Immune control of mammalian gamma-herpesviruses: lessons from murid herpesvirus-4

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Many acute viral infections can be controlled by vaccination; however, vaccinating against persistent infections remains problematic. Herpesviruses are a classic example. Here, we discuss their immune control, particularly that of gamma-herpesviruses, relating the animal model provided by murid herpesvirus-4 (MuHV-4) to human infections. The following points emerge: (i) CD8+ T-cell evasion by herpesviruses confers a prominent role in host defence on CD4+ T cells. CD4+ T cells inhibit MuHV-4 lytic gene expression via gamma-interferon (IFN-γ). By reducing the lytic secretion of immune evasion proteins, they may also help CD8+ T cells to control virus-driven lymphoproliferation in mixed lytic/latent lesions. Similarly, CD4+ T cells specific for Epstein–Barr virus lytic antigens could improve the impact of adoptively transferred, latent antigen–specific CD8+ T cells. (ii) In general, viral immune evasion necessitates multiple host effectors for optimal control. Thus, subunit vaccines, which tend to prime single effectors, have proved less successful than attenuated virus mutants, which prime multiple effectors. Latency-deficient mutants could make safe and effective gamma-herpesvirus vaccines. (iii) The antibody response to MuHV-4 infection helps to prevent disease but is suboptimal for neutralization. Vaccinating virus carriers with virion fusion complex components improves their neutralization titres. Reducing the infectivity of herpesvirus carriers in this way could be a useful adjunct to vaccinating naive individuals with attenuated mutants.

Herpesviruses present complicated and difficult immune targets

Parasitism is ubiquitous and the immune systems of multicellular organisms work mainly to reduce their parasite loads. Vaccination can improve immune protection further by pre-empting natural infection; a major aim of immunology is to deliver safe and effective vaccines. Epidemic viruses rely on rapid lytic replication to transmit before host immunity becomes effective. Blunting their lytic replication can therefore protect both individuals and populations. Neutralizing antibodies provide the best defence (Zinkernagel & Hengartner, 2006). Since these generally act by blocking receptor binding (Skehel & Wiley, 2000), a vaccine that reproduces viral receptor binding epitopes generally elicits good protection. We have much less idea how to protect against viruses that persist: because they use immune evasion to extend host colonization and transmission far beyond the narrow window that precedes adaptive immunity, blunting lytic replication confers only limited protection.

The herpesviridae account for many persistent mammalian infections. Gamma-herpesviruses persist mainly in lymphocytes, beta-herpesviruses in monocytes and alpha-herpesviruses in neurons. These different reservoirs have some bearing on immune recognition; for example, gamma-herpesvirus persistence requires intermittent viral protein synthesis (Moorman et al., 2003; Fowler et al., 2003). Nevertheless, the immune control of different herpesviruses reveals many common themes. Human experiments are difficult to perform and rarely reveal mechanisms, so much of our knowledge of immune control has come from other species, predominantly mice. Unfortunately, viral immune evasion critically affects the host–parasite balance and tends to be host-restricted, making murine infections with human herpesviruses problematic. Equivalent pathogens that behave more normally in mice provide a useful alternative. These cannot be used to test specific reagents but, by revealing basic principles, they allow us to plan better clinical trials.

Alpha-herpesvirus study has the longest history, as herpes simplex virus (HSV) is readily isolated from patients, propagates well in vitro and can infect mice. The mouse model played a major role in the development of antiviral chemotherapy (Klein, 1982). However, it has limitations, notably in immunological analysis; viral immune evasion is an integral part of host colonization (Yewdell & Hill, 2002) and many HSV immune evasion mechanisms simply do not work in mice. Without an equivalent murid alpha-
Adoptively transferred, EBV-stimulated T cell populations, its narrow species tropism has severely constrained mammalian herpesviruses. Although Epstein–Barr virus continues to provide a major challenge. The gamma-individual host and viral genes to this equilibrium continues to provide a major challenge. The gamma-herpesviruses were, for many years, the least understood mammalian herpesviruses. Although Epstein–Barr virus (EBV) was discovered almost 50 years ago (Epstein et al., 1964), its narrow species tropism has severely constrained functional analysis. This all changed with the discovery of murine herpesvirus-4 (MuHV-4; archetypal strain MHV-68) (Blaskovic et al., 1980); its relative simplicity (compared with MCMV) and its capacity to establish a realistic infection in inbred mice have enabled rapid progress, and our knowledge of gamma-herpesvirus pathogenesis is now at least as complete as for the alpha- and beta-herpesviruses. This review therefore concentrates on the gamma-herpesviruses and MuHV-4.

**Gamma-herpesviruses: does CD8+ T-cell-mediated control explain the pathogenesis of human infections?**

Two human gamma-herpesviruses are known, EBV and the Kaposi’s sarcoma-associated herpesvirus (KSHV), both of which can cause disease. However, EBV is both more prevalent and more frequently pathogenic. It has also been studied much more intensively than KSHV. Thus, the broad picture of EBV is well-defined – transmission via saliva, an acute, self-limiting, lymphoproliferative illness and persistence in circulating memory B cells – while that of KSHV, while probably similar, is not. Of the human gamma-herpesviruses, therefore, we consider mainly EBV.

Latent EBV can cause tumours in vivo and transforms primary B cells in vitro. Immunological analysis has consequently focussed on the recognition of viral latency gene products expressed in transformed B cells, mainly by CD8+ T cells (Rickinson & Moss, 1997). B-cell differentiation seems to terminate the proliferation of many EBV-infected B cells in vivo (Babcock et al., 1999; Hadinoto et al., 2008), but the pathological consequences of T-cell deficiency imply that immune mechanisms are also important. An important unknown is how far in vivo immune control matches the CD8+ T-cell-mediated killing that is defined in vitro.

