

Natural history of murine γ -herpesvirus infection

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Murine γ -herpesvirus 68 (MHV-68) is a natural pathogen of small rodents and insectivores (mice, voles and shrews). The primary infection is characterized by virus replication in lung epithelial cells and the establishment of a latent infection in B lymphocytes. The virus is also observed to persist in lung epithelial cells, dendritic cells and macrophages. Splenomegaly is observed two weeks after infection, in which there is a CD4⁺ T-cell-mediated expansion of B and T cells in the spleen. At three weeks post-infection an infectious mononucleosis-like syndrome is observed involving a major expansion of V β 4⁺CD8⁺ T cells. Later in the course of persistent infection, ca. 10% of mice develop lymphoproliferative disease characterized as lymphomas of B-cell origin.

The genome from MHV-68 strain g2.4 has been sequenced and contains ca. 73 genes, the majority of which are collinear and homologous to other γ -herpesviruses. The genome includes cellular homologues for a complement-regulatory protein, Bcl-2, cyclin D and interleukin-8 receptor and a set of novel genes M1 to M4. The function of these genes in the context of latent infections, evasion of immune responses and virus-mediated pathologies is discussed.

Both innate and adaptive immune responses play an active role in limiting virus infection. The absence of type I interferon (IFN) results in a lethal MHV-68 infection, emphasizing the central role of these cytokines at the initial stages of infection. In contrast, type II IFN is not essential for the recovery from infection in the lung, but a failure of type II IFN receptor signalling results in the atrophy of lymphoid tissue associated with virus persistence. Splenic atrophy appears to be the result of immunopathology, since in the absence of CD8⁺ T cells no pathology occurs. CD8⁺ T cells play a major role in recovery from the primary infection, and also in regulating latently infected cells expressing the M2 gene product. CD4⁺ T cells have a key role in surveillance against virus recurrences in the lung, in part mediated through 'help' in the genesis of neutralizing antibodies. In the absence of CD4⁺ T cells, virus-specific CD8⁺ T cells are able to control the primary infection in the respiratory tract, yet surprisingly the memory CD8⁺ T cells generated are unable to inhibit virus recurrences in the lung. This could be explained in part by the observations that this virus can downregulate major histocompatibility complex class I expression and also restrict inflammatory cell responses by producing a chemokine-binding protein (M3 gene product).

MHV-68 provides an excellent model to explore methods for controlling γ -herpesvirus infection through vaccination and chemotherapy. Vaccination with gp150 (a homologue of gp350 of Epstein–Barr virus) results in a reduction in splenomegaly and virus latency but does not block replication in the lung, nor the establishment of a latent infection. Even when lung virus infection is greatly reduced following the action of CD8⁺ T cells, induced via a prime–boost vaccination strategy, a latent infection is established. Potent antiviral compounds such as the nucleoside analogue 2'-deoxy-5-ethyl-beta-4'-thiouridine, which disrupts virus replication *in vivo*, cannot inhibit the establishment of a latent infection. Clearly, devising strategies to interrupt the establishment of latent virus infections may well prove impossible with existing methods.

Keywords: murine γ -herpesvirus (MHV-68); latency; pathogenesis; vaccination; interferon; T and B lymphocytes

1. INTRODUCTION

γ -Herpesviruses have been identified in a range of animals from mice to man. They are host-range specific and have a propensity to establish a latent infection in lymphocytes. Much of our knowledge of the biology of γ -herpesviruses stems from studies of Epstein–Barr virus (EBV), including the molecular basis of latency and

transformation. However, despite recent progress on the molecular virology of Kaposi's sarcoma herpesvirus (KSHV) and *Herpesvirus saimiri* (HVS), there remains a great deal unknown about the activity of these viruses in their natural host. In particular, there is little known about the early events of γ -herpesvirus infection *in vivo*, the function of viral genes in the primary infection and in the transition to latency, the significance of sites of infection other than lymphocytes, and the interplay between the immune response and virus. With the introduction of

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murine γ -herpesvirus 68 (MHV-68), it is now possible to address many of these questions. The features of this virus make it an ideal small-animal model for understanding γ -herpesvirus infection of man and domestic animals (for reviews see Nash & Sunil-Chandra 1994; Simas & Efstathiou 1998; Virgin & Speck 1999).

2. THE ORIGINS AND ECOLOGY OF MURINE γ -HVs

MHV-68, or murine HV-4, was originally isolated from bank voles (*Clethrionomys glareolus*) in Slovakia during a study on the ecology of arboviruses (Blaskovic *et al.* 1980). During the survey two other related herpesviruses (MHV-60 and MHV-72) were isolated from bank voles and two viruses (MHV-76 and MHV-78) were isolated from wood mice (*Apodemus flavicollis*). One other isolate has been reported in a shrew (*Crocidura russula*), MHV-Brest (Chastel *et al.* 1994). This family of viruses is probably geographically widespread and may occur throughout the mouse and vole subfamilies. A recent survey of captured voles and mice on the Wirrell, Liverpool, UK identified herpesvirus DNA in 13 out of 14 wood mice and 6 out of 12 bank voles (J. P. Stewart and A. A. Nash, unpublished data). A more extensive survey in Slovakia identified the virus by antibody assays in five species of small rodents, fallow deer (*Dama dama*), wild boar (*Sus scrofa*) and sheep. Interestingly they also identified neutralizing antibodies in 8 out of 20 employees of the Institute of Virology, Slovak Academy of Sciences and the Comenius University, Bratislava (Mistrikova *et al.* 2000). This is the first evidence for 'infection' of man by murine γ -herpesvirus; however, caution is needed in interpreting such data in view of the potential for cross-reactive antibodies. It will now be important to investigate whether the sero-positive individuals carry the virus.

3. VIRUS ISOLATION AND GROWTH CHARACTERISTICS

The five viruses were originally isolated following the inoculation of diluted suspensions of various tissues (lung, spleen, liver, kidney and heart) into the brain of newborn mice. Different virus isolates were obtained from the brain of mice following either the first, second or third intracranial passage from mouse to mouse. These 'Slovakian' viruses caused cytopathic effects in epithelial and fibroblast cell lines from a variety of species ranging from chickens to primates (Svobodova *et al.* 1982). MHV-68 establishes a latent infection in NS0, a mouse myeloma cell line, but not in the thymoma cell line BW5147 (Sunil-Chandra *et al.* 1993). The virus is maintained in NS0 cells indefinitely as a latent infection, with *ca.* 5% of the cells undergoing reactivation and expressing lytic-cycle proteins. This scenario is similar to that seen for lymphoblastoid cell lines infected with EBV. Other murine B-cell lines can also be infected including B-cell hybridomas, the commonly used A20 cell line and S11, a B-cell line derived from a lymphoma obtained from an MHV-68-infected mouse (see § 10).

