent lytic infection and latency.

## The $\gamma$ HV68 model

Infection with  $\gamma$ HV68 presents the opportunity to evaluate the role of specific immune system genes and individual  $\gamma$ -herpesvirus genes in a small animal model amenable to both genetic and pathogenetic studies (reviewed in [11–15]). Analysis of the  $\gamma$ HV68 genome demonstrates that this virus is closely related to primate  $\gamma$ -herpesviruses — including EBV, KSHV and HVS [16–18].  $\gamma$ HV68 is a natural pathogen of wild rodents [19,20] and is capable of infecting both outbred and inbred mice [19,21–23].  $\gamma$ HV68 infects multiple organs of inbred mice and can establish a latent infection [21–25].

Chronic infection with  $\gamma$ HV68 has been associated with three important pathologies: tumors, vasculitis of the great arteries

and splenic fibrosis. In one study, a significant portion of mice infected with  $\gamma$ HV68 developed tumors or lymphoproliferative disorders and treatment with cyclosporin A increased the frequency of lymphoproliferative disease [26]. This important study has not yet been reproduced by other groups although it holds out significant hope that this system will be useful for mechanistic studies of  $\gamma$ HV68 induced tumors. Molecular and transgenic studies have recently demonstrated that the  $\gamma$ HV68 v-Cyclin is the product of a bona fide oncogene with the capacity to induce cell cycle progression in primary lymphocytes [27••]. Studies using mutants of  $\gamma$ HV68 that lack oncogenes, such as that for v-Cyclin, in a model of tumor development would be very exciting. Studies with one mutant  $\gamma$ HV68 isolate have been published, demonstrating that genetic approaches to the role of individual viral genes will be practical in this system [28].

In addition to inducing tumors,  $\gamma$ HV68 causes a severe chronic vasculitis of the great elastic arteries [29]. Vasculitis induced by  $\gamma$ HV68 is regulated by IFN- $\gamma$  and B cells [29].  $\gamma$ HV68 also causes T cell dependent chronic splenic fibrosis in mice lacking the IFN- $\gamma$  receptor [30]. Thus — in addition to allowing mechanistic studies of  $\gamma$ HV68 latency and chronic infection —  $\gamma$ HV68 infection provides a small-animal model for analysis of the role of the immune system in tumor development, vasculitis and chronic inflammation.

### Questions that must be answered to permit mechanistic studies of immunity during chronic $\gamma$ -herpesvirus infection

The  $\gamma$ HV68 system is very new and the fundamentals of the model are only now being described. To understand how the immune system operates during chronic infection, several aspects of  $\gamma$ HV68 biology must be defined; four of these are outlined below. First, the nature of  $\gamma$ HV68 infection in chronically infected tissues must be defined. Recent studies have shown that it is essential to simultaneously quantitate the frequency of cells carrying  $\gamma$ HV68 genome, the frequency of cells that reactivate  $\gamma$ HV68 and the amount of preformed infectious  $\gamma$ HV68 in chronically infected tissues. Second, the gene programs that operate during latent infection must be identified. Latency genes may encode antigens that play a role in chronic immunity. In addition, multiple latent gene programs may exist (as is true for EBV). Third, cells that harbor latent  $\gamma$ HV68 infection must be identified. These cells are probably the antigen-presenting cells that present (or fail to present) epitopes for immune recognition. Effects of the immune system on individual latent cell populations will also need to be defined. Fourth, the immune components that regulate acute infection, chronic productive infection and latent infection must be identified. Extensive progress has been made on all of these fronts over the past year by several groups (as detailed below), demonstrating that this system has great potential for future studies.