Adoptively transferred, EBV-stimulated T cell populations, which are for the most part CD8+, can suppress some forms of EBV-induced lymphoproliferative disease (Gottschalk et al., 2005), which supports the idea that CD8+ T cells are important. However, the direct evidence for CD8+ T-cell-mediated control is limited: as with alpha- and beta-herpesviruses, CD4+ T-cell deficiencies lead to disease (Carneiro-Sampaio & Coutinho, 2007) while CD8+ T-cell deficiencies do not (Cerundolo & de la Salle, 2006); an increased proportion of CD4+ T cells in EBV adoptive transfers seems to correlate with a better therapeutic effect (Haque et al., 2007), and the only attempt at priming CD8+ T cells to date, although weak, failed to reduce the rate of EBV seroconversion (Elliott et al., 2008).

The contribution of CD8+ T cells to MuHV-4 control is limited

MuHV-4 provided the first opportunity to test directly the role of CD8+ T cells in gamma-herpesvirus pathogenesis. MuHV-4 is genetically closer to KSHV than to EBV (Virgin et al., 1997), but is B-cell tropic like EBV and shows a similar approach to host colonization (Nash et al., 2001); for example, both MuHV-4 (Bowden et al., 1997) and EBV (Roughan and Thorley-Lawson, 2009) exploit host germlinal centres for B-cell colonization. Although MuHV-4 naturally infects Apodemus flavicollis mice (Kozuch et al., 1993), it also behaves much like a natural parasite in Mus musculus-derived laboratory strains, persisting without disease unless there is immune suppression (Virgin & Speck, 1999). Crucially, its immune evasion genes still seem to work (Stevenson & Efstathiou, 2005).

An early finding with MuHV-4 was that high dose lung infection is acutely lethal to CD8+ T-cell-depleted BALB/c mice (Ehtisham et al., 1993). A chronic wasting disease in CD4+ T-cell-deficient C57BL/6 mice was consequently interpreted as CD8+ T-cell exhaustion without the help of CD4+ T cells (Cardin et al., 1996). However, CD8+ T cells were found to be fully functional in the ill mice (Stevenson et al., 1998; Belz et al., 2003) and boosting them to high levels did not affect survival much (Belz et al., 2000). Also, MuHV-4-infected, CD8+ T-cell-deficient C57BL/6 mice remained healthy, as did CD8+ T-cell-deficient BALB/c mice given a low dose infection, whereas mice lacking both CD4+ and CD8+ T cells always succumbed (Stevenson et al., 1999a). Therefore, CD8+ T cells help to limit MuHV-4 lytic replication, but are generally neither necessary nor sufficient to prevent lytic-based pathologies. Instead, there is an important role for CD4+ T cells as either direct effector cells or helpers of the antibody response (Table 1).

MuHV-4-infected β2-microglobulin-deficient BALB/c mice, which largely lack classical CD8+ T cells, do show a threefold increase in lymphoproliferative disease compared with uninfected controls (Tarakanova et al., 2005). However, such disease does not occur in β2-microglobulin-deficient C57BL/6 mice; β2-microglobulin-deficient mice have several other deficits, including a lack of natural killer (NK) T cells, low serum IgG and altered NK cell repertoires, and the tumours observed largely lacked viral genomes. Therefore, whether CD8+ T cells are required to limit MuHV-4-driven lymphoproliferation remains unclear.

**Latent antigen recognition by CD8+ T cells**

After intranasal MuHV-4 infection of immunocompetent mice, the extent of lymphoid infection depends mainly on
latent antigen, an H2Kd-restricted epitope in M2 (Husain et al., 1999c). However, in immunodeficient mice, lytic and latent control are readily confused. Thus, MuHV-4 latent infections to which K3 could contribute. In support of such an idea, MuHV-4 lacking its M11 apoptosis inhibitor has a latency defect (de Lima et al., 2005), even though M11 expression is minimal in B cells (Marques et al., 2003). One way K3 might act is by downregulating latency-associated lymphoproliferation (Stevenson et al., 1999c). However, in immunodeficient mice, lytic and latent control are readily confused. Thus, MuHV-4 latent loads increase when CD8+ T cells are lacking (Stevenson et al., 1999a; Tibbetts et al., 2002), but lytic replication also increases and could seed more latency as a secondary event. This occurs particularly with intraperitoneal infections, because infectious virus seeds directly to the spleen.

A clearer picture of the function of latent antigen-specific CD8+ T cells has come from analysing a defined MuHV-4 latent antigen, an H2Kd-restricted epitope in M2 (Huśain et al., 1999). M2 interferes with B cell receptor signal transduction (Rodrigues et al., 2006) and is therefore functionally equivalent to the EBV LMP-2A (Burkhardt et al., 1992) and the KSHV K1 (Lee et al., 1998) proteins. Mouse strains not recognizing M2, such as C57BL/6 (H2b), have higher long-term latent loads than strains that do, such as BALB/c (H2b); mutating the M2 epitope anchor residues increases H2d mouse latent loads and restoring epitope expression returns them to normal (Marques et al., 2008). Therefore, latent antigen-specific CD8+ T cells help to regulate long-term latent loads. Interestingly, H2b mice show a massive expansion of non-classical Vβ4+CD8+ T cells (Braaten et al., 2006). This could be considered a back-up defence when classical CD8+ T cell recognition fails; the higher latent loads of H2b mice imply that the control Vβ4+CD8+ T cells exert is relatively weak.

**Herpesvirus immune evasion limits CD8+ T cell function**

Although M2-specific CD8+ T cells clearly exert some control over MuHV-4, it is difficult to estimate whether their effect is proportionate to their numbers, that is whether CD8+ T cell recognition is efficient. This is an important concept for vaccination: if CD8+ T cells are poorly efficient, then boosting their numbers is likely to give only limited returns. An obvious consideration is viral evasion (Stevenson, 2004).

