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Immune Control of γ-Herpesviruses

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Abstract

Vaccination against γ-herpesviruses has been hampered by our limited understanding of their normal control. Epstein–Barr virus (EBV)-transformed B cells are killed by viral latency antigen-specific $CD8^+$ T cells *in vitro*, but attempts to block B cell infection with antibody or to prime anti-viral $CD8^+$ T cells have protected poorly *in vivo*. The Doherty laboratory used Murid Herpesvirus-4 (MuHV-4) to analyze γ-herpesvirus control in mice and found $CD4^+$ T cell dependence, with viral evasion limiting $CD8^+$ T cell function. MuHV-4 colonizes germinal center (GC) B cells via lytic transfer from myeloid cells, and $CD4^+$ T cells control myeloid infection. GC colonization and protective, lytic antigen-specific $CD4^+$ T cells are now evident also for EBV. Subunit vaccines have protected only transiently against MuHV-4, but whole virus vaccines give long-term protection, via $CD4^+$ T cells and antibody. They block infection transfer to B cells, and need include no known viral latency gene, nor any MuHV-4-specific gene. Thus, the Doherty approach of *in vivo* murine analysis has led to a plausible vaccine strategy for EBV and, perhaps, some insight into what $CD8^+$ T cells really do.

Keywords: gammaherpesvirus, vaccine, immune control, T cells

Understanding γ-Herpesvirus Control

Epstein–Barr virus (EBV) and the Kaposi's sarcoma-associated herpesvirus (KSHV) are widespread and cause cancers, so cognate vaccines would improve human health (18). Analysis has focused on *in vitro* EBV-transformed B cells, which can be killed by $CD8^+$ T cells recognizing viral latency antigens (74). However, genetic deficiencies in $CD4^+$ T cells or NK cells, not $CD8^+$ T cells, predispose to EBV disease (16); protecting immunocompromised patients correlates better with $CD4^+$ than $CD8^+$ T cell transfer (39); and vaccines to block virion attachment to B cells or prime latent antigen-specific $CD8^+$ T cells have worked poorly (18).

Species restrictions make EBV and KSHV hard to study *in vivo*. The discovery of Murid Herpesvirus-4 (MuHV-4, archetypal strain MHV-68) by Slovakian virologists (7) opened γ-herpesvirus infection control to the Doherty approach of comprehensive murine analysis. MuHV-4, KSHV, and EBV share obvious genetic homology and persist in B cells (79). MuHV-4 normally infects yellow-necked mice (50), but intact immune evasion (84) and sexual transmission (29) in laboratory strains argue against significant attenuation.

Using mice to answer questions about human γ-herpesvirus infections has a simple evolution-based rationale. γ-Herpesvirus infections long preceded human speciation (63). Herpesviruses can evolve rapidly but are selected only by host change: any loss of transmission selects viral compensation. Thus, viral evolution tracks host evolution; the most virus-diverse genes interact with the most host-diverse genes; and they counteract host diversity to keep outcomes the same. Immune evasion provides a well-documented example. With MuHV-4, Doherty research program provided an opportunity to understand generically *in vivo* γ-herpesvirus control.

MuHV-4: Surprises from the Start

Preceding Doherty involvement, the Nash group showed that $CD8^+$ T cells control acute MuHV-4 lung infection (22) and that $CD4^+$ T cells promote acute B cell infection (103). Cancers were noted in old infected mice (93). The Doherty group focused more on long-term infection control and identified chronic illness in $CD4^+$ T cell deficiency (15). The *zeitgeist* predicted $CD8^+$ T cell exhaustion without $CD4^+$ T cell help. However, while $CD8^+$ T cell deficiency increased lytic infection, it did not cause chronic illness (88); and

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$CD8^+$ T cell responses in $CD4^+$ T cell-deficient mice were intact, even elevated (85), yet unable to stop chronic lytic infection (5). The reason was viral $CD8^+$ T cell evasion (89), subsequently identified also for EBV (42). $CD8^+$ T cells controlled acute lytic infection in epithelial cells, (88); but viral evasion protected myeloid cell infection.

Virus-infected cancers were found to be rare, even in immunocompromised mice. Ubiquitous EBV yet geographically restricted Burkitt's lymphoma and nasopharyngeal carcinoma indicates the importance of cofactors in γ-herpesvirus disease. Selection for host and virus survival conserves normal infection but not unconnected cofactors. Consequently, animals model human γ-herpesvirus infections much better than disease. This applies generally. For example, papillomavirus infection is conserved across species, so although disease varies (14), vaccinating against infection translated (94).