Primary mouse B cells are also efficiently infected with MHV-68 resulting in the appearance of clumps of enlarged, activated cells within three to four days (Dutia *et al.* 1999b; Stevenson & Doherty 1999). The cells do not

become transformed and usually die within two weeks. Dutia *et al.* (1999b) explored a number of protocols in order to enhance cell proliferation and transformation. These included addition of cytokines (murine interleukins IL-2, IL-4, IL-10), and/or lectins (phytohaemagglutinin and lipopolysaccharide) to conditioned medium, depletion of CD8⁺ T cells, infection of lymphocytes from transgenic mouse strains with a propensity to develop tumours (p53^{-/-}, Rb^{+/-}) and growth on CD40⁺ ligand-expressing fibroblasts. Some of these techniques prolonged the life of the cultures to six to eight weeks, but none resulted in established or immortalized cell lines.

Infection with MHV-68 did, however, cause measurable phenotypic changes in primary lymphocytes. In addition to increased cell proliferation, there was also an increase in B-cell survival compared with mock-infected cultures. The expression of B220 and major histocompatibility complex (MHC) class I was also increased in infected cultures. Transcription of virus-encoded transfer RNA-like structures (vtRNAs) was observed in all infected B cells, but there was no evidence of the lytic-cycle transcripts, e.g. TK or gH, or lytic-cycle antigens as determined by a polyclonal MHV-68 antiserum. Surprisingly, analysis of the DNA conformation in infected cells revealed that the virus DNA was linear. This linear DNA was nuclear and unencapsidated, since it was sensitive to DNase I treatment. Circularization of HV DNA normally occurs early after infection and is regarded as a prerequisite for viral DNA replication and establishment of latency. The failure of MHV-68 DNA to circularize in primary lymphocytes may underlie the inability of the virus to transform and/or replicate in these cell cultures.

4. FEATURES OF THE MHV-68 GENOME

The genome of MHV-68 consists of 118 kbp of unique DNA flanked by variable numbers of 1.23 kbp terminal repeat (TR) regions (Efstathiou *et al.* 1990a,b). The genome has been sequenced by two laboratories and contains *ca.* 73 protein-coding open reading frames (ORFs), the majority of which are collinear and homologous to those of other γ -herpesviruses (figure 1).

The complete DNA sequence of MHV-68 strain g2.4 and a comprehensive interpretation of genetic content have been determined by S. Milligan, S. Efstathiou, J. Stewart, A. Nash and A. Davison (unpublished data) and deposited in the GenBank database under accession number AF105037. The total size of the unique region (118 311 bp) plus one copy of the TR (1239 bp) is 119 550 bp.

Virgin *et al.* (1997) have published the genome sequence of MHV-68 strain WUMS (accession number U97553). Despite its name, this 'strain' was derived by limited passage and plaque purification of the g2.4 strain. As a consequence, the sequences of g2.4 and WUMS are very similar. The sequence of strain WUMS differs from that of strain g2.4 as follows. (i) Lack of a T residue at 4945, 2 bp upstream from the vtRNA-7 gene. This residue is also present in the sequence reported by Bowden *et al.* (1997). (ii) Lack of one copy of the 100 bp repeat element at 98 982–99 091. This region appears to be non-coding. Variability in the number of repeats in tandem reiterations is a well-known feature of herpesvirus genomes. (iii) Differences in the lengths of two tracts of G residues in TR at 118 355–118 366 and 118 903–118 904. These

