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Modeling Oncogenic Herpesvirus Infections in Humanized Mice

Philip T. Lange¹, Blossom Damania¹

¹Department of Microbiology and Immunology and Lineberger Comprehensive Cancer Center, The University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, United States

Abstract

The creation of humanized mice generally involves the reconstitution of immunodeficient mice with human immune constituents. Different methodologies have been employed, and significant progress has been made towards the development of robustly humanized mouse models. Some of the techniques used include the injection of mature human immune cells, the injection of human hematopoietic stem cells (HSCs) capable of reconstituting radiation-depleted murine bone marrow, and the implantation of human fetal liver and thymus fragments under the kidney capsule to create a thymic organoid that can support thymopoiesis. This review will serve as a brief introduction to the three most commonly utilized humanized mouse models for the study of gammaherpesvirus-driven pathogenesis, and highlight some of the critical discoveries these models have enabled.

Keywords

KSHV; EBV; mice; oncogenic viruses; immune system

Introduction to gammaherpesviruses

Gammaherpesviruses, like all herpesviruses, are ubiquitous dsDNA viruses that establish lifelong infections. Unlike most alpha- and betaherpesviruses, gammaherpesviruses are associated with many different malignancies. There are two known human gammaherpesviruses, Epstein-Barr virus (EBV) and Kaposi sarcoma-associated herpesvirus (KSHV), which are each associated with significant human cancer burden [1,2]. EBV chronically infects >90% of the adult population, whereas KSHV exhibits unequal global distribution with estimates ranging from <5% to 70% depending on the geographic location and the population examined [3–6]. As herpesviruses, EBV and KSHV exhibit biphasic lifecycles, capable of both lytic replication and latency. Lytic replication is defined by the production of infectious viral progeny, whereas latency is a quiescent phase characterized by limited viral gene expression and the maintenance of the viral episome. ‘Reactivation’ refers to the programmatic switch from the latent to lytic life cycle, and is believed to occur

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Conflict of Interest statement

The authors declare no conflict of interest

periodically throughout the lifetime of the host. The specific frequency of reactivation varies depending on the time post infection, relative competency of the host's immune system, and other factors [7–10]. Both EBV and KSHV demonstrate lymphotropism, but are capable of infecting a variety of cell types, as emphasized by the numerous non-B cell malignancies with which these viruses are associated.

The host tropism of gammaherpesviruses is exquisitely species-specific, hampering *in vivo* animal studies of EBV and KSHV pathogenesis. The natural rodent pathogen, murine gammaherpesvirus 68 (MHV68), has been employed extensively to study the biology of *de novo* gammaherpesvirus infection, and the role of the host immune response in control of gammaherpesvirus replication, latency, and reactivation. MHV68 does not efficiently promote oncogenesis in most wild-type mouse strains, however defects in antigen presentation or interferon gamma signaling allow for the development of lymphoproliferative disease and oncogenesis [11–13]. Xenograft tumor models have long been employed in order to examine growth of gammaherpesvirus-positive tumors in an *in vivo* system. However, these models do not capture the initial virus-driven cellular transformation, and often utilize mice with little to no immune constituents, making immunotherapeutic studies unfeasible. Additionally, many transgenic mouse models have also been developed to study the effect of expressing one or more viral gene in an intact animal host [14,15]. However, these models may not accurately convey the temporal and cell type-specific regulation of viral gene expression, nor the immune response to intact viral particles. Thus, in order to better model the complex virus-host interactions that underpin gammaherpesvirus-driven oncogenesis, a number of groups have pioneered the use of humanized mice.

Infected human cord blood mononuclear cells

A powerful model of EBV-driven lymphomagenesis involves the engraftment of CD34-depleted cord blood that has been infected with EBV *ex vivo* (Figure 1A) [16]. As the CD34⁺ cell population comprises the majority of hematopoietic stem cells, the CD34-depleted cord blood contains primarily fully differentiated immune cells. As such, the T cells present in the CD34-depleted cord blood have been selected in the fetal thymus and exhibit human MHC restriction. The cord blood is infected with EBV, then intraperitoneally injected into 3–5-week-old NOD-*scid*IL2Rg^{null} (NSG) mice, which maintain human lymphocytes for more than four weeks. This is in contrast to mice injected with uninfected cord blood, under which conditions B cells do not readily engraft [16]. This model exhibits a remarkably high penetrance of lymphomagenesis, with nearly 100% of the mice succumbing to EBV⁺ B cell lymphomas within 4–6 weeks of injection [16]. Analysis of EBV gene expression has shown that the virus exists primarily in type III latency, in which all of the latent viral genes are expressed [16]. The arising malignancies display characteristics of human diffuse large B cell lymphomas (DLBCL), and most commonly involve the pancreas, biliary tract, and mesentery, but can also invade other tissues [16,17]. These tumors are highly aggressive, and show high expression of PD-L1 and PD-L2 [14]. Consistent with the expression of inhibitory ligands by the tumor cells, treatment with anti-PD-1 and anti-CTLA4 antibodies significantly slows tumor growth, however all mice eventually succumb

to DLBCL-like disease [18]. Thus, the CD34-depleted cord blood model may be used as a powerful model for study of immunotherapeutic treatments for EBV-driven lymphomas.