The lag between human γ-herpesvirus infections and cancer generally exceeds murine longevity, and many murine cancers involve retroviruses, which are rare in humans. Therefore, while humans and mice share oncogenes, equating genetically undefined cancers between them is problematic. Few cancers in MuHV-4-infected mice are MuHV-4-infected (98), and even the MuHV-4-infected S11 cancer also produces retrovirus (PGS, unpublished data). Thus, the Doherty group focused on viral loads rather than disease.

A Reassessment of EBV Control

The likely source of discrepancy between $CD8^+$ T cell-dependent EBV control *in vitro* and $CD4^+$ T cell-dependent MuHV-4 and EBV control *in vivo* was revealed by the Thorley-Lawson group. They showed that *in vivo* EBV colonizes not proliferating blasts, as *in vitro*, but germinal center (GC)-experienced resting memory B cells (82), like MuHV-4 (27) (Fig. 1). This explained why type 2 EBV, which transforms B cells poorly, nonetheless causes the same disease as type 1 EBV (48). Immunocompromised patients accumulated infected resting memory B cells, not blasts, and showed more lytic infection (3). Thus, *in vivo* EBV-driven B cell proliferation seemed to be self-limiting, suggesting that infected GC initiation is the key immune target.

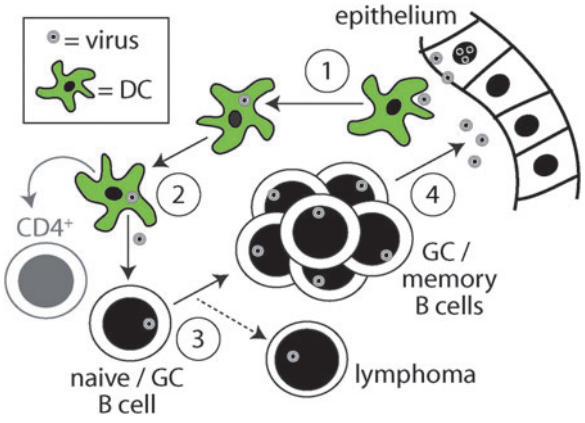

FIG. 1. The γ-herpesvirus GC cycle. ① Incoming epithelial infection reaches DC. They migrate to lymph nodes. ② Infected DC pass virus to naive B cells. DC seem also to recruit antigen-independent $CD4^+$ T cell help for infected B cell proliferation in GC. EBV-driven GC B cell proliferation may be less $CD4^+$ T cell-dependent through LMP-1 substituting for CD40 engagement. ③ Infected B cells emerge from GC as resting memory cells. Host mutations acquired in GC can cause lymphomas. ④ Memory B cells do not enter new GC. They reactivate virus in submucosal sites, feeding transmission, and transfer infection back to new naive B cells via DC. This lytic component of infection provides a target for immune control. DC, dendritic cells; EBV, Epstein–Barr virus; GC, germinal center.

MuHV-4 colonizes splenic GC via at least three rounds of myeloid/lymphoid virus transfer: from dendritic cells (DC) to lymph node B cells (34), from splenic marginal zone macrophages to marginal zone B cells, and then from follicular DC to GC B cells (30). Infected memory B cells cannot enter new GC (53); their virus must reactivate and transfer back to new naive B cells, via DC. Therefore, myeloid to B cell virus transfer is repeated and ongoing, making it a feasible target for infection control.

GC colonization is relevant to disease, as Burkitt's lymphoma has a GC origin. It is driven by host—not viral—oncogenes (99). It seems to result from EBV continually forcing B cells through the mutagenic GC setting, then upon chance mutation inhibiting regulatory mechanisms such as apoptosis and immune attack. Cancers routinely accumulate secondary mutations, so with host genes driving proliferation, viral genes could easily become redundant for cancer survival. Immune recognition might then select for viral genome loss.

γ-Herpesviruses have not evolved to persist in cancer cells, and MuHV-4 engineered to mutate defined host oncogenes is rapidly lost after transformation (90). Therefore, EBV might initiate many more cancers than those retaining viral genomes. Encouragingly, despite viral genome loss in the MuHV-4 model, vaccination protected against disease. This provides another argument for vaccination against EBV.

Vaccination and γ-Herpesvirus Host Entry

Gp350 vaccine aimed to stop EBV infection by blocking virion attachment to B cells (100). It protected tamarins against disease but did not stop human B cell infection (18). As xenogenic settings compromise viral evasion, tamarin infection may be unrealistically easy to suppress.