### Definition of $\gamma$ HV68 latency

Latency is the presence of  $\gamma$ HV68 viral genome occurs in a form that can be reactivated in the absence of ongoing

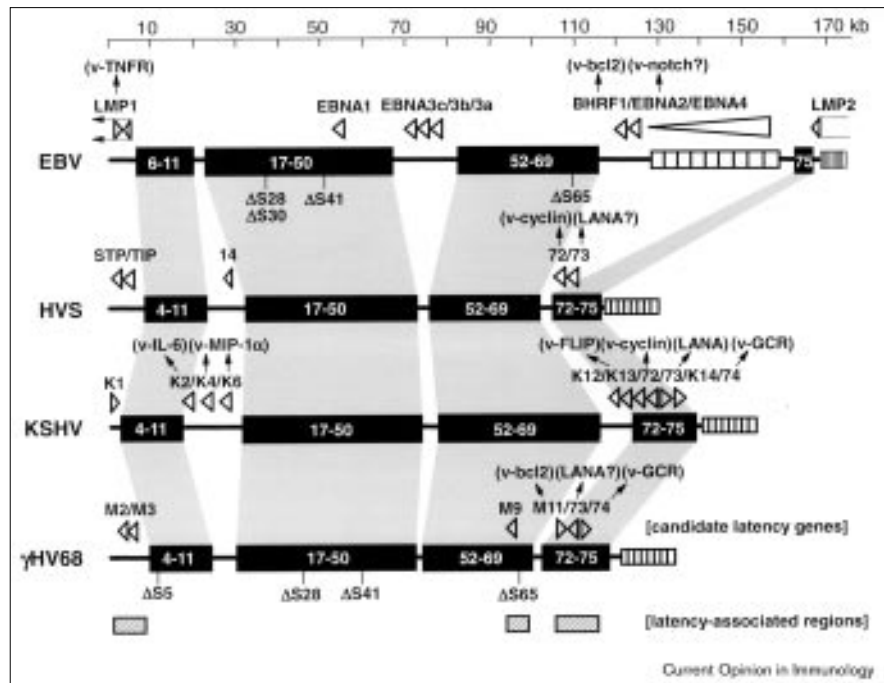
production of preformed infectious virus. Thus chronic  $\gamma$ HV68 infection differs from chronic infection with lymphocytic choriomeningitis virus or HIV (in which continuous productive infection occurs). This difference is likely to have significant impact on mechanisms of immunity. Antigens expressed in latently infected cells may stimulate an immune response. Intermittent reactivation from latency probably occurs at some level [31••], providing a potential source of chronic stimulation with lytic viral antigens. The physiologic importance of intermittent reactivation to latency and immune cell activation will have to be assessed experimentally.

Latency and persistent, productive infection can coexist in the same animal. For example, B cell deficient mice have demonstrable latency in spleen and peritoneal cells [24,32••,33••] when persistent productive infection has been clearly demonstrated in lung [34••] and in the aortic wall ([29]; HW Virgin IV, SH Speck, unpublished data). Since latency and persistent productive infection can coexist, at least in immunocompromised mice, it is critical that latency be measured using assays that quantitate both latently infected cells and preformed infectious virus (which may confound interpretation of assays that measure reactivation from latency) in chronically infected tissues.

Initial studies used plaques detected by 5–7 days in an infectious center assay as a measure of latency. Infectious center assays involve plating out intact cells onto permissive monolayers and assessing the generation of plaques over time. It was presumed that the plaques found in infectious center assays were due to reactivation from latency since standard plaque assays failed to show preformed infectious virus [22,25,30,35–37]. However, studies in the murine cytomegalovirus (MCMV) system from multiple groups have shown that plaque assays can lack sufficient sensitivity for preformed infectious virus in disrupted organs to be diagnostic for latency [38–40]. Importantly, two findings in the  $\gamma$ HV68 system invalidate the plaque assay for ruling out the presence of preformed infectious  $\gamma$ HV68. First, using a more sensitive limiting dilution assay, studies in mice deficient in  $\beta$ 2-microglobulin ( $\beta$ 2m<sup>-/-</sup>; these have decreased numbers of CD8<sup>+</sup> T cells) revealed preformed infectious  $\gamma$ HV68 after ‘clearance’ of infectious virus as measured by plaque assays [24]. Persistence of preformed infectious virus in  $\beta$ 2m<sup>-/-</sup> mice has recently been confirmed by another group [41•]. Second, a recent study used a sensitive PCR assay to demonstrate lytically derived linear  $\gamma$ HV68 genomes in lungs of B cell deficient mice [34••] when plaque assays fail to detect infectious virus [22,24,37]. These studies show that, as already shown for MCMV, plaque assays are not optimal for detecting preformed infectious  $\gamma$ HV68 in chronically infected tissues. Other assays — including limiting dilution analysis, measurement of latent gene expression and quantitation of cells carrying  $\gamma$ HV68 genome — are now available and will provide a firmer foundation for experimentally measuring  $\gamma$ HV68 latency.