Herpesvirus genes that inhibit major histocompatibility complex (MHC) class I-restricted antigen presentation are typically expressed early in lytic infection, presumably to allow reactivation and transmission in the face of established CD8+ T-cell immunity. The MuHV-4 K3 degrades MHC class I heavy chains (Boname & Stevenson, 2001; Lybarger et al., 2003) and the TAP peptide transporter (Boname et al., 2004a). KSHV has two homologues, K3 and K5, that degrade MHC class I heavy chains and other substrates (Früh et al., 2002), and the EBV BNLF2a inhibits TAP (Hislop et al., 2007) (Table 2). Surprisingly, K3− MuHV-4 (Stevenson et al., 2002) showed an additional, CD8-dependent defect in latency-associated lymphoproliferation. This implied that K3 normally alleviates the acute, CD8+ T-cell-mediated control of viral latency, and would explain the limited impact of M2-based vaccination (Usherwood et al., 2001).

K3 mRNA is detectable in latently infected germinal centre B cells, and K3 could therefore protect B cells directly against CD8+ T cell attack. However, like most trans-acting herpesvirus evasion genes, K3 is predominantly a lytic transcript (Coleman et al., 2003a); prominent early lytic gene expression in sites of MuHV-4 latency (Milho et al., 2009) suggests extensive interplay between lytic and latent infections to which K3 could contribute. In support of such an idea, MuHV-4 lacking its M11 apoptosis inhibitor has a latency defect (de Lima et al., 2005), even though M11 expression is minimal in B cells (Marques et al., 2003). One way K3 might act is by downregulating latency.

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**Table 1. Key lessons on MuHV-4 immune control from knockout mice**

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Immunodeficiency</th>
<th>Virus load</th>
<th>Disease*</th>
</tr>
</thead>
<tbody>
<tr>
<td>β2M−/−C57BL/6</td>
<td>CD8+ T cells †</td>
<td>Increased</td>
<td>No</td>
</tr>
<tr>
<td>β2M−/−BALB/c</td>
<td>CD8+ T cells</td>
<td>Increased</td>
<td>Yes</td>
</tr>
<tr>
<td>I-Ab−/−C57BL/6</td>
<td>CD4+ T cells</td>
<td>Increased†</td>
<td>Yes</td>
</tr>
<tr>
<td>μMT C57BL/6</td>
<td>B cells</td>
<td>Increased†</td>
<td>No</td>
</tr>
<tr>
<td>IFN-γ−/−C57BL/6</td>
<td>IFN-γ−</td>
<td>Normal</td>
<td>No</td>
</tr>
<tr>
<td>IFN-γ/R−/−BALB/c</td>
<td>IFN-γ− receptor</td>
<td>Increased</td>
<td></td>
</tr>
</tbody>
</table>

*The disease of β2M−/− BALB/c mice is mainly lymphoproliferative; that of I-Ab−/− and IFN-γ/R−/− mice reflects chronic lytic replication. †β2M−/− mice also have no CD1-restricted NKT cells, reduced serum IgG concentrations and altered NK cell repertoires. §Despite an acute latency amplification deficit, chronic lytic replication is increased. §Normal B cell latency is abolished but virus loads are increased in myeloid cells. ||Mainly greater chronic lytic replication.

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**Table 2. MuHV-4 CD8+ T cell evasion mechanisms**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Site of action</th>
<th>Mechanism</th>
<th>Human gamma-herpesvirus equivalents</th>
</tr>
</thead>
<tbody>
<tr>
<td>K3</td>
<td>Lytic + latent infections</td>
<td>MHC class I + TAP degradation</td>
<td>KSHV K3, K5 and EBV BNLF2a</td>
</tr>
<tr>
<td>M3</td>
<td>Lytic + latent infections</td>
<td>Chemokine binding</td>
<td>Possibly KSHV vMIPs and EBV vIL-10*</td>
</tr>
<tr>
<td>ORF73</td>
<td>Latency</td>
<td>Poor antigen presentation</td>
<td>KSHV ORF73 and EBV EBNA-1</td>
</tr>
<tr>
<td>M2</td>
<td>Latency</td>
<td>Selection of virus variants</td>
<td>KSHV K1 and possibly EBV EBNA-3C</td>
</tr>
</tbody>
</table>

*These are functionally different from M3 but conform to the same scheme of a secreted lytic cycle protein that can provide immune evasion in trans in a mixed lytic/latent lesion.
MHC class I-restricted antigen presentation in lytically infected myeloid cells (Smith et al., 2007). These probably carry virus to lymphoid tissue and, once there, express early lytic evasion genes such as M3 (Marques et al., 2003), which binds chemokines (Parry et al., 2000; van Berkel et al., 2000) and inhibits CD8+ T cell function (Bridgeman et al., 2001; Rice et al., 2002). Therefore, lytic K3 expression could both increase virus seeding to B cells and help to protect B cells via M3. EBV and KSHV lack M3, but encode other secreted evasion proteins (Nicholas, 2005) and are unlikely to be any less complex than MuHV-4.

**Cis-acting evasion protects gamma-herpesvirus episome maintenance**

Viral evasion increases the extent of acute, virus-driven lymphoproliferation and probably allows it to continue long-term at a low level. Nevertheless, by 2–3 months after infection with EBV or MuHV-4, lymphoproliferation is largely controlled. The prevalent latent state is then quiescence without viral protein expression (Thorley-Lawson, 2001). While this is presumably inaccessible to immune attack, when latently infected cells divide, their viral episomes must be replicated and segregated by a viral episome maintenance protein. Gamma-herpesviruses have consequently evolved mechanisms to keep episome maintenance immunologically silent. The EBV episome maintenance protein, EBNA-1, limits its epitope presentation to CD8+ T cells through transcriptional (Sample et al., 1992) and translational (Levitskaya et al., 1995; Yin et al., 2003) auto-regulation. The MuHV-4 ORF73 episome maintenance protein is similar. If its cis-acting evasion is bypassed, CD8+ T cells ablate latency (Bennett et al., 2005), indicating that episome maintenance is normally protected.