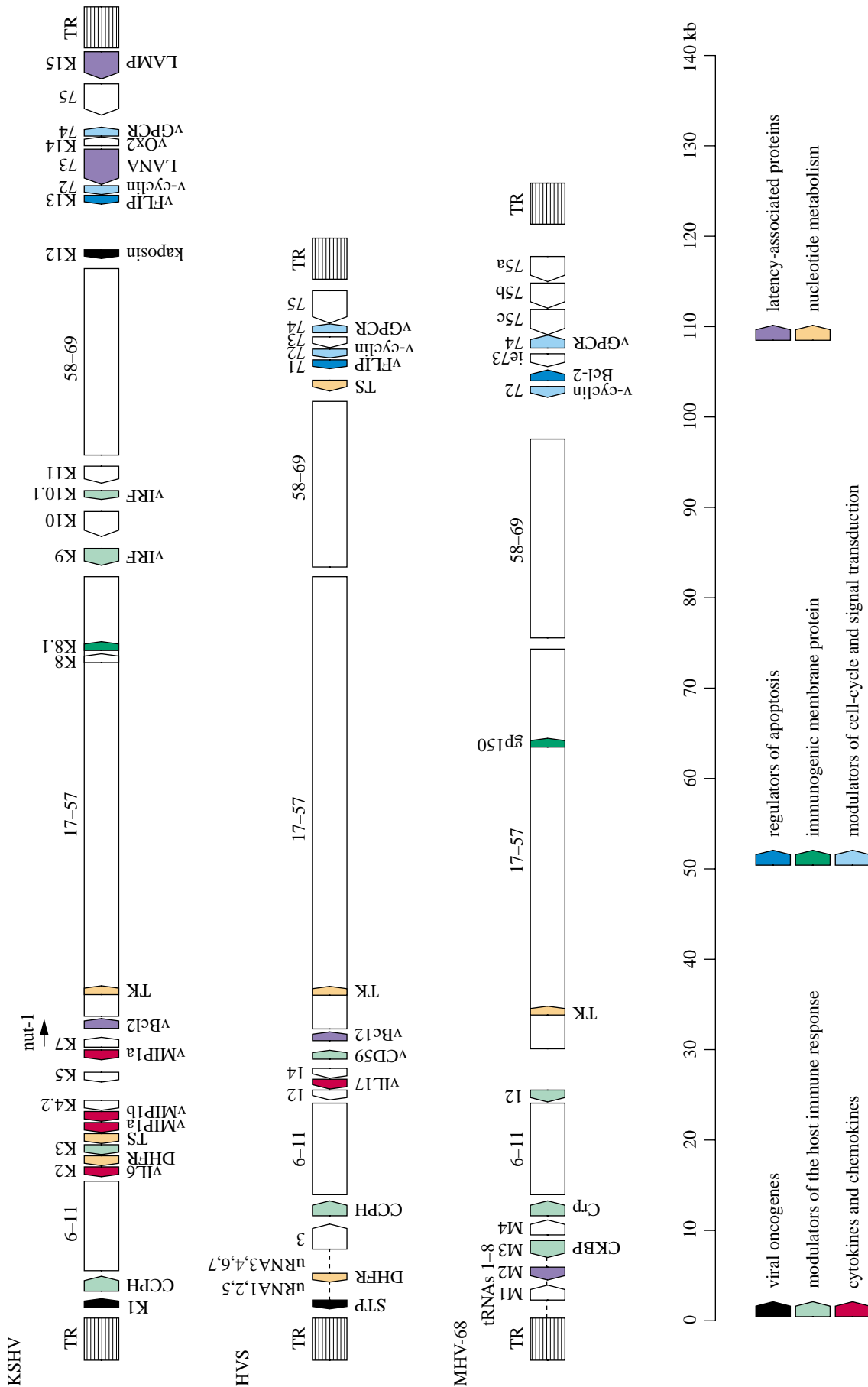


Figure 1. The organization of the MHV-68 genome is compared with that of HVS (Albrecht *et al.* 1992) and KSHV (Russo *et al.* 1996). The open boxes show herpesvirus core gene blocks. These are genes involved in DNA replication and virus structure. TR elements are shown by open bars. γ -herpesvirus-specific genes and functions are indicated above and below the genome structures. Functions are abbreviated as follows: CcPH, complement-control protein homologue; Crp, chemokine-binding protein; Crp, complement-regulatory protein; DHFR, dihydrofolate reductase; LAMP, latency-associated membrane protein; LANA, latent nuclear antigen; nut-1 is an abundant polyadenylated nuclear RNA; STP, saimiri-transforming protein; TK, thymidine kinase; TS, thymidine kinase; vFLIP, viral FLICE inhibitor protein; vGPCR, viral G-protein-coupled receptor; vIL17, viral IL-17; vIL6, viral IL-6; vIRF, viral IFN-regulatory protein; vMIP, viral macrophage inflammatory factor; vOx2, viral Ox2.

regions are variable in length in strain g2.4 and would cause frameshifts in ORFs M12 and M13.

Interpretation of the genome sequence of strain g2.4 resulted in identification of 73 strong candidates for viral genes. Differences from the interpretation of Virgin *et al.* (1997) are as follows. (i) The TR was identified as 1239 bp rather than 1213 bp. This interpretative error appears to have resulted from a lack of recognition of a direct repeat at the ends of the TR. (ii) Identification of four additional genes (17.5, 28, 57 and 67A). Gene 57 overlaps ORF M8 in an alternative frame, and was correctly identified by Mackett *et al.* (1997). (iii) Redefinition of the initiation codons of genes 47, 62, 63 and 68 based on sequence comparisons. The initiation codon of gene 57 identified by Mackett *et al.* (1997) was similarly redefined. (iv) Definition of splice sites for genes 29, 50 and 57. Splice sites for gene 57 were identified previously by Mackett *et al.* (1997). (v) Reclassification of genes M9, M7 and K3 as genes 65, 51 and 12, respectively, based on similarities with HVS counterparts. (vi) Downgrading of ORFs M5, M6, M8, M10a, M10b, M10c, M12, M13 and M14 to entities unlikely to encode proteins.

In order to add weight to the interpretation of the genetic content of MHV-68 at the left end of the unique region, cosmid libraries were derived from two related viruses, MHV-76 (host *Apodemus flavicollis*; Blaskovic *et al.* 1980) and MHV-Brest (host *Crocidura russula*; Chastel *et al.* 1994). The DNA sequence of the left end of the MHV-76 genome is identical to that of MHV-68, aside from a deletion of 9538 bp precisely at the left end, which results in loss of ORFs M1 to M4 and the eight vtRNAs. In contrast, the sequence of the corresponding region in the genome of MHV-Brest is similar to that of MHV-68, but sufficient differences exist to warrant classification of this virus as a separate species (murine HV-7).

5. MHV-68 GENOME CONTAINS HOMOLOGUES OF CELLULAR GENES AND A SET OF NOVEL VIRULENCE GENES

There are several functional homologues of cellular genes, such as bcl-2 (M11), IL-8 receptor (IL-8R; ORF 74), cyclin D (ORF 72) and a complement-regulatory protein (ORF 4). A bcl-2 homologue is associated with all the γ -herpesviruses so far studied. In the case of KSHV, EBV, MHV-68 and HVS the vBcl-2 is effective at preventing apoptosis when transfected into mammalian cell lines (Bellows *et al.* 2000; Henderson *et al.* 1993; Nava *et al.* 1997; Sarid *et al.* 1997; Wang *et al.* 1999). This gene appears early in the infectious cycle, suggesting that it is likely to be active during the productive infection. Recent data from our laboratory indicate that M11 is transcribed during the latent infection in lung and spleen (Roy *et al.* 2000). As with the other γ -herpesviruses, MHV-68 also has a number of genes unique to the virus. These are located at the left-hand end of the genome and include the genes M1 to M4 and eight vtRNAs (Bowden *et al.* 1997; Husain *et al.* 1999; Simas *et al.* 1998). The vtRNAs are transcribed during virus latency and serve as a useful marker for latently infected cells. The function of these products remains to be determined.

Sequence analysis of the MHV-76 genome reveals a deficiency in ORFs M1 to M4 and the eight vtRNAs.