**Figure 1**

$\gamma$ -herpesvirus latency-associated and transforming genes. The map positions of viral genes implicated in latency and/or cellular transformation by  $\gamma$ HV68 are shown at the bottom; the human and primate  $\gamma$ -herpesviruses EBV, HVS and KSHV are also shown. The grid at the top indicates length in kilobasepairs (kb). The blocks of conserved viral genes present in the genomes of all, or most,  $\gamma$ -herpesviruses are indicated by black rectangles. Genes not present in these blocks in all four viruses are designated as  $\Delta$ S below the black rectangles. In addition, the candidate  $\gamma$ HV68 latency genes identified by RT-PCR analysis of latently infected PECs and splenocytes (and latency genes in other viruses) are shown by open arrowheads; latency-associated regions of  $\gamma$ HV68 are shown by hatched boxes [43\*\*]. Genes for viral homologs (v-) of host proteins such as GCR, macrophage inflammatory protein (MIP) and tumor necrosis factor receptor (TNFR) are present, as are genes for viral proteins such as EBV nuclear antigens (EBNAs), latency-associated nuclear antigen (LANA) and latent membrane proteins (LMPs). See [43\*\*] and references therein for details of the identification and characterization of EBV, KSHV and HVS gene transcription in latently infected cells and associated tumors.



Limiting dilution analysis provides a different assay for simultaneously quantitating both preformed infectious virus and cells that reactivate latent  $\gamma$ HV68 [24,32\*\*,33\*\*]. In this assay latent cells are serially diluted onto permissive monolayers and the presence of infectious virus is detected by cytopathic effect over 2–4 weeks in culture, allowing prolonged time for reactivation or outgrowth of preformed infectious virus. To distinguish preformed infectious virus from latent virus, cells are mechanically disrupted (latent virus cannot reactivate from killed cells) and diluted in parallel with live cells. Importantly, controls show that preformed infectious  $\gamma$ HV68 is not inactivated by the mechanical disruption procedure [24,32\*\*,33\*\*]. The major advantages of this assay are that it allows quantitation both of cells reactivating  $\gamma$ HV68 and of preformed  $\gamma$ HV68 in the same samples, it is easy to perform and quite reproducible and it is 5–10-fold more sensitive than plaque assays [24,32\*\*,33\*\*]. Notwithstanding the sensitivity of this assay, and others such as PCR detection of linear genomes [34\*\*], a positive molecular definition of  $\gamma$ HV68 latency has been lacking (see below).

#### Genome regions transcribed during $\gamma$ HV68 latency: towards a molecular definition of $\gamma$ HV68 latency

One candidate molecular marker for latent infection has been *in situ* hybridization with a probe specific for the  $\gamma$ HV68 tRNA like transcripts [42]. Whereas cells that were positive for  $\gamma$ HV68 tRNA were observed in this study on day 21, a lack of preformed infectious virus was indicated by the failure to demonstrate viral DNA by *in situ*

hybridization using a probe within the terminal repeats; there was also a reliance on previous studies showing that preformed infectious virus was absent, as measured by plaque assay [42]. However, the absence of signal in *in situ* hybridization does not rule out lytic replication and the sensitivity of *in situ* hybridization is hard to measure. For example, *in situ* hybridization for transcripts from the glycoprotein H and thymidine kinase genes failed to demonstrate lytically infected cells even when sensitive PCR analysis demonstrated the presence of linear genomes and lytic replication [34\*\*]. In addition, the  $\gamma$ HV68 tRNA like genes are abundantly transcribed during lytic infection and thus are not specific for latency [42]. Notwithstanding these concerns, the recent demonstration that cells expressing  $\gamma$ HV68 tRNA transcripts can be found as late as 70 days after infection holds out great promise that, when validated using newly available methods for quantitating preformed infectious virus (see above), tRNA expression may well be a useful tool for defining latent cells *in vivo* [31\*\*].