CD4+ (Nikiforow et al., 2003) and CD8+ (Voo et al., 2004) T cells can recognize EBNA-1 epitopes on in vitro-transformed B cells. However, such recognition is dose-dependent (Mautner et al., 2004). EBNA-1 expression is linked to the cell cycle (Davenport & Pagano, 1999) and in vitro cell proliferation rates tend to be very high. Thus, in vitro cultures or EBNA-1 expressed from heterologous promoters (Fu et al., 2004) do not necessarily yield physiologically relevant results. Tumours with high proliferation rates might present EBNA-1 in vivo, but such tumours are unlikely to grow out in the first place because EBV carriers generally have EBNA-1-specific T cells (Blake et al., 2000). For MuHV-4, even the optimized expression of a CD4+ T cell epitope in latency made no difference to host colonization (Smith et al., 2006). It therefore seems unlikely that T cells control gamma-herpesvirus latency through the recognition of episome maintenance.

**Direct CD4+ T cell effector function in MuHV-4 infection**

Given the limits imposed by viral evasion, how might CD4+ T cells contribute to host defence? One possibility is CD8+ T cell help, as described for lymphocytic choriomeningitis virus (LCMV) infection (Battegay et al., 1994). However, herpesviruses and LCMV are ecologically quite different: herpesviruses evade CD8+ T cell recognition, whereas LCMV persists by inducing a partially tolerant carrier state, like hepatitis B virus. MuHV-4-specific CD8+ T-cell responses seem not to require CD4+ T cell help (Belz et al., 2003). Even for LCMV, CD4+ T cells may be needed more for antibody to reduce viral loads than for direct CD8+ T cell help (Thomsen et al., 1996). Thus, while CD8+ T-cell responses can be CD4-dependent, this seems not to apply in herpesvirus infections.

Other possibilities are B cell help and direct CD4+ T cell effector function. The latter was addressed in MuHV-4 by depleting T cell subsets from B-cell-deficient mice (Christensen et al., 1999). CD4+ T cells were found to be at least as important as CD8+ for controlling acute lytic replication in this setting. Therefore, CD4+ T cells can be directly antiviral, independent of CD8+ T cells or B cells. This contrasts with influenza virus infection, in which CD4+ T cells act almost entirely through B cell help (Topham & Doherty, 1998).

Depleting CD8+ T cells plus gamma-interferon (IFN-γ) additively increased viral titres, whereas depleting CD4+ T cells plus IFN-γ did not. Therefore, CD4+ but not CD8+ T cells act via IFN-γ. CD4+ T cell antiviral effector function was later confirmed to be CD8-independent and IFN-γ-dependent (Sparks-Thissen et al., 2005), and IFN-γ was found to suppress viral lytic gene expression directly in myeloid cells (Steed et al., 2007). The importance of IFN-γ in protecting B-cell-deficient mice is consistent with MuHV-4 causing disease in IFN-γ receptor-deficient mice (Weck et al., 1997; Dutia et al., 1997). Why MuHV-4 should remain sensitive to IFN-γ rather than evolving an evasion mechanism is unclear. Its long-term transmission may benefit from viral replication being suppressed when there is significant inflammation, so as not to deplete the latent pool non-productively or cause excessive harm to the host. Whatever the evolutionary explanation, the end result, as with MCMV (Polić et al., 1996), is that IFN-γ-producing CD4+ T cells contribute significantly to viral control.

**CD4+ T cells and the control of MuHV-4 latency**

The survival of MuHV-4-infected, CD8+ T cell-deficient mice (Stevenson et al., 1999a) suggests that CD4+ T cells might also help to limit virus-driven lymphoproliferation. However, such a function has been hard to define as MuHV-4 exploits CD4+ T cells to drive its lymphoproliferation in the first place (Usherwood et al., 1996). IFN-γ receptor-deficient mice show impaired control of latency (Dutia et al., 1997), but IFN-γ-deficient mice do not (Sarawar et al., 1997) (the difference could reflect the different genetic backgrounds of these knockouts or additional complexity in the interaction between MuHV-4 and IFN-γ). Probably the main arguments for CD4+ T
cells being important in the control of latency are that priming mice with MuHV-4 mutants (Tibbetts et al., 2003; Boname et al., 2004b; Fowler & Efstathiou, 2004), which primes both CD4+ and CD8+ T-cells (Boname et al., 2004b), reduces wild-type latency much more effectively than priming just CD8+ T cells (Stevenson et al., 1999c; Usherwood et al., 2001), and that CD4+ T cells are an important component of the protection induced by priming with MuHV-4 (McClellan et al., 2004).

Although CD4+ T cells seem to control MuHV-4 via IFN-γ, what they recognize is unclear. Most cells supporting MuHV-4 lytic replication lack MHC class II expression. Therefore, CD4+ T cells probably control lytic replication indirectly, with antigen presentation by MHC class II+ cells inducing IFN-γ that then protects other cell types (that the presenting cells need not be infected reduces the scope for viral evasion). CD4+ T cells are also unlikely to combat latency directly, as the deliberate latent expression of a CD4+ T cell target failed to reduce viral loads (Smith et al., 2006). However, the low-level, non-cytopathic expression of viral latent antigens also makes bystander protection unlikely. Therefore, the effects of CD4+ T cells on latency may reflect lytic antigen recognition. This would be consistent with latency-deficient mutants inducing good protection against wild-type latency (Boname et al., 2004b; Fowler & Efstathiou, 2004).

When MuHV-4 is given intraperitoneally or when normal antibody production is lacking, CD4+ T cells can reduce the seeding of latency by reducing the extent of viral lytic replication (Sparks-Thissen et al., 2004). However, after intranasal infection of immunocompetent mice, blocking lytic replication is insufficient to control latency (Stevenson et al., 1999c), so another explanation of how lytic antigen-specific CD4+ T cells might normally act is required. One possibility is that CD4+ T-cell-derived IFN-γ blocks the expression of viral early lytic genes in myeloid cells (Steed et al., 2007). By reducing the secretion of evasion gene products such as M3, this could expose latently infected B cells to CD8+ T cell attack (Stevenson, 2004). Thus, CD4+ and CD8+ T cells would work together, but in a way dictated by viral evasion rather than by pure immunology. A need for both T cell subsets to achieve optimal control is consistent with several points: MuHV-4 causes disease in CD4+ T-cell-deficient mice (Cardin et al., 1996), MuHV-4 titres remain elevated in CD8-deficient C57BL/6 mice (Stevenson et al., 1999a), MuHV-4 causes tumours in β2-microglobulin-deficient BALB/c mice (Tarakanova et al., 2005) and CD8+ or CD4+ T-cell priming alone is poorly protective (Stewart et al., 1999; Liu et al., 1999).