This virus grows *in vitro* with the same efficiency as MHV-68. *In vivo*, MHV-76 is cleared more rapidly from the lung, compared with MHV-68, and does not induce splenomegaly. However, MHV-76 does establish a latent infection, albeit with a reduced efficiency (A. Macrae, unpublished data). This implies that this region of the genome has important determinants of pathogenicity. M1 has some limited homology to the poxvirus serpins and also to M3. Deletion of this gene has little or no obvious effect on the primary infection or on the establishment of latency in the spleen (Simas *et al.* 1998). However, there appears to be enhanced reactivation from a latent infection, implying that this gene is able to regulate the appearance of reactivating virus (Clambey *et al.* 2000). Interestingly, the M1-deleted virus does not mediate splenic pathology; however, in the absence of an M1 revertant in these experiments it is difficult to exclude the involvement of other gene(s) in this pathological process (see § 11(a)). M3 is an abundant protein found in the lytic and latent stages of MHV-68 infection. It is secreted from infected cells in large quantities and has a high affinity for specific members of the chemokine family (Parry *et al.* 2000; Van Berkel *et al.* 2000). This novel protein binds to all classes of chemokines, CC, CXC, C and CX3C, and functionally inhibits the ability of chemokines to signal through the host G-protein-coupled receptor. Whereas this factor has dramatic effects on inflammatory chemokine responses, the ability of B cells to home to germinal centres is not impeded. Less is known about the other two genes in this region, M2 and M4. M4 is another secreted protein that appears early in the lytic cycle, and preliminary data suggest that it is able to bind selectively to B cells and promote the survival of these cells *in vitro* (F. Wan, B. M. Dutia and A. A. Nash, unpublished data). M2 is a latency-associated antigen of unknown function (Husain *et al.* 1999). However, the observation that M2 is expressed in B cells only during splenomegaly may indicate a role for this gene product in the amplification of latently infected B cells and in the genesis of splenomegaly (Usherwood *et al.* 2000).

6. ENTRY AND SPREAD: FROM THE LUNG TO THE LYMPHOID SYSTEM

The natural route of infection is uncertain, but by analogy with other animal γ -herpesviruses the respiratory tract is likely to be a primary target. As noted above (§ 2) a recent survey of bank voles and wood mice in the UK revealed that all wood mice and 70% of bank voles harboured virus in the respiratory tract. Introducing virus intranasally into five- to six-week-old inbred mice results in a productive infection of alveolar epithelial cells, with some mononuclear cells in the lung also positive for late virus gene expression (Sunil-Chandra *et al.* 1992a). Infection of the lung is accompanied by bronchiolitis. During MHV-68 infection inflammatory responses evolve slowly in terms of the lung infection. In contrast, during MHV-76 infection there is a rapid inflammatory cell localization at sites of infection in the lung (A. Macrae, unpublished data). The difference between the two viruses could be attributed to the chemokine-binding activity of the M3 gene product delaying the onset of inflammation.

During MHV-68 infection the components of the inflammatory response, as determined by bronchoalveolar lavage (BAL), include an initial wave of macrophages, peaking at day 3, followed by a wave of CD8⁺ T cells peaking at day 7. Inflammation resolves by the second week, although focal accumulations of mononuclear cells are seen as late as day 30 in the lung, indicating persistence of antigen (Sunil-Chandra *et al.* 1992a).

From the lung the virus enters the local lymph node (the mediastinal lymph node (MLN)). Here dendritic cells, macrophages and B cells are infected and we believe that dendritic cells are responsible for initially transporting virus to the draining lymph node, where they seed B cells. Evidence supporting a role for dendritic cells in this process comes from studies using an MHV-68-green fluorescent protein recombinant virus and tracking infected cells in normal and in B-cell-deficient (μ MT) mice (S. Selvarajah, unpublished data). In the presence or absence of B cells, virus is detected in CD11c-positive dendritic cells and F4/80-positive macrophages in the MLN. Infection appears to be transient in the absence of B cells and there is little or no spread to other lymphoid compartments. We speculate that the MLN is the primary site for B-cell infection and that B cells are the principal cell population responsible for disseminating virus within the host. Tropism of MHV-68 for B cells may be related to the presence of gp150 on the virion envelope. Experiments using gp150-His fusion protein demonstrated binding to CD19⁺ (B cells) and to some CD19⁻ spleen cells. However, there was no interaction between gp150 and murine epithelial cells (I. Atkin, unpublished data). Upon infection in the MLN, B cells undergo a rapid expansion accompanied by an increase in the number of latently infected B cells. From the MLN, B cells traffic to the spleen and other lymphoid compartments carrying the virus. By the second week of infection a similar rapid expansion of latently infected B cells is observed in the spleen. The number of latently infected cells increases from 1 per 10⁷ to 1 per 10⁴ spleen cells in the space of a few days and then the numbers return to around 1 per 5 × 10⁵ to 1 per 10⁶ by the third or fourth week of infection (Sunil-Chandra *et al.* 1992a; Usherwood *et al.* 1996a).

B-cell proliferation and hence the number of latently infected cells observed during lymphadenopathy and splenomegaly is controlled by CD4⁺ T cells (Usherwood *et al.* 1996a). This observation argues in favour of the virus exploiting T-B-cell collaboration to its advantage, for example by using B-cell proliferation as a means to maximize the number of latently infected B cells. However, the virus clearly does not have to rely on CD4⁺ T-cell expansion of B cells to establish and maintain virus latency. In the absence of CD4⁺ T cells there is no splenomegaly or amplification of latently infected B cells; however, the virus still engages B cells and establishes a latent infection. As with EBV it is likely that the virus has the capability to directly manipulate B-cell growth and differentiation in order to establish a latent infection. A remarkable feature of MHV-68 latency is the constant number of latently infected cells (*ca.* 1 per 5 × 10⁵) found in the spleen for the life of the animal. This number is established whether CD4⁺ T cells are present or not in the host.

We know that B cells and CD4⁺ T cells are absolutely required for the evolution of splenomegaly, which suggests that cognate interactions occur between these cells, similar to that for any other antigenic response. During splenomegaly there are large increases in both B- and T-cell populations (Usherwood *et al.* 1996c). Germinal centres increase in number and size and act as the principal location for latently infected cells. By the third week of infection there is an increase in the number of circulating lymphocytes, dominated by V β 4⁺CD8⁺ T cells (Tripp *et al.* 1997). This phase of the infection is similar to infectious mononucleosis caused by EBV. The mechanism for this selective increase in V β 4 usage is not known, but indicates a form of superantigen-driven proliferation (Hardy *et al.* 2000).