On the basis of the limitations of the above assays, we have begun to establish a molecular definition of  $\gamma$ HV68 latency. Transcription from 19 regions of the  $\gamma$ HV68 genome was evaluated in latently infected spleen and peritoneal cells using nested, reverse-transcriptase (RT)-PCR assays with PCR sensitivity at between 1 and 10 copies of target DNA (Figure 1; [43\*\*]). Tissues contained cells that reactivated  $\gamma$ HV68 but did not contain significant levels of preformed infectious virus as measured by limiting dilution analysis.

Minimal transcription from seven regions of the genome encoding known lytic genes was detected in latently infected tissues [43\*\*]. Whereas the absence of transcription of a single lytic gene might be explained by assay failure, lack of transcription of seven different lytic genes strongly argues against the presence of significant lytic gene expression in latently infected peritoneal exudate cells (PECs) and splenocyte populations derived from B cell deficient mice.

Additional regions were selected for analysis as candidate latency genes, based on three criteria: first, homology to known latency or latency/lytic switch genes; second, homology to tumor-associated genes or candidate oncogenes of KSHV, EBV or HVS; third, location in the viral genome. Latency- and tumor-associated genes of  $\gamma$ -herpesviruses tend to be present in regions of the genome between large conserved blocks generally thought to contain lytic cycle genes (Figure 1). Notably, transcripts from several genome regions (gene 73, gene 74, gene M11 and gene M2) were detected in latent tissues — despite the fact that these regions are inefficiently transcribed (as measured by northern blots of total RNA) in lytically infected fibroblasts [43\*\*]. Transcripts from the regions of the genome encoding M3 and M9 were also detected in latent tissues. However, these genome regions — like the viral tRNAs — are abundantly transcribed in lytically infected fibroblasts [31\*\*,43\*\*] and thus these genome regions are not latency specific.

The identity of genes within the regions which are actively transcribed in latent tissues is informative. Gene 73 is homologous to gene 73 of KSHV that encodes the latency associated nuclear antigen detected in Kaposi's sarcoma cells [44,45]. Gene 74 is homologous to the IL-8-receptor-like, viral G-protein coupled receptor (v-GCR) gene of KSHV and HVS [16]. The KSHV v-GCR signals constitutively and causes cell growth [46]. M11 encodes a v-Bcl2 protein homologous to the EBV, KSHV and HVS v-Bcl2 proteins [16]. The active transcription of  $\gamma$ HV68 genome regions containing homologs of known latency- or tumor-associated genes of EBV, KSHV and HVS provides a strong argument for the relevance of studies in the  $\gamma$ HV68 system for understanding the pathogenesis of chronic infection with EBV and KSHV.

Another approach to defining latent transcripts using *in situ* hybridization [31\*\*] failed to detect transcripts in latent tissues aside from viral tRNAs and transcripts from the region of the genome encoding M3 (M3 was detected until day 21 of infection). The failure to detect latent transcription of gene 73, v-GCR (gene 74), v-Bcl2 (gene M11) and gene M2 using *in situ* hybridization — these regions were shown to be transcribed in latent tissues using RT-PCR [43\*\*] — is not surprising given the sensitivity of nested RT-PCR compared with *in situ* hybridization. Interestingly, the M3 region of the genome was detected in spleen at times when 14 other *in situ* probes (all of which could detect transcription in acutely infected lung) gave negative

results [31\*\*]. This striking finding suggests either that M3 is transcribed *in vivo* at levels higher than any other lytic gene evaluated or that M3 has a role independent of lytic infection. We have recently shown that M3 encodes an abundant, secreted protein of unknown function [47\*]. It is possible that expression of a secreted protein is important for establishment of splenic infection by  $\gamma$ HV68. Further studies of the function of M3, which shows significant homology only to the M1 gene of  $\gamma$ HV68 (which has some homology to poxvirus serpins), will be of great interest.