The same may apply to immunodeficient mice transplanted with human hematopoietic progenitor cells, as their infection similarly requires EBV injection and results in virus-driven transformation rather than memory B cell colonization (32). The Doherty approach was to keep infection control realistically difficult, by preserving a natural context.

Context starts with host entry. Gp350 vaccination assumed that the tonsillar B cell infection of infectious mononucleosis is EBV host entry. Yet the naive B cells EBV targets are normally segregated from free antigen, as antigen without costimulation triggers B cell apoptosis. Infectious mononucleosis occurs at least a month after transmission (43), and prospective analysis detected oral EBV only after systemic infection (20), implying not entry but a distinct route of host exit. Although EBV poorly infects epithelial cells *in vitro* (46), the right cells may not have been tested (26) and the EBV used has come from cancer cells, which may counter-select normal fitness. For example, the standard B95 strain has a large genomic deletion (68). Thus, there are good reasons to question the idea of direct B cell infection by incoming cell-free EBV.

The MHV-68 isolate of MuHV-4 appears to be intact, as a related virus is genetically colinear (44). It is noninfectious orally (66). The lungs can be infected by inoculation under sedation, but MuHV-4 enters alert mice via the olfactory epithelium (65). B cells first become infected in lymph nodes, via DC (34). Submucosal lymphoid tissue is colonized only after systemic spread (31). Oral rhesus lymphocryptovirus (RhLCV) can infect macaques. However, no oral RhLCV entry site is known, and the macaques are given a high virus dose under sedation (106). When oral MuHV-4 infects sedated mice, viral luciferase imaging shows not oral but respiratory infection, reflecting inoculum aspiration (66). Therefore, a natural γ-herpesvirus entry route other than olfactory or genital (29) is yet to be shown.

Gp350-specific antibodies abound in EBV carriers without stopping transmission or selecting antigenic variants (109). After gp350-independent epithelial entry (47), B cell infection via cell-to-cell spread might resist neutralization, and a failure of such spread might explain gp350 vaccine efficacy in tamarins. A gp350-type vaccine was reported to protect macaques against RhLCV (78). However, the result was infection in 2/4 rather than 4/4 animals ($p > 0.4$ by Fisher's exact test). Vaccination with the equivalent gp150 of MuHV-4 did not protect (76).

Antibody restricts MuHV-4 less by neutralization than by engaging IgG Fc receptors, and gp150-specific antibodies have failed to protect in this way (107). They also fail to neutralize (36). Therefore, *in vivo* analysis of γ-herpesvirus host entry and spread provides no clear rationale for a gp350 vaccine. Nor is there a clear precedent for blocking epithelial host entry: the IgA response to MuHV-4 is weak (95), and protection against superinfection operates after entry (37).

Understanding the Impact of Antiviral CD8$^+$ T Cells

Priming latent antigen-specific CD8$^+$ T cells has protected poorly against EBV (18), suggesting that *in vitro*-type transformation is not how *in vivo* infected B cell proliferation works. Some role for the viral latency genes seems likely, but a GC context may limit their immunological accessibility, for example, through reduced expression or associated viral evasion. Nor has CD8$^+$ T cell priming protected against MuHV-4. Priming lytic antigen-specific CD8$^+$ T cells potently reduced acute lytic infection, but B cells were still infected and their proliferation soon restored viral loads (55,87). Priming latent antigen-specific CD8$^+$ T cells also failed to reduce long-term infection (104). Disrupting viral CD8$^+$ T cell evasion severely curtails infection (6,12,91), so effector function seems more limiting than priming. Viral evasion notably protects the myeloid gateway to B cells (80), making its control CD4$^+$ T cell-dependent (96) (Fig. 2).

The immunological approach to vaccines identifies protective responses and then delivers their targets in recombinant form. Because human infections are hard to analyze functionally, attention often focuses on numerically large responses, particularly for T cells. However, in complex infections, large T cell responses are not necessarily protective: mainly they identify abundant antigen. Protective responses must target an infection's self-renewing source. For example, the large CD8$^+$ T cell responses to EBV (13) and MuHV-4 (86) lytic antigens in infectious mononucleosis imply poor control of upstream B cell proliferation.