7. VIRAL GENE EXPRESSION DURING LATENT INFECTION

Viral gene expression changes dramatically from infection in the lung to infection in the spleen. As the virus enters into the latent state there is a progressive shut down of gene expression. In MHV-68 infection this depends to a large extent on the cellular basis of latency. Four cell types have been implicated in maintaining the latent state: B cells, dendritic cells, macrophages and epithelial cells in the lung (Flano *et al.* 2000; Stewart *et al.* 1998; Sunil-Chandra *et al.* 1992b; Weck *et al.* 1999). In the lymph node and spleen, M2 expression appears to be transient, appearing only when the virus enters the lymphoid compartment and lasts for around two weeks. M3 is expressed for much longer (up to ten months) in the spleen, but is also highly expressed during the productive infection where it plays a key role in disrupting inflammatory cell responses. Other latency-associated genes expressed include ORF 73 (homologous to latent nuclear antigen of KSHV), ORF 74 (G-protein-coupled receptor, GCR) and M11 (Bcl-2 homologue) (Virgin *et al.* 1999). These three genes are also expressed in lung epithelial cells for well over 100 days post-infection (Roy *et al.* 2000; D. Roy and J. P. Stewart, unpublished data). This argues strongly in favour of the respiratory tract as a major site of persistence for this virus.

8. MANIPULATION OF THE PRODUCTIVE AND LATENT INFECTION USING ANTIVIRAL CHEMOTHERAPY

MHV-68 is susceptible to antiviral agents that target thymidine kinase and DNA polymerase enzymes (Barnes *et al.* 1999; Sunil-Chandra *et al.* 1994b; Neyts & De Clercq 1998). This includes acyclovir and the thiopyrimidine analogue 2'-deoxy-5-ethyl-beta-4'-thiouridine (4'-S-EtdU). This compound exhibits potent antiviral activity *in vitro* and rapidly inactivates MHV-68 replication in the lungs of mice when given three days post-infection (Barnes *et al.* 1999). However, as with other nucleoside-based antiviral compounds, 4'-S-EtdU was unable to prevent the establishment of viral latency, despite delaying the onset of the latent infection in the spleen.

In order to explore further the effect of antivirals on the establishment of virus latency, Barnes *et al.* (1999) investigated the prophylactic use of 4'-S-EtdU during

MHV-68 infection. Mice were given 4'-S-EtdU prior to infection and then divided into two groups. One group was maintained on the antiviral for the course of the experiment and the second group had the antiviral treatment withdrawn at day 12. In both groups of mice there was no evidence of a productive infection in the lung, no splenomegaly and no latent infection in the spleen detected up to day 20. In the absence of any productive infection it was important to determine whether the virus was able to persist in the lung. Interestingly, using a sensitive co-cultivation assay, virus was recovered from the lungs of mice from day 20 onwards in the group withdrawn from 4'-S-EtdU after 12 days, but not from the group of mice receiving continuous drug treatment. However, despite the failure to recover infectious virus, viral DNA was detected in the lungs of mice undergoing continuous drug treatment for up to at least 54 days after the primary infection. Latent virus was also detected at day 31 in spleen cells from mice withdrawn from drug treatment and these mice developed a delayed antibody response. The implication of this study is that virus can infect the lung and be retained at this site indefinitely, despite the presence of the antiviral compound. However, when the antiviral block is released then the infection proceeds and persistence or latency is established in the lung and spleen.

9. INFECTION IN THE IMMUNOCOMPROMISED HOST

When young (two- to three-week-old) or immunocompromised mice are infected experimentally the virus can spread via the bloodstream to other tissues, where it undergoes a productive infection (Kulkarni *et al.* 1997; Sunil-Chandra *et al.* 1992a). This includes the heart, kidney, liver, adrenal gland and peripheral nervous system, e.g. trigeminal ganglion. The central nervous system can also become infected when virus is introduced intracranially or following infection of mice deficient in the type I interferon (IFN) receptor (R) (IFNR^{-/-}) (Terry *et al.* 2000). Both glial and neuronal cells are observed to undergo a productive infection. These data argue in favour of a promiscuous virus infection, which is supported by recent observations on the infection of peritoneal exudate cells (Weck *et al.* 1999) and epithelial cells in the gut (Peacock & Bost 2000).

10. MHV-68-INDUCED LYMPHOPROLIFERATIVE DISORDERS

BALB/c mice infected with MHV-68 for periods of nine months or longer have been shown to develop lymphomas. In one study, *ca.* 10% of infected mice developed tumours in both lymphoid and non-lymphoid tissue (lung, liver, kidney and heart), of which 50% were classified as high-grade lymphomas (Sunil-Chandra *et al.* 1994a). In a separate investigation the frequency of mice with tumours increased to over 50% following treatment with the immunosuppressive drug, cyclosporin A. However, despite the increase in the number of positive animals the kinetics of induction did not change with a median time of 14 months to detect tumours. The tumours in both experiments were of mixed cell phenotype with CD3⁺ T cells interspersed amongst B220⁺ B

cells. The B cells were either κ or λ light chain restricted, suggesting a clonal origin of the B-cell population. MHV-68 DNA-positive lymphocytes were found interspersed in the tumour cell mass or on the fringes of lymphomas. In some animals the number of virus genome-positive cells was low, whereas in others there were huge numbers of genome-positive cells. The infected cells were not positive for lytic-cycle proteins, suggesting that virus reactivation was not occurring in these mice. Studies with more sensitive probes for latent transcripts are now possible and may clarify the role of the virus further.

BALB/c mice infected with MHV-72 also develop tumours with a frequency similar to those infected with MHV-68. The number of tumour-bearing mice increased following immunosuppression with the antifungal agent, FK-506 (Mistrikova *et al.* 1996, 1999). In 5 out of 13 neoplasia-positive mice, virus was isolated directly from the tumours.