#### Cellular sites of $\gamma$ HV68 latency

B lymphocytes are latently infected with  $\gamma$ HV68 [25]. However, other studies suggested the existence of additional cellular sites of  $\gamma$ HV68 latency. For example, mice lacking B cells establish splenic and peritoneal cell latency efficiently after intraperitoneal inoculation with  $\gamma$ HV68 [24,32\*\*,33\*\*] and even after intranasal infection, a few latent cells can be detected by infectious center assays in B cell deficient mice [13]. Furthermore,  $\gamma$ HV68 persists in the lung of B cell deficient mice after intranasal inoculation [38] and lung epithelial cells may be latently infected in these mice [34\*\*]. Lastly, an adherent cell population in spleen harbors latent  $\gamma$ HV68 [25].

Following up on the suggestion that B cells are not the only cellular site of  $\gamma$ HV68 latency, we have recently shown that latent  $\gamma$ HV68 can be found in spleen, bone marrow and PECs [24,32\*\*]. Interestingly, macrophages are the predominant cells carrying latent  $\gamma$ HV68 in PECs [33\*\*]. Consistent with previous analyses [25], we confirmed that CD19<sup>+</sup> B cells carry the  $\gamma$ HV68 genome during latent infection [33\*\*]. Quantitation of the frequency of genome<sup>+</sup> cells using a limiting dilution, nested PCR assay revealed that B cells were a relatively minor reservoir (about 10% of genome<sup>+</sup> cells) of  $\gamma$ HV68 latency in PECs.

Since the natural route of infection for  $\gamma$ HV68 infection has not been experimentally defined, we compared latency using two different routes — intraperitoneal and intranasal [32\*\*]. Regardless of route, PECs carried latent  $\gamma$ HV68 [32\*\*]. As previously reported [37], splenic latency was inefficiently established in B cell deficient mice after intranasal inoculation — consistent with a role for B cells in spread of  $\gamma$ HV68 infection to the spleen (see below). Thus, route of inoculation is not the primary determinant of cellular sites of  $\gamma$ HV68 latency.

The existence of multiple cellular reservoirs for latent  $\gamma$ HV68 (e.g. B cells, macrophages and perhaps lung epithelial cells) is similar, first, to the observed latency of EBV in both B cells and perhaps epithelial cells [1,2], second, to the fact that KSHV can latently infect both B cells and endothelial or spindle cells [4,44,45,48–54] and, third, to the MCMV latency in both macrophages and endothelial cells [55,56]. The availability of a murine model in which a  $\gamma$ -herpesvirus is latent in multiple cell types provides for the first time an opportunity to directly address the physiologic importance

of various latent reservoirs. For example, we found that latently infected macrophages can be recruited (presumably from latently infected bone marrow) to sites of inflammation [33••]. This suggests the hypothesis that systemic latency could be maintained by continuous emigration of latent cells from the bone marrow. It remains to be determined whether macrophages are a major reservoir of  $\gamma$ HV68 latency in other latently infected tissues (e.g. spleen and lung); the possibility that additional sites of latency will be identified over time must be kept in mind. It will be important for future studies of latency, and in particular studies of how the immune system regulates latency, to define which cellular sites of latency are being studied. In addition, studies of immune responses during chronic infection must take into account the presence of latent  $\gamma$ HV68 in macrophages because these cells are known to present antigen and to regulate immune induction via secretion of cytokines.

### Role of specific immune components in regulating chronic and latent $\gamma$ HV68 infection

#### *Role of B cells*

B cells serve multiple roles during  $\gamma$ HV68 infection. First, they are a site of latency (see above). In addition they probably play a role in spread of the virus through the host. There are several lines of evidence that support this. First, infection of B cell deficient mice by the intraperitoneal route results in inefficient acute splenic infection [24]. Second, infection of B cell deficient mice via the intranasal route results in inefficient latent infection of the spleen [32••,37]. Last, experimental data in support of a role for B cells in spread of the virus were supplied by the demonstration that adoptive transfer of T cell depleted spleen cells into B cell deficient mice allowed spread of virus to the spleen [34••]. The importance of B cells in infection with  $\gamma$ HV68 is also suggested by the finding of  $\gamma$ HV68-tRNA<sup>+</sup> cells in germinal centers [31••,42]. Perhaps germinal center B cells are involved in  $\gamma$ HV68 latency. The identity of the tRNA<sup>+</sup> cells in germinal centers will be very informative.