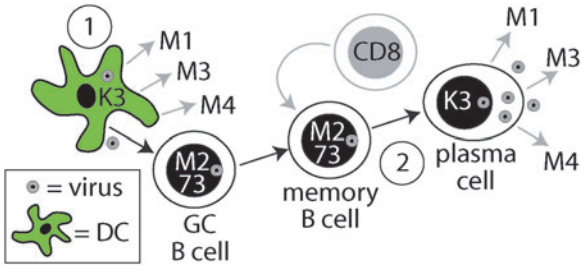

FIG. 2. Viral CD8$^+$ T cell evasion. ① Infected DC entering lymph nodes pass virus to B cells. They also secrete viral evasins: M1, M3, and M4 (58). EBV and KSHV secrete their own evasin sets. M1 promotes an expansion of Vβ4$^+$CD8$^+$ T cells (24). M4 promotes lymphoid colonization (23,35). M3 binds chemokines (70) to evade CD8$^+$ T cells (12) and can provide bystander protection (73). Thus, lytically infected DC create an evasive milieu for latently infected B cell proliferation. K3 degrades MHC class I heavy chains (10) and the associated peptide transporter (9), further protecting infected DC against CD8$^+$ T cells (80) and promoting B cell proliferation (91). ② Infected B cells make ORF73, which maintains the viral episome, and M2, which like the EBV LMP-2 and KSHV K1 mimics antigen receptor signaling (75). Like EBNA-1, ORF73 minimizes its entry into the MHC class I presentation pathway and bypassing this evasion terminates lymphoid infection at an early stage (6). Linking a well-presented epitope to M2 also attenuates infection (38). KSHV, Kaposi's sarcoma-associated herpesvirus.

When the cis-acting CD8$^+$ T cell evasion of MuHV-4 episome maintenance is subverted, B cell proliferation is ablated by a T cell population that remains small because the viral antigen load remains low (6). The MuHV-4 M2 latency gene is a prominent CD8$^+$ T cell target in BALB/c mice (45). It shows selection for antigenic change (39), and M2-specific CD8$^+$ T cells limit long-term viral loads (57). However, M2-specific CD8$^+$ T cell priming reduced host colonization only transiently (104). The site of normal M2 epitope recognition is unclear, and recognition after GC-associated proliferation, for example, during reactivation (40), could reduce viral loads and select antigenic variants without providing a good basis for vaccination.

Vaccine Targets for MuHV-4 and EBV

The virological approach to vaccines disrupts pathogenic functions to make immunogenic yet harmless strains. This has worked against some acute diseases caused by α-herpesviruses, including T cell lymphoma in chickens (11), and live-attenuated MuHV-4 protects against wild-type infection, via CD4$^+$ T cells and antibody (62,101).

How CD4$^+$ T cell contribute is still unclear. While they have been reported to recognize latent EBV infection via EBNA-1 (69), others did not reproduce this result (60). EBNA-1 limits its recognition through low turnover (110). As cell division increases EBNA-1 synthesis (19), rapid *in vitro* division may lead to breakthrough recognition. EBV-infected cells normally divide only intermittently (67), so such recognition seems unlikely to work *in vivo*. MuHV-4 forced to present a CD4$^+$ T cell target in latency showed

no *in vivo* attenuation (81), and further EBV studies concluded that protective $CD4^+$ T cells recognize lytic antigens (59). $CD4^+$ T cells suppress chronic MuHV-4 replication in myeloid cells (96). As myeloid cells transfer MuHV-4 to B cells (31), this fits with $CD4^+$ T cell-dependent vaccine protection acting upstream of B cell infection (37).

Lytic infection suppression by $CD4^+$ T cells requires interferon (IFNγ) (17,83), and human immunodeficiency phenotypes support an important role for IFNγ in herpesvirus control (16). Mechanisms remain unclear. In MuHV-4-infected mice that lack IFNγ signaling, $CD8^+$ T cells drive multiorgan fibrosis and splenic atrophy (21). When perforin is also lacking, there is instead massive splenomegaly (4). Perforin plus fas deficiency also causes dramatic disease, even though these deficiencies are individually well tolerated (102). Partial redundancy between effector molecules, and each functioning in multiple cell types makes, useful conclusions hard to draw, and the sudden shifts from coping to catastrophe with combined mutations hard to unravel. The Doherty focus on T cells as complex but coherent functional units has proved easier to relate to vaccination.