A number of B-cell lines have been established from MHV-68-infected tumour-positive mice, of which S11 is the best characterized. This IgM⁺, MHC class II⁺ B-cell line harbours the virus in a latent form, as demonstrated by the predominance of a circular genome (Usherwood *et al.* 1996b). As with lymphoblastoid cell lines derived from EBV infection, S11 has around 2–5% of cells expressing lytic antigens. *In situ* hybridization of S11 revealed that vtRNA and M2, but not M3, were expressed in virtually all latently infected cells (Husain *et al.* 1999). S11 establishes tumours when transferred to nude mice and has been used to dissect the immunological mechanisms involved in targeting tumour cell growth. In a series of adoptive transfer experiments of MHV-68-specific CD8⁺ and CD4⁺ T cells into S11 tumour-bearing nude mice, regression of tumour cell growth was effectively achieved by CD4⁺ T cells but, surprisingly, not with CD8⁺ T cells. The mechanism of protection probably involves a delayed-type hypersensitivity (DTH) response, since macrophages feature prominently in the tumour mass (Robertson *et al.* 2001).

The molecular basis for tumour cell induction is not known. Cell lines adapted from lymphomas in mice have multiple chromosome rearrangements, and in the situation where viral DNA was detected in such cells, it is tempting to speculate that virus could initiate tumorigenesis by a hit-and-run mechanism. A number of candidate viral genes exist which could initiate cell transformation, including ORF 72 (cyclin D homologue), ORF 74 (IL-8 receptor/GCR homologue) and M11 (Bcl-2 homologue). Transgenic mice expressing the cyclin D homologue under the control of the *lck* promoter, which is active early in thymocyte development, showed increased numbers of immature thymocytes. Forty-five per cent of these mice develop high-grade lymphoblastic lymphomas, the majority of which are localized in the thymus. These mice also show decreases in the numbers of mature T cells and an increase in thymic apoptosis, supporting the notion that v-cyclin may require the involvement of other factors to promote cell survival and tumour formation (Van Dyk *et al.* 1999). Transfection of the GCR homologue into 3T3 cell lines leads to the establishment of stable transformed cells. These cells do not, however, develop into tumours in nude mice. Bcl-2 is expressed during the latent phase and is highly efficient at

preventing cell death via such immunological mechanisms as tumour necrosis factor- α (TNF- α) and Fas–Fas ligand interaction (Wang *et al.* 1999; Roy *et al.* 2000). It seems likely that *in vivo* a number of genes act in concert to promote cellular proliferation, survival and tumour formation.

11. INNATE AND ADAPTIVE IMMUNE RESPONSE TO MHV-68

Both the innate and adaptive immune responses are involved in the recovery from MHV-68 infection. As noted above (§6) the virus presents a moving target for host defences as it traffics from the lung to lymphoid tissue and from a productive infection to latency. The virus's survival strategy also makes it difficult for the immune system to intervene during virus latency, due to a shut down of virus gene expression and through the use of decoy and other virus proteins that subvert host defences during the productive infection. In studying the immune response to MHV-68 infection, transgenic mice have been used with selective defects in the immune system. This powerful approach has been instrumental in defining key immunological mechanisms active against the virus.

(a) *The role of IFNs in MHV-68 infection*

As with many virus infections the type I IFN system is important in controlling the initial stages of MHV-68 infection. Using type I IFNR^{-/-} mice, an aggressive infection of the lung ensues with a rapid dissemination to lymph nodes and the bloodstream (Dutia *et al.* 1999a). Mice succumbed to the infection at doses of 10⁵ plaque-forming units with a mortality of 80–90%. At post-mortem the lungs of mice at eight days post-infection showed signs of focal haemorrhage and patchy necrosis. The spleens of the same mice showed a marked increase in the number of productively infected cells in the red and white pulp, indicative of an uncontrolled spread of virus from the lung. A similar picture was observed using mice deficient in interferon regulatory factor (IRF)-1, an important factor in the activation of the type I IFN response. The dramatic events seen in type I IFNR^{-/-} mice indicate a crucial role for IFN in checking the initial infection and in limiting spread to other tissues. The mechanism whereby this protection is achieved is currently not known.

In mice deficient in the type II IFNR an entirely different course of infection is observed (Dutia *et al.* 1997). In contrast to the events in the lung of infected type I IFNR^{-/-} mice, there is no difference between normal and type II IFNR^{-/-} mice in terms of virus growth and lung pathology. However, there are dramatic changes in the spleen and MLN of infected type II IFNR^{-/-} mice, culminating in atrophied tissue, a loss of lymphoid architecture and extensive fibrosis. Between days 14 and 21 there is a dramatic reduction in the number of B- and T-cell populations in the spleen. At the same time there is a four- to fivefold increase in the number of lymphocytes entering the blood. In defining the underlying causes of this pathology the following observations were made. (i) Not surprisingly there is a major perturbation of the cytokine and chemokine response. There are very high

levels of IFN- γ and little or no production of the chemokines IP-10 and Mig. The pro-inflammatory cytokines IL-1 β and TNF- α are also increased. However, despite the loss of IFN- γ signalling there is no preferential induction of Th2 responses. The fact that IP-10 is important in Th1-mediated DTH responses could account in part for the splenomegaly seen in the normal infection. Conversely, loss of this chemokine will influence cell trafficking into the spleen and may contribute to the lymphocytosis in the blood (B. Ebrahimi, unpublished observations). (ii) There is an increase in the number of latently infected cells in the spleen but there is no apparent productive infection that might result in damage through virus-mediated cytotoxic effects. (iii) There is an increase in the number of apoptotic cells, but cell death alone does not account for the loss of cells seen in the spleen between days 14 and 21. (iv) Depletion of either CD8⁺ or CD4⁺ T cells prior to infection of type II IFNR^{-/-} mice results in a complete reversal of the pathology, clearly suggesting an immunopathological component underlying splenic atrophy (Dutia *et al.* 1997). How T cells contribute to the pathology is unclear, particularly as the numbers of both CD4⁺ and CD8⁺ T-cell subsets are reduced from day 14 onwards. (v) The search for viral proteins that could contribute towards the pathology has revealed a role for genes in the left-hand end of the genome. Recombinant viruses lacking either M1 or vtRNA-3 fail to induce pathology, despite showing normal growth characteristics in the lung and comparable levels of latent infection and splenomegaly to wild-type virus. In summary, there appears to be no clear-cut answer to the genesis of splenic atrophy. The failure to respond to IFN- γ responses in the lymphoid compartment results in CD8⁺ T cells mediating auto-aggressive behaviour.