Relating MuHV-4 immune control to EBV and KSHV

The MuHV-4 M2 recognition data imply that CD8+ T cells exert some control over long-term gamma-herpesvirus latent loads, but that their effectiveness is limited by genetics: a host of a given MHC class I haplotype can only recognize viruses with presentable epitopes. Both M2 (Marques et al., 2008) and the KSHV K1 (Stebbing et al., 2003) show evidence of strong positive selection for amino acid diversity, presumably because less well-recognized viruses achieve higher latent loads and therefore transmit better to new hosts. Thus, a combination of human leukocyte antigen (HLA) typing and viral latent gene sequencing may be sufficient to predict EBV and KSHV latent loads. Certainly, ignoring virus–HLA interactions could miss an important source of individual variation.

Even with a small pool of latently expressed viral proteins, some CD8+ T-cell recognition is likely in outbred humans because the large number of MHC class I molecules available (typically five or six rather than two or three in genetically homozygous mice) increases the chance of finding a presentable epitope. Therefore, it makes sense to restore latent antigen-specific CD8+ T cells to immunodeficient patients. However, the impact of these cells will be limited acutely by viral evasion and in the longer term by HLA class I haplotype. Therefore, priming or boosting latent antigen-specific CD8+ T cells in immunocompetent patients seems less likely to be worthwhile.

Adoptively transferred T-cell populations are typically biased towards CD8+ T cells because their numbers are expanded with in vitro-transformed B cells that present mainly HLA class I-restricted epitopes. If EBV lytic cycle evasion proteins promote in vivo latency amplification, as with MuHV-4 (Bridgeman et al., 2001), then increasing the representation of EBV lytic antigen-specific CD4+ T cells (Adhikary et al., 2006) in adoptive transfers might well improve their effectiveness.

Antibody in host defence against gamma-herpesviruses

Although CD4+ T cells can be antiviral through IFN-γ production, they might also protect immunocompetent hosts by promoting antibody responses. Antibody certainly helps to contain persistent MuHV-4 (Gangappa et al., 2002; Kim et al., 2002). However, MuHV-4 tends to cause disease in antibody-deficient mice by chronic lytic replication and is, therefore, different from EBV in this regard. A role for antibody in host defence against EBV has been hard to define because B-cell-deficient patients, lacking the main viral latency reservoir, do not support normal persistence (Faulkner et al., 2000).

The failure of acyclovir to reduce latent loads during infectious mononucleosis (Yao et al., 1989a) or in EBV carriers (Yao et al., 1989b) suggests that established EBV latency is impervious to antibody, as viral lytic replication is presumably the only viable antibody target. However, the acyclovir experiment (Yao et al., 1989b) lasted only 2 weeks – the half-life of EBV-infected B cells even in acute infection is approximately 1 week (Hadinoto et al., 2008) – so latent load reductions may have been hard to detect. Also, acyclovir was given relatively late in infection:
infectious mononucleosis only occurs once EBV latency is well established (Hoagland, 1964). Passive antibody can reduce acute lytic replication by MuHV-4 (D.E. Wright & P.G. Stevenson, unpublished data). Therefore, the use of passive antibody to protect immunocompromised patients exposed to primary EBV should probably be explored further. The protection against acute MuHV-4 by antibody is largely IgG Fc receptor-dependent and is therefore likely to involve antibody-dependent cytotoxicity (D.E. Wright & P.G. Stevenson, unpublished data), consistent with similar findings for HSV (Hayashida et al., 1982).

**Gamma-herpesvirus neutralization**

Virion neutralization by pre-formed antibody has the potential to block herpesvirus infection completely. However, *in vivo* neutralization is not well understood for any herpesvirus, because host entry remains ill-defined (Table 3). It has been hypothesized that incoming EBV virions directly infect B cells in tonsillar crypts (Faulkner et al., 2000). However, direct evidence for this is lacking. B cell infection by cell-free virions is blocked by anti-gp350 antibodies (Thorley-Lawson & Geilinger, 1980). Therefore, the failure of gp350 vaccination to reduce the rate of EBV seroconversion (Sokal et al., 2007) argues that B cell infection by cell-free virions may not be essential for *in vivo* host colonization. Incoming virions could instead infect epithelial cells without engaging gp350 (Janz et al., 2000) and then reach B cells through antibody-secluded cell–cell contacts.

The possibility that incoming MuHV-4 virions might reach B cells directly was addressed with replication-deficient mutants. Viral DNA persisted in the spleen after intraperitoneal inoculation (Tibbetts et al., 2006; Kayhan et al., 2007) but did not reach the spleen after intranasal inoculation under anaesthesia (Moser et al., 2006; Kayhan et al., 2007) and persisted in lung B cells in one study (Moser et al., 2006) but not in another (Kayhan et al., 2007). The conservative conclusion would be that circulating B cells are not directly accessible to incoming virions. *In vivo* infection provides a crucial test of any neutralization because it presents both more barriers than *in vitro* infections, for example mucinous secretions, and more opportunities, for example a multiplicity of cell types. However, it is clearly important to use a realistic inoculation. Intraperitoneal inoculation bypasses important features of natural infection and even intranasal infections are unsatisfactory if general anaesthesia and large inoculation volumes are used to deliver virions directly to the lung. When mice spontaneously inhale a small volume of MuHV-4 inoculum without anaesthesia, acute lytic replication occurs only in the nose, not in the lung (Milho et al., 2009). Systemic persistence is nevertheless robustly established. In contrast, oral virus is very poorly infectious (Milho et al., 2009). Therefore, natural transmission is likely to involve only the upper respiratory tract.