$\gamma$ HV68 infection has striking effects on B cell activation.  $\gamma$ HV68 infection results in a considerable expansion of splenic B cells, a process requiring CD4<sup>+</sup> T cells [57,58••]. This expansion is associated with significant hypergammaglobulinemia [58••,59•] and much of the antibody is not apparently directed to  $\gamma$ HV68 antigens [58••,59•]. The role of antiviral antibody in controlling  $\gamma$ HV68 infection has not been formally addressed. Interestingly, infection of spleen cultures *in vitro* results in striking activation of B cells although — unlike the situation *in vivo* — CD4<sup>+</sup> T cells are not required for this activation [58••]. The specific role of B cell activation (both dependent on and independent of CD4<sup>+</sup> T cells) in  $\gamma$ HV68 infection and latency promises to be a very interesting future area of investigation.

A specific role for B cells in regulating  $\gamma$ HV68 latency has only recently been established [32••]. B cell deficient,  $\gamma$ HV68-infected mice develop arteritis involving the great elastic arteries [29], have ongoing productive infection in

the lung [34••] and die after prolonged infection of unknown causes [32••]. In addition, one early study demonstrated an increased frequency of cells reactivating  $\gamma$ HV68 in spleens of B cell deficient mice compared with normal mice after intraperitoneal infection [24]. These observations led us to undertake a detailed kinetic analysis of  $\gamma$ HV68 latency in normal B6 mice compared with B cell deficient mice. The number of cells that reactivate  $\gamma$ HV68 is similar in B cell deficient and normal mice early after infection (days 10–15). However, the impressive decline in cells reactivating  $\gamma$ HV68 that occurs in normal mice between 15 and 40 days of infection does not occur in B cell deficient mice [32••]. This is not explained by the lack of latently infected B cells (at least in PECs) since B cells account for only about 10% of genome<sup>+</sup> cells [33••].

There were several potential explanations for the maintenance of a large population of cells that efficiently reactivate  $\gamma$ HV68 in B cell deficient mice. We tested one hypothesis — that the number of cells carrying latent  $\gamma$ HV68 genome was higher in B cell deficient mice than normal mice — using a limiting dilution, nested PCR assay to quantitate cells positive for the genome [32••]. This assay, when run in parallel with limiting dilution reactivation assays, allows a direct comparison of the frequency of cells reactivating  $\gamma$ HV68 and the frequency of cells carrying  $\gamma$ HV68 genome. Surprisingly, this analysis showed that normal and B cell deficient mice maintain similar frequencies of genome<sup>+</sup> cells over time — despite the fact that cells from normal mice reactivated  $\gamma$ HV68 at a lower frequency than cells from B cell deficient mice. Thus the efficiency of reactivation, rather than the number of genome<sup>+</sup> cells, is regulated by B cells [32••]. This is a very important result since it shows that alterations in immunity can alter the nature of reactivation without changing the number of cells carrying  $\gamma$ HV68 genome. To obtain mechanistic information, future studies of  $\gamma$ HV68 latency will have to measure both the frequency of cells that reactivate  $\gamma$ HV68 and the frequency of cells that carry the  $\gamma$ HV68 genome.

The mechanism by which B cells regulate  $\gamma$ HV68 latency is not known. Initial experiments argue against the possibility that a factor secreted by normal cells (but not cells from B cell deficient mice) inhibits reactivation *in vitro* [24,32••]. Notably, B cell deficient mice harbor persistent productive  $\gamma$ HV68 infection in the lung [34••] and in the aorta ([29]; AJ Dal Canto, HW Virgin IV, SH Speck, unpublished data) — raising the possibility that persistent productive infection in some sites can continually generate newly latent cells in the spleen or PECs. Newly latent cells would be likely to reactivate efficiently, just as cells from both B cell deficient and normal mice do between 10 and 15 days after infection [32••]. Studies in B cell deficient mice infected with lymphocytic choriomeningitis virus have shown that these mice have abnormal T cell responses to viral infection [60]. This raises the alternative possibility that T cells normally cause the decline in the

frequency of cells that reactivate  $\gamma$ HV68 in normal mice, but are lacking in B cell deficient mice.