(b) *The role of CD4⁺ T cells in protective immunity and immunopathology*

CD4⁺ T cells occupy a dominant role in the immune response by aiding the evolution of protective antibody responses and in Th1-mediated inflammatory responses. While the absence of CD4⁺ T cells during MHV-68 infection makes little difference to the control of virus replication in the lung, it has a major effect on the development of splenomegaly (Ehtisham *et al.* 1993; Usherwood *et al.* 1996a). Both splenomegaly and the amplification of latently infected B cells appear to be driven by CD4⁺ T cells. Aside from aiding the virus in establishing a latent infection in the appropriate lymphoid compartments, CD4⁺ T cells are also needed to mature the antibody response to the virus by driving B-cell proliferation and maturation (Sangster *et al.* 2000). During this active phase of proliferation in the spleen there is a marked increase in cytokine levels, notably IFN- γ and IL-6 (Sarawar *et al.* 1996). IFN- γ has a dramatic effect on maintaining the integrity of the spleen during infection, whereas IL-6 does not have a vital role to play since in its absence splenomegaly develops normally, along with the antiviral antibody response (Sarawar *et al.* 1998).

CD4⁺ T cells play an important role in immune surveillance, as highlighted in studies on MHV-68-infected MHC class II-deficient mice (Cardin *et al.* 1996).

Whereas these animals are able to control an acute infection of the lung, they fail to make IgG antiviral antibodies, they do not induce splenomegaly and consequently have a reduced incidence of latently infected B cells. MHC class II-deficient mice are prone to recurrent viral infection in the lung. The infection is largely unresolved and progresses towards a chronic lung disorder. This disease occurs despite the presence of an active CD8⁺ T-cell response. Even augmenting the cytotoxic T lymphocyte (CTL) response by immunizing mice with the protective T-cell epitopes p56 and p79 failed to inhibit virus reactivation and the subsequent chronic lung disease (Belz *et al.* 2000). Two explanations for the evolution of virus recurrences and chronic disease are possible. One involves the importance of antibody in suppressing the emergence of reactivating virus. The second involves a direct role for CD4⁺ T cells in maintaining the persistent state through antiviral cytokine activity and/or the recruitment of macrophages. Support for antiviral antibodies comes from experiments comparing levels of virus reactivation or recurrences in lungs of μ MT mice with those seen in normal mice. Removal of CD8⁺ T cells from μ MT mice led to a low level of recurrent virus, whereas removal of both CD4⁺ and CD8⁺ T cells led to a large increase in infectious virus in the lung (Stewart *et al.* 1998). In contrast, depletion of CD4⁺ and CD8⁺ T cells in normal infected mice did not lead to recurrence of virus. As there is no antiviral antibody response in μ MT mice then it is logical to conclude that antibody was a key factor in immune surveillance against the persistent lung infection. It is worth noting that CD4⁺ T cells do remain in an activated state during the latent infection as determined by the frequency of CD62^{lo}-positive cells, indicating a continual turnover of this cell population (Stevenson *et al.* 1999c).

(c) **CD8⁺ T cells as regulators of the acute and latent infection**

The importance of CD8⁺ T cells in controlling MHV-68 infection in the lung was demonstrated in mice deficient in CD8⁺ T-cell responses. Depletion of CD8⁺ T cells prior to infection resulted in a severe infection in the lung and dissemination of virus via the blood to infect spleen, liver and adrenal gland (Ehtisham *et al.* 1993). The timing of CD8⁺ T-cell depletion during the primary infection influences the extent of infection in the lung and spleen (K. A. Robertson and A. A. Nash, unpublished data). Removal of CD8⁺ T cells at three or five days after infection resulted in a delayed elimination of infectious virus from the lung and a productive infection in the spleen, with higher levels of latent virus infection recorded. Depleting CD8⁺ T cells at day 7 did not influence the course of the primary infection in the lung or the infection in the spleen; however, a recurrent infection was noted in the lung at day 22 and persisted to at least day 30. This observation is similar to the recurrent infection seen in MHC class II-deficient mice, but these mice have an efficient CD8⁺ T-cell response and a deficient CD4⁺ T-cell response. Not surprisingly depletion of both CD4⁺ (before infection) and CD8⁺ T cells (at either three or seven days post-infection) resulted in a lethal infection. In experiments on CD8⁺ T-cell-deficient mice, recovery

from infection with MHV-68 depends upon the dose of virus inoculated intranasally and the age of mice (Stevenson *et al.* 1999c).

The evolution of the CD8⁺ T-cell response to MHV-68 has been investigated by Stevenson *et al.* (1999a,c), using a combination of limiting dilution analysis, MHC class I tetramer staining and IFN- γ assays to determine the frequency of virus-specific T cells. They identified two MHC class I peptides involved in specific T-cell responses in infected tissues: p56 (AGPHNDMEI) from ORF 6 (single-stranded DNA-binding protein) is H-2D^b-restricted and p79 (TSINFVKI) from ORF 61 (large ribonucleotide reductase subunit) is H-2K^b-restricted. CD8⁺ T cells recognizing p56 and p79 were identified at high frequencies in the lung up to 22 days post-infection, following BAL. These peptide-specific T cells were also present at day 81, indicating retention or a constant trafficking of virus-specific CD8⁺ T cells to monitor the persistent or latent MHV-68 infection. CD8⁺ T cells recognizing p56 and p79 were also observed in mice deficient in MHC class II, implying that CD8⁺ T-cell responses are induced and maintained in the absence of CD4⁺ T-cell help (Stevenson *et al.* 1998). A surprising feature of this study was the modest increase in CD8⁺ T cells in the lung of class II-deficient mice undergoing viral recurrence. As noted above, despite the continued presence of a memory CTL response, this alone was insufficient to prevent virus from re-emerging and causing disease. A possible explanation for the failure to target recurrent infections could be related to virus-evasion strategies. As already noted the M3 gene product could inhibit the migration of T cells to target areas by inhibiting chemokine expression. Another possibility is the reduction of MHC class I expression linked to the inhibitory properties of the K3 gene product (Stevenson *et al.* 2000).

The M2 gene product is expressed during latency in the MHV-68-infected B-cell line, the S11 B-cell line and in latently infected B cells in the spleen. S11 has been used to generate CD8⁺ T-cell lines, one of which recognized an epitope from the M2 gene product, M2₉₁₋₉₉ (GFNKLRLSTL). CTLs taken from the spleen 18 days post-infection were also shown to recognize the M2 epitope. This indicates that T-cell surveillance of latent MHV-68 antigens occurs and may be an important factor in regulating the numbers of latently infected B cells during the second and third weeks of infection (Husain *et al.* 1999).