In contrast with lower respiratory tract infection (Coleman et al., 2003b), thymidine kinase-deficient MuHV-4 fails to establish a significant infection via the upper respiratory tract (Gill et al., 2009). Virions entering by this route may therefore have not only to replicate lytically but also to do so in terminally differentiated cells before reaching B cells. This is an important consideration for antiviral therapy as well as for neutralization: acyclovir could potentially have some effect against EBV if given very early after exposure.

A striking finding with MuHV-4 – although so far only applied to lung infection – has been that immune sera neutralize virions much less well for host entry than for infection *in vitro* (Gillet et al., 2007a). Thus, it seems that *in vitro* assays greatly overestimate neutralization, presumably because they rely largely on blocks to cell binding (Gill et al., 2006; Gillet et al., 2009a) that can be bypassed *in vivo*. Notably, MuHV-4 virions exposed to immune sera bind poorly to fibroblasts, but show enhanced infectivity for IgG Fc receptor<sup>+</sup> cells (Rosa et al., 2007).

**Table 3.** Gamma-herpesvirus neutralization

<table>
<thead>
<tr>
<th>Glycoprotein target</th>
<th>EBV neutralization</th>
<th>MuHV-4 neutralization</th>
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<tbody>
<tr>
<td></td>
<td><em>In vitro</em></td>
<td><em>In vivo</em></td>
</tr>
<tr>
<td>gB</td>
<td>Yes</td>
<td>Unknown</td>
</tr>
<tr>
<td>gH/gL (+ gp42) (fusion)&lt;sup&gt;†&lt;/sup&gt;</td>
<td>Yes</td>
<td>Unknown</td>
</tr>
<tr>
<td>gH/gL (+ gp70) (binding)&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>No</td>
<td>Unknown</td>
</tr>
<tr>
<td>gp350/gp150&lt;sup&gt;§&lt;/sup&gt;</td>
<td>Yes</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

*B cell infection. The neutralization of epithelial infection remains largely unexplored.

†EBV uses gH/gL/gp42 to fuse with B cells. MuHV-4 has no known equivalent to gp42.

‡Here we distinguish gH/gL antibodies that block membrane fusion from those that block cell binding: for MuHV-4 to heparan sulfate and for EBV to an unknown epithelial ligand. The MuHV-4 gp70 also binds to heparan sulfate, so a combined binding block must target both gH/gL and gp70. EBV has no gp70 homologue.

§The EBV major variable glycoprotein, gp350, binds to CR2 on B cells. The MuHV-4 equivalent, gp150, does not bind to B cells and functions mainly in virion release.
In contrast with cell binding blocks, fusion blocks should be universal and antibodies to gH/gL or gB – the conserved, essential components of herpesvirus fusion (Turner et al., 1998) – can act post-binding (Gill et al., 2006; Gillet et al., 2006) to inhibit both IgG Fc-independent and IgG Fc-dependent MuHV-4 infections (Gillet et al., 2007a). However, post-binding neutralization is far from straightforward. An association between gH/gL and gB (Gillet & Stevenson, 2007a) provides mutual shielding (Gillet & Stevenson, 2007b; Gillet et al., 2009b) until post-endocytic low pH triggers gL dissociation and gB/gH conformation changes (Gillet et al., 2008a, b). Neutralization is consequently forced to act by gH/gL stabilization or steric hindrance, neither of which is easy to achieve. This may nevertheless be the best chance of blocking host entry.

The major gamma-herpes virion glycoprotein as a neutralization target

All gamma-herpesviruses share a positionally conserved but sequence-variable major glycoprotein: gp350 in EBV, K8.1 in KSHV and gp150 in MuHV-4. None is required for fusion, arguing that they are unlikely to be good neutralization targets. Unlike gp350, the MuHV-4 gp150 does not bind to B cells. Instead it regulates epithelial infection by making cell binding heparan sulfate-dependent (de Lima et al., 2004). Interestingly, the EBV gp350 also negatively regulates epithelial cell binding (Shannon-Lowe et al., 2006), suggesting that these proteins share a common function of epithelial virion release.

Gp150 also helps MuHV-4 evade neutralization, an important function for released virions. Antibodies to gp150 do not neutralize. Instead they are responsible for immune sera enhancing infection via IgG Fc receptors (Gillet et al., 2007b). Gp150 is the immunodominant MuHV-4 antibody target (Gillet et al., 2007b), so MuHV-4 virions that are blocked for normal cell binding by immune sera can remain infectious via a gp150–antibody–IgG Fc receptor link (Rosa et al., 2007). The most immunogenic region of gp150 is shared by gp350 and the KSHV K8.1, suggesting that this evasion mechanism is conserved. Thus, gB and gH/gL may be better in vivo EBV neutralization targets than gp350.

All mammalian herpesviruses probably conform to similar pathogenetic schemes

The phenotypes of genetic and acquired human immunodeficiencies (Carneiro-Sampaio & Coutinho, 2007) suggest that the key elements of alpha-, beta- and gamma-herpesvirus host defence are broadly similar. There is also remarkable immunological consistency between MCMV (Polic et al., 1998) and MuHV-4, the best explored experimental herpesvirus infections. For each, multiple effector mechanisms contribute to control, and CD4+ T cells are at least as important as CD8+ T. The main difference between MCMV and MuHV-4 is that NK cells contribute less to controlling MuHV-4 (Usherwood et al., 2005). This presumably reflects that more comprehensive T-cell evasion by MCMV forces more reliance on NK function. It might be speculated that MCMV and MuHV-4 are following different evolutionary strategies: immune evasion genes tend to be species-specific, so MCMV, with many such genes, is being as successful as possible in one host while losing the flexibility to switch to others; MuHV-4 has fewer evasion genes but appears able to infect more species (Kozuch et al., 1993); a close relative of MuHV-4 (Andrew Davison, personal communication) has even been isolated from a shrew (Chastel et al., 1994). Few hosts comparable to humans have survived, so the evolutionary pressures on human herpesviruses may be subtly different. However, the problems alpha- and beta-herpesviruses cause when NK cell function is defective (Carneiro-Sampaio & Coutinho, 2007) argue that immune evasion limits viral control by T cells.