#### Role of IFN- $\gamma$

IFN- $\gamma$  is secreted during acute  $\gamma$ HV68 infection [61]. After infection with doses of  $\gamma$ HV68 that kill mice lacking the IFN- $\alpha/\beta$  receptor or the cytoplasmic transcription factor STAT1, mice lacking either IFN- $\gamma$  or the IFN- $\gamma$  receptor show only a small defect in  $\gamma$ HV68 clearance [29,30,35]. However, under the correct experimental conditions, lack of IFN- $\gamma$  can predispose to death from acute  $\gamma$ HV68 infection (AJ Dal Canto, HW Virgin IV, SH Speck, unpublished data; [62]).

The lack of dramatic effects of IFN- $\gamma$  during acute infection is in contrast to effects seen during chronic infection. Lack of either IFN- $\gamma$  or the IFN- $\gamma$  receptor predisposes  $\gamma$ HV68 infected mice to a severe vasculitis of the great elastic arteries ([29]; AJ Dal Canto, HW Virgin IV, SH Speck, unpublished data). This arteritis is also seen in mice deficient in CD4<sup>+</sup> T cells and MHC class II, an interesting observation given recent studies showing that CD4<sup>+</sup> T cells utilize IFN- $\gamma$  to protect against lethal infection in the absence of B cells and CD8<sup>+</sup> T cells [63\*\*].

Interestingly, mice deficient in the IFN- $\gamma$  receptor develop a striking splenic fibrosis 14 days post infection and this is dependent on the presence of CD8<sup>+</sup> or CD4<sup>+</sup> T cells [30]. This latter study is of great interest as it is the first demonstration of potential immunopathology in the  $\gamma$ HV68 system and it will be important to pursue it further since very little is known about possible immunopathologic events during  $\gamma$ -herpesvirus infection. IFN- $\gamma$  may regulate establishment of latency [30] with  $\gamma$ HV68 but this issue requires further investigation using additional assays to distinguish latent cells from low levels of persistent, preformed infectious virus and to quantitate the frequency of cells carrying  $\gamma$ HV68 genome.

#### Role of CD4<sup>+</sup> T cells

CD4<sup>+</sup> T cells play important roles in  $\gamma$ HV68 infection. CD4<sup>+</sup> T cells are involved in the induction of splenomegaly and B cell activation after  $\gamma$ HV68 infection [36,57,58\*\*]. In addition, CD4<sup>+</sup> T cells can play an important role in protection against acute infection either in combination with CD8<sup>+</sup> T cells [64] or in the absence of functional CD8<sup>+</sup> T cells [41\*,63\*\*]. The response of CD4<sup>+</sup> T cells persists for months after acute infection, with many of the cells of activated phenotype as determined by staining them for CD62 [65\*] and by measuring their capacity to secrete IFN- $\gamma$  upon activation [59\*]. The mechanisms underlying this persistent activation are as yet undefined. Whereas early studies argued for a critical role for CD4<sup>+</sup> T cells in maintaining CD8<sup>+</sup> responses [36], subsequent studies have clearly shown that the CD8<sup>+</sup> T cell response is robust in mice deficient in MHC class II [66\*\*]. The protective role of CD4<sup>+</sup> T cells is mediated at least in part via secretion of IFN- $\gamma$  [63\*\*]. In this interesting study, B cell

deficient mice were used to study the role of CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the absence of any contribution of B cells to resistance. These data are interesting in view of studies of MCMV that demonstrated that IFN- $\gamma$  regulates chronic infection *in vivo* as well as reactivation from latency [67] and that CD4<sup>+</sup> T cells, via IFN- $\gamma$ , regulate persistent infection of salivary glands with MCMV [68–70]. The importance of IFN- $\gamma$  and CD4<sup>+</sup> T cells is also suggested by the fact that both IFN- $\gamma$  unresponsive mice and mice deficient in CD4<sup>+</sup> cells and MHC class II are highly susceptible to  $\gamma$ HV68 induced arteritis [29]. The downstream mediators of IFN- $\gamma$  effects have not been defined although inducible nitric oxide synthase (iNOS) is a candidate because of studies demonstrating increased mortality in iNOS deficient mice after intranasal inoculation of  $\gamma$ HV68 [62].

