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-Herpesviruses 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...
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 invaries (14), vaccinating against infection translated (94). Thus. 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. 

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.

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.

**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.
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 rhino-lympho-cryptovirus (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.

**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 γ-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 onco-
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