How does poor CD8+ T-cell efficacy fit with the sizeable CD8+ T-cell responses that herpesviruses often elicit? A key point is whether these responses target cells central to host colonization or only their derivatives. For example, do the large populations of EBV lytic antigen-specific CD8+ T cells found in infectious mononucleosis (Callan et al., 1996) recognize the source of the problem (latency amplification) or merely chase its tail (lytic reactivation)? The latter could help to limit disease, but is unlikely to achieve infection control, MuHV-4 elicits large lytic antigen-specific CD8+ T-cell responses (Stevenson et al., 1999b) that clearly do not control latency amplification, since priming them fails to prevent it (Stevenson et al., 1999c). Indeed, the expansion of these cells reflects a failure to control latency: when the cis-acting immune evasion of MuHV-4 episome maintenance is bypassed, the lytic antigen-specific CD8+ T cell population remains small, as does that responsible for latency ablation (Bennett et al., 2005). Beta-herpesviruses show a similar discrepancy between large T cell numbers and effective immunity: large CD8+ T-cell responses to cytomegalovirus infection (Khan et al., 2002; Karrer et al., 2003) are not associated with better control. Limited viral evasion in some cell types (Hengel et al., 2000) could stimulate CD8+ T cells that make only a limited contribution to infection control, or waning CD4+ T-cell control of an earlier checkpoint could elicit downstream, compensatory responses. Essentially, large CD8+ T-cell responses to herpesviruses should raise suspicions of immune inadequacies elsewhere.

Alpha-herpesviruses and T-cell function in the brain

Our knowledge of T-cell immunity to alpha-herpesviruses lags somewhat behind that of the beta- and gamma-herpesviruses. A particular issue with alpha-herpesviruses has been whether their latency in neurons mandates distinct mechanisms of immune control. Many unusual
phenomena have been reported for T cells in the brain. However, the only really consistent finding, first reported for transplantation (Medawar, 1948), has been some intracerebral immune privilege due to poor priming. Thus, a viral infection confined to the brain parenchyma elicits little immune response (Stevenson et al., 1997a). However, if infection reaches the cerebrospinal fluid or if priming occurs outside the brain – as it would for HSV – then the intracerebral infection is readily recognized (Stevenson et al., 1997b). Neurons have higher thresholds for MHC class I-restricted epitope presentation than most other cell types and may resist some pro-apoptotic stimuli, but antiviral CD8\(^+\) T-cell function in the brain does not seem to be impaired (Stevenson et al., 1997b).

Post-mortem studies of human trigeminal ganglia have found HSV-specific CD8\(^+\) T cells near infected neurons (Verjans et al., 2007). While the functional importance of these cells remains unclear, their presence has been taken as evidence that CD8\(^+\) T cells might control neuronal HSV infection. HSV infection of mice has been used to explore how this might work. It was assumed early on that a lack of neuronal MHC class I expression would necessitate HSV control by a mechanism other than conventional cytotoxicity (Simmons and Tscharke, 1992). Histological data – the VP16 staining in acutely infected trigeminal ganglia exceeded subsequent neuronal loss – were consequently interpreted as non-cytolytic clearance. However, neurons can express MHC class I (Redwine et al., 2001). Moreover, neurons can express VP16 with immediate-early kinetics (Thompson et al., 2009), and infected cell tagging (Proença et al., 2008) has shown that HSV immediate-early promoters can be active before neuronal latency is established. Therefore, a loss of VP16 expression need not reflect immune clearance. Non-cytolytic HSV suppression by granzyme B-mediated degradation of the ICP4 viral transactivator was recently described (Knickelbein et al., 2008). However, such a mechanism is hard to reconcile with the (H\textsuperscript{b} restricted) HSV-specific CD8\(^+\) T-cell response focussing on gB, a late lytic gene (Wallace et al., 1999): by the time gB is made, the need for ICP4 should have passed. It is also unclear how a murine enzyme could target a human viral protein while sparing its normal cellular substrates. Thus, whether CD8\(^+\) T cells might mediate non-cytolytic control of HSV in neurons remains controversial.

**Immune evasion probably limits CD8\(^+\) T-cell recognition of HSV-infected human neurons**

Regardless of how HSV-specific CD8\(^+\) T cells might act, can they efficiently recognize HSV-infected human neurons? Alpha-herpesvirus latency classically involves no viral protein expression and should therefore be inaccessible to attack by T cells. When reactivation occurs, viral epitopes can potentially be presented. However, the HSV ICP-47 blocks the TAP peptide transporter (York et al., 1994). ICP-47 does not block murine TAP (Tomazin et al., 1998) and introducing a gene capable of inhibiting murine MHC class I-restricted antigen presentation into HSV significantly impairs CD8\(^+\) T-cell-mediated protection (Orr et al., 2007). These results raise doubt as to whether CD8\(^+\) T-cell-mediated control of HSV in mice will translate simply to humans. Recent data suggest that even in mice, CD4\(^+\) T cells might contribute significantly to HSV control in the nervous system (Johnson et al., 2008).

**Herpesvirus vaccines for naive subjects**

Where does herpesvirus immune evasion leave vaccination? Persistent, evasive viruses are clearly not straightforward targets. Disease and transmission will not necessarily be prevented in the same way, nor will acute and chronic infection syndromes. Therefore, strategic aims must be considered.

The best-established herpesvirus vaccines are veterinary: live-attenuated pseudorabies virus and Marek’s disease virus (MDV). Both can prevent disease but neither prevents transmission; this is achieved by quarantine and culling (Bouma, 2005; Baigent et al., 2006) (Table 4). The only human herpesvirus vaccine in widespread use is live-attenuated varicella-zoster virus (VZV) (Takahashi, 2001). This similarly prevents the symptoms of acute infection (chickenpox), but whether it can prevent the establishment and transmission of wild-type VZV, and whether the same approach could be applied to beta- and gamma-herpesviruses, where persistence causes more problems than acute infection, remain unclear. The other difficulty with applying the VZV approach more widely is that empirical attenuation is now considered a significant risk; the implementation of VZV vaccination was based on safety testing from 30 years earlier.