12. EXPLORING VACCINATION STRATEGIES AGAINST γ -HERPESVIRUSES USING THE MHV-68 SYSTEM

MHV-68 represents an important model in defining novel vaccination strategies against human and animal γ -herpesviruses. At least it could establish the efficacy of glycoprotein vaccines such as MHV-68-gp150, a homologue of gp350 (Stewart *et al.* 1996) the EBV vaccine currently in clinical trials, as well as candidate CTL vaccines targeted at the acute or latent infection.

To investigate the role of gp150 as an immunogen, Stewart *et al.* (1999) developed a gp150-vaccinia recombinant. A neutralizing antibody was induced, but the immunized mice failed to control the infection in the lung. However, vaccination did dramatically inhibit

splenomegaly (mononucleosis) and reduced the peak of latently infected B cells seen during the second week of infection. It is possible that antibody is a major factor in limiting the spread of virus to B cells, thereby reducing the number of latently infected cells trafficking to the spleen and consequently reducing the stimulus important in the induction of splenomegaly.

CD8⁺ T cells are important for the rapid elimination of the productive MHV-68 infection in the lung and presumably may have an important role to play in monitoring latently infected cells. A knowledge of the two CTL epitopes, p56 and p79, has led to investigations on the efficacy of these epitopes at inducing protective immunity. In one study, dendritic cells were pulsed *in vitro* with the peptides and used to immunize mice (Liu *et al.* 1999). Upon challenge with a low dose of MHV-68, a 10- to 100-fold reduction in the lung virus titres was noted at day 6, indicating an effector T-cell response was generated. Although this immunization protocol did not prevent the establishment of a latent infection, the results from one time-point indicated a reduction in the level of virus latency. In a similar study, this time using recombinant vaccinia or influenza viruses expressing the peptides' epitopes, a similar outcome was achieved. However, by using a prime-boost strategy (vaccinia followed by influenza virus recombinants) a massive p56 CTL response was achieved, which afforded almost complete protection in the lung. Even with this overwhelming protection in the lung the virus was still able to establish a latent infection, and to mediate the infectious mononucleosis-like syndrome (Stevenson *et al.* 1999b). Interestingly in neither of these studies was the persistent or latent infection examined in the lung.

It is apparent from these studies that successful vaccination strategies against γ -herpesviruses will rely on identifying CTL epitopes of latent antigens. A candidate antigen is the M2 gene product, from which a dominant CTL epitope has already been identified (Husain *et al.* 1999), and infusion of M2 epitope-specific CTLs results in a dramatic decrease in latent virus load in the spleen (Usherwood *et al.* 2000). The way forward may lie in coupling this epitope with those of the lytic-cycle antigens (ORFs 6 and 6l) and possibly an antibody target (gp150) and again examining the frequency of latent infection and of the mononucleosis syndrome.

13. CONCLUSIONS

MHV-68 has provided an important model system to explore many facets of the biology of γ -herpesvirus infections in the host. From the studies to date a number of conclusions can be made that impact on other γ -herpesvirus infections. The early events in MHV-68 infection indicate a targeting of the virus to alveolar epithelial cells, which undergo a productive infection, but which also have the ability to sustain a persistent or latent virus infection. There may be parallels with nasopharyngeal EBV infections where the mechanism of viral persistence still remains largely unexplored. Evidence from MHV-68 indicates a prominent role for viral Bcl-2 during the alveolar infection, possibly preventing epithelial cell death by immunological mechanisms. This may be one reason why, in the absence of a CD4⁺ T-cell-antibody

response, virus recurrences arise and remain unchecked by CTLs that normally function by delivering apoptotic death signals. The failure of CD8⁺ T cells to control a recurrent infection contrasts with their ability to control virus replication in the lung during the primary infection. In order to resolve these differences more information is needed on the mechanism of virus persistence in the lung.

The interaction of virus with B cells results in the establishment of a latent infection. In the lymph node and spleen, B cells undergo proliferation as part of the ongoing immune response to the virus. As a consequence B cells latently infected with MHV-68 also expand, ensuring that the virus is carried along the B-cell differentiation pathway towards memory B cells and/or plasma cells. Interestingly, latent infections in the spleen are still established in the absence of CD4⁺ T-cell responses indicating that the virus may directly influence B-cell proliferation and differentiation. The fact that the M2 gene product is transiently expressed during splenomegaly implies a key role for this process in the establishment of virus latency. Transient expression may be related to the fact that M2 is a target for CTL responses, which may indicate why there is a dramatic decline in the numbers of latently infected B cells following the decline in splenomegaly. The vtRNAs are another marker for latency and clearly highlight germinal centres in the spleen and lymph node as the main sites of latent infection. The establishment of latency in B cells is presumably a dynamic process relying on reinfection of the B-cell pool, most probably from the lung, to reinforce reservoirs of latency in the host. The lung could certainly fulfil this role since reintroducing normal B cells into μ MT mice that have a persistent lung infection, results in the establishment of latently infected B cells. The converse is also true, namely introducing latently infected B cells into non-infected μ MT mice results in infection of the lung. This reinforces the idea that B cells can disseminate virus to other tissues during the primary and latent infection.

During the primary infection, and presumably during recurrences, the virus has evolved some novel strategies to dampen immune responses. One strategy involves inhibiting the function of inflammatory chemokine responses through the activity of a chemokine-binding protein. The failure of memory CD8⁺ T cells to localize to sites of virus recurrences could involve this protein. Equally, downregulation of MHC class I molecules by the K3 gene product could also impact on the failure of CD8⁺ T cells to recognize recurrent lesions. These subversive anti-host defence molecules give the virus an important window of opportunity to breach the immune response and spread to other compartments. Assuming that similar events occur with other γ -herpesviruses, then it makes vaccination against latent infections a difficult goal to achieve. The evidence to date suggests that neither immunization with gp150 nor p56 and/or p79 CTL peptides prevents the establishment of a latent infection.

The challenges ahead with this important virus model lie in defining viral gene expression *in vivo* using knock-out or knock-in recombinant viruses. Only then will it be possible to define host-cell functions that can be harnessed to counter virus persistence via antiviral compounds or through vaccination.

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