An underlying assumption has been that protective $CD4^+$ T cells directly recognize infected cells (59,92). However, this remains questionable. Major histocompatibility complex (MHC) class II glycoproteins present mainly cell exogenous antigens; not all infected MuHV-4-infected myeloid cells express MHC class II (96); and in acutely infected lungs, MuHV-4 replicates mainly in MHC class II^- alveolar epithelial cells (54). Murine cytomegalovirus (MCMV), which also shows control by $CD4^+$ T cells (49) and IFNγ (56), removes MHC class II from infected cells (111). Therefore, $CD4^+$ T cells seem likely to control herpesviruses indirectly. Antibody contributing via Fc receptor engagement (107) suggests a role for innate effector cells, and NK cell recruitment by $CD4^+$ T cells (71) is important to control MuHV-4 (PGS, unpublished). Thus, the best vaccine antigens may be those presented by uninfected rather than by infected MHC class II^+ cells.

How EBV reaches B cells is unknown. Direct tonsillar B cell infection by incoming virions now seems unlikely, and diverse viruses besides MuHV-4 exploit DC to reach lymphocytes (1,51,72), so this route is certainly plausible. MuHV-4 entry into splenic GC matches immune complex transport (30), so complement receptor binding by the EBV gp350 (97) also suggests close parallels. Infected Reed-Sternberg cells have many DC features (77). Based on single-cell polymerase chain reaction (PCR), they have been identified as GC B cells (52). Yet they express neither immunoglobulin nor any other B cell-specific marker, and in a complex GC setting, PCR may poorly distinguish nuclear from endocytosed DNA. Immunostaining olfactory epithelium for known EBV receptors and analyzing early RhLCV infection might usefully extend the leads from MuHV-4.

Vaccine Delivery for MuHV-4 and EBV

The Doherty approach of analyzing a realistic murine infection has given a coherent overview of γ-herpesvirus control that *in vivo* EBV data seem to fit, and live-attenuated MuHV-4 has demonstrated effective vaccination. Poor protection by recombinant MuHV-4 glycoproteins (61) suggests that multiple viral targets are needed. The oncogenic potential of EBV latency genes precludes their inclusion in a live vaccine. However, MuHV-4 crippled for latent infection still protects (8,28,108); and this remains effective when the vaccine virus lacks genes *M1*, *M2*, *M3*, *M4*, *ORF4*, and *ORF73* (PGS, unpublished). Therefore, no known MuHV-4 latency gene, and no gene without an obvious homolog in EBV is needed, suggesting that EBV lacking latency genes might also be effective.

EBV virus-like particles have protected SCID-Hu mice against lymphoproliferative disease (105). However, protection by inactivated MuHV-4 (2) requires large antigen amounts, and EBV virus-like particle production depends on transfection-induced reactivation from latency that is likely to be limited by genome silencing, making it difficult to scale up. MuHV-4 data suggest that removing the EBV latency genes might, with repair of the known genomic defects (68) and transactivator complementation (66), make a safe vaccine that could be propagated *in vitro*; and nasal EBV lacking just its latency genes might prime safely and effectively via local lytic replication.

While MuHV-4 experiments have demonstrated effective vaccination, they have not encompassed the host and viral diversity of natural EBV and KSHV infections. Also the short life span of mice gives limited scope for testing protection longevity. Human T cell memory declines over 3–5 years (64), so EBV vaccine efficacy may depend on antibody alone—enhancing attack by IgG Fc $receptor^+$ cells and accelerating new T cell priming through opsonization. Then, wild-type viral loads must be kept low for many years. Mainly MuHV-4 has provided a rational basis for EBV clinical trials, identifying key mechanisms and a vaccine approach that can suppress wild-type infection to an apparently new steady state.

What Do $CD8^+$ T Cells Really Do?

One Doherty aim with MuHV-4 was to relate a molecular understanding of $CD8^+$ T cell recognition to an important *in vivo* function. $CD8^+$ T cells had been assumed to control γ-herpesviruses. However, while they have some role, viral evasion makes $CD4^+$ T cells the key subset. MuHV-4 (and MCMV) suggests by their evasion that an important normal role of $CD8^+$ T cells is to kill pathogen-infected myeloid cells, which might otherwise recirculate (25). A study of vaccinia virus showed inflammatory myeloid cells killing infected keratinocytes, whereas $CD8^+$ T cells killed infected myeloid cells (41). Thus, T cells may have evolved in part to regulate an existing myeloid defense: $CD4^+$ T cells turning it on through MHC class II, and $CD8^+$ T cells turning it off through MHC class I. Intracellular bacteria typically reside in endosomes rather than the cytoplasm, but cross-presentation pathways could lead to their recognition via MHC class I; and a failure to kill bacterially infected or activated myeloid cells might explain transporter associated with antigen processing-deficient human phenotypes (33). This aspect of $CD8^+$ T cell function needs further exploration.

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