**Table 4. Herpesvirus vaccines: the problem is transmission**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Vaccine</th>
<th>Disease control</th>
<th>Infection control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pseudorabies virus</td>
<td>Live attenuated</td>
<td>Yes</td>
<td>No*</td>
</tr>
<tr>
<td>MDV</td>
<td>Live attenuated</td>
<td>Yes</td>
<td>No*</td>
</tr>
<tr>
<td>VZV</td>
<td>Live attenuated</td>
<td>Yes</td>
<td>Unknown</td>
</tr>
<tr>
<td>EBV</td>
<td>Gp350</td>
<td>Unlikely</td>
<td>No*</td>
</tr>
<tr>
<td>MuHV-4</td>
<td>Live attenuated</td>
<td>Unknown</td>
<td>Unknown†</td>
</tr>
</tbody>
</table>

*Field studies showed reduced but not abolished transmission.
†Superinfection is reduced but there has been no field test of transmission.
For safety reasons, a recombinant, subunit vaccine would be preferred. However, viral evasion gives the limited range of immune effectors primed by such vaccines limited scope for infection control. Experience with MuHV-4 has borne this out: lytic antigen-specific CD8$^+$ T cells (Stevenson et al., 1999c), latent antigen-specific CD8$^+$ T cells (Usherwood et al., 2001) and gp150 (Stewart et al., 1999) have failed to reduce steady-state latent loads by themselves. Each reduced peak latent loads, but this would seem unlikely to prevent long-term disease. Only attenuated whole virus vaccines have reduced long-term latent loads (Tibbetts et al., 2003; Boname et al., 2004b; Fowler & Efstathiou, 2004). The best route to an effective gamma-herpesvirus vaccine may therefore be genetically targeted

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Fig. 1. A schematic view of the gamma-herpesvirus life cycle for MuHV-4. T cells are shown in red, B cells in blue, epithelial cells in white, neuronal cells are brown and relevant others are in green. Arrows show the movement of virus. Numbers 1–5 indicate definable steps. Key intervention points are latency establishment in naive hosts (steps 2 and 3) and antibody binding to the virions shed by carriers (step 1). Step 1: virions enter a naive host via secretions such as saliva. MuHV-4 probably enters via the nose. In vivo entry presents both more opportunities and more challenges than in vitro infection. For example, epithelial surfaces have an abundant covering of mucus from goblet cells that provides a physical barrier, yet some cells extend cilia through this. Since virions come from immune carriers, they are likely to have attached antibody. The virions are not normally neutralized, but neutralization may be possible through boosting fusion-complex-specific antibodies in virus carriers. Non-neutralized MuHV-4 could first infect epithelial cells, IgG Fc receptor-bearing dendritic cells or olfactory neurons. There is no good evidence for direct B cell infection after a non-invasive inoculation. Step 2: local lytic spread could potentially be targeted by antiviral drugs or antibody. Latency establishment seems to occur mainly in draining lymph nodes. Infection may reach these via dendritic cells or via cell-free virions captured by subcapsular sinus macrophages. Step 3: infection next spreads to B cells, which proliferate. Lysically infected myeloid cells secrete evasion proteins such as the M3 chemokine binding protein to protect B cells against CD8$^+$ T-cell attack. K3 protects myeloid cells and perhaps also B cells against CD8$^+$ T-cell recognition. IFN-$\gamma$ produced by CD4$^+$ T cells may limit M3 production and thereby help latent antigen-specific CD8$^+$ T cells to attack B cells. Step 4: B cells exit germinal centres and differentiate into long-lived, resting memory B cells. Episome maintenance by ORF73 remains below the threshold of antigen presentation. Step 5: virus reactivation probably occurs in submucosal sites, accompanied by B-cell differentiation to a plasma cell phenotype (Sun & Thorley-Lawson, 2007; Wilson et al., 2007). There may be further lytic replication in epithelial cells prior to virion shedding. Lytic antigen-specific CD8$^+$ T cells can potentially inhibit reactivation, but K3 and M3 limit their impact. Consequently, CD4$^+$ T cells seem to protect better against lytic spread. Gp150 promotes virion release and helps to limit neutralization.
mutants, backed up by a detailed knowledge of pathogenesis to ensure safety. The most promising MuHV-4 vaccine to date has been a mutant lacking its ORF73 episome maintenance protein (Fowler & Efthathiou, 2004). The EBV equivalent, an EBNA-1-EBNA-2 mutant, should fail to either persist or transform cells.

Herpesvirus vaccines for virus carriers

Any vaccination strategy must respect the ecology of the target parasite. Fig. 1 illustrates relevant features of the gamma-herpesvirus life cycle for MuHV-4. Many details remain poorly defined, but two distinct hosts are clear: naive subjects and virus carriers. The viral life cycle could be interrupted at latency establishment in the former or transmission from the latter.

MDV illustrates a potential drawback of a vaccine focussed only on naive subjects. Vaccination protects against disease but not against wild-type transmission, and the natural selection for a more transmissible wild-type seems also to have selected for greater virulence (Biggs, 1997). Therefore, a useful supplement to reducing herpesvirus latent loads in naive subjects through live attenuated vaccines would be to target viral transmission from carriers. MuHV-4 induces antibody responses that are suboptimal for in vivo neutralization (Gillet et al., 2007a), in part because the key neutralization target, the virion membrane fusion complex, is only weakly immunogenic. Neutralization can be improved by vaccinating virus carriers with recombinant fusion complex components (Gillet et al., 2007a). Such an approach could help to eliminate herpesviruses from populations by reducing the infectivity of carriers.

In summary, the control of herpesvirus infections by vaccination alone remains a major challenge, principally because viral immune evasion makes transmission hard to block. A dual vaccination strategy, distinguishing the need to protect naive subjects against disease and to reduce the spread of infection from existing carriers, probably provides the best chance of eliminating these difficult and complicated pathogens.

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