MHC class II deficient mice develop progressive productive  $\gamma$ HV68 infection over time, clearly demonstrating the key role for MHC class II dependent processes in maintaining latency as the predominant state of infection during chronic infection [36]. Two studies argue that CD4<sup>+</sup> T cells may regulate the number of latently infected cells [36,41\*]. This is an intriguing observation but interpretation is complicated by at least two caveats. First, the high levels of preformed infectious  $\gamma$ HV68 found in MHC class II deficient mice [36] indicate that distinction between latent cells and low levels of preformed infectious virus in tissues will be particularly important to quantitate in MHC class II deficient mice. Second, since CD4<sup>+</sup> T cells regulate B cell function, and efficiency of reactivation is regulated by B cells (see above), effects of CD4<sup>+</sup> T cells on the number of genome<sup>+</sup> cells and the efficiency of reactivation will need to be determined.

#### Role of CD8<sup>+</sup> T cells

CD8<sup>+</sup> T cells play an important role in clearing acute  $\gamma$ HV68 infection [41\*,64]. However, CD8<sup>+</sup> T cells are not required for survival of acute infection although this may depend on the mouse strain and conditions of infection [41\*]. One striking aspect of the response of CD8<sup>+</sup> cells to  $\gamma$ HV68 infection is the dramatic expansion of V $\beta$ 4<sup>+</sup> CD8<sup>+</sup> T cells [71]. The mechanism underlying this selective V $\beta$  expansion and the physiologic importance of this phenomenon are currently undefined. Whereas several CD8<sup>+</sup> T cell epitopes have been identified in lytic cycle gene products [66\*\*,72] none of them selectively stimulates V $\beta$ 4<sup>+</sup> T cells. In addition, V $\beta$ 4<sup>+</sup> T cell expansion occurs in  $\beta$ 2m<sup>-/-</sup> mice, suggesting that this expansion does not require high-level surface expression of MHC class I [72]. The epitopes recognized by CD8<sup>+</sup> T cells shift over time, a phenomenon potentially related to different cell populations presenting epitopes with different efficiencies [72]. Interestingly, and consistent with B cells playing a major role in spread of  $\gamma$ HV68 and regulation of  $\gamma$ HV68 latency, B cells play a role in generating responses by CD8<sup>+</sup> T cells [72]. The mechanism by which CD8<sup>+</sup> T cells act is not known although one recent study suggests that perforin is

not required for control of acute  $\gamma$ HV68 infection [73]. However, cytotoxic CD8<sup>+</sup> T cells have been demonstrated during infection [59\*,66\*\*].

## Conclusions

Experimental advances over the past year have significantly altered our view of  $\gamma$ HV68 infection and latency and validate this system as a small-animal model that can be manipulated genetically and that will continue to provide novel insights into  $\gamma$ -herpesvirus pathogenesis. The fact that  $\gamma$ HV68 may share latency genes with KSHV and/or EBV bolsters the argument that studies in this small animal model will, in addition to being of fundamental interest in their own right, provide insights relevant to human  $\gamma$ -herpesviruses. The broad outlines of the nature of immunity during acute infection are now in place, with many mechanisms yet to be defined. The first information on the regulation of chronic infection by specific parts of the immune system (especially B cells and IFN- $\gamma$ ) is now available and opens an entirely new field of experimentation in which mechanistic studies relating chronic immune function to persistent and latent  $\gamma$ -herpesvirus infection can be performed. Recent advances in the experimental methodology for quantitating latent  $\gamma$ HV68 will provide a newly sophisticated approach to understanding mechanisms of latency. Identification of multiple cellular sites of latency provides both new questions and new models for understanding the dynamics of systemic latency *in vivo*. Identification of the functions of individual viral genes is in its infancy but will provide additional avenues for exploring mechanisms of chronic infection. Lastly, the diseases induced by  $\gamma$ HV68 — vasculitis [29], tumors [26] and splenic fibrosis [30] — provide the opportunity to link understanding of immunity and viral genetics to disease processes in a novel way.

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