Two latent EBV genes that have long been implicated in EBV-driven B cell transformation and proliferation are latent membrane protein 1 (LMP1) and LMP2A. LMP1, which mimics constitutively active CD40 signaling, is required for *in vitro* transformation of primary B cells [20], and is sufficient to promote B cell lymphomagenesis when driven by a B cell specific promoter in transgenic mice [21,22]. LMP2A, which mimics B cell receptor (BCR) signaling, is not necessary or sufficient to promote lymphomagenesis, although its expression in transgenic mouse models drives B cell proliferation and survival [23,24], and can substitute for p53 mutations to facilitate transformation in a MYC model of lymphomagenesis [25]. Furthermore, LMP2A has been demonstrated to prevent apoptosis in B cells that fail to express a functional B cell receptor following somatic hypermutation in the germinal center [26]. Confoundingly, transgenic mice expressing both LMP1 and LMP2A exhibit a “normalized” phenotype, in which the expression of LMP2A attenuates LMP1 driven hyperproliferation and rescues the impaired germinal center formation observed in LMP1 transgenic mice [27]. Interestingly, LMP1- and LMP2A-deficient viruses, as well as a virus lacking both LMP1 and LMP2A, are able to promote lymphomagenesis in this cord blood humanized mouse model [16,28], albeit with slower kinetics. Thus, *in vitro* transformation studies and xenograft models may not accurately recapitulate the nuances that exist in the context of humanized mouse infection using intact virus.

Engraftment of CD34⁺ cells

A similar humanized mouse model relies on the self-renewing HSCs within the cord blood to engraft a humanized immune system prior to viral infection. In this model, the CD34⁺ HSCs from cord blood or fetal liver are isolated and transferred to sublethally irradiated immunodeficient mice, typically via intrahepatic or intravenous injection (Figure 1B). Mice reconstituted with human immune cells in this way are typically referred to using the prefix hu- and the strain mouse used (e.g. huNSG). Most often, newborn mice are used as the recipients of human HSCs, with the assumption that the murine thymic tissue will allow for robust maturation of the HSC-derived human T cells. Notably, as these human T cells are educated in a murine thymus, they will be restricted to murine MHC, an important factor to consider when attempting to model human disease. Nevertheless, the engraftment of human HSCs prior to viral infection allows for a more physiological timeline of infection than the cord blood model discussed above. Furthermore, as in the CD34-depleted cord blood model, the EBV⁺ tumors that arise in this model often resemble human DLBCL [29–31].

All EBV latency genes are expressed in latency III, whereas latency II is defined by the expression of just EBNA-1, EBNA-LP, LMP1, LMP2A, LMP2B, and the EBV-encoded small RNAs (EBERs). Depending on the precise details of the model used and the anatomic site examined, EBV typically exists in either latency II or latency III in HSC humanized mice [29,30,32,33]. Notably, while EBV mutants lacking the oncogenes EBNA3A or EBNA3C were able to establish persistent latency in huNSG mice, these viruses did not promote lymphomagenesis [34]. Additionally, it has been demonstrated that the EBV microRNAs encoded by the BHRF1 locus do not contribute to lymphomagenesis [35],

however EBV lacking all microRNAs was reported not to induce lymphomas in huNSG mice due to the role of EBV microRNAs in immune evasion [36].

There are two subtypes of EBV that circulate in the human population, type 1 and type 2. Type 1 EBV (EBV-1) shows tropism for B cells, and readily transforms primary B cells in cell culture. On the other hand, type 2 EBV (EBV-2) transforms B cells less efficiently in cell culture, but readily infects primary T cells and has been found at high levels in circulating T cells from Kenyan infants [37,38]. Researchers used a huBRGS model to study the biology of EBV-2 in humanized mice. Unlike EBV-1, EBV-2 infection of huBRGS mice results in high viral loads in human T cells, as well as B cells [32]. Interestingly, both EBV-1 and EBV-2 induce similar B cell lymphomas that resemble human DLBCL, and, despite robust T cell infection, no T cell lymphomas are observed in EBV-2 infected animals [32].

Finally, the CD34⁺ HSC humanized mouse model has proven to be a powerful tool for the examination of immune responses to primary EBV infection. Despite EBV having been discovered more than 50 year ago, the immunological mechanisms that underly asymptomatic infection, infectious mononucleosis, viral oncogenesis, and other EBV-related clinical presentations have remained elusive and difficult to study in a basic science setting. Through the use of huNSG mice, investigators have confirmed the critical role of CD8⁺ T cell function in restricting EBV lytic replication and reactivation [39–41], as well as explored innate immune recognition of EBV in the context of an intact host [42,43]. Furthermore, huNSG mice have been employed to explore potential EBV vaccination strategies with promising results [43–45],

Use of fetal liver and thymus

The most comprehensive humanized mouse model combines the use of fetal liver and thymic tissue, as well as CD34⁺ HSCs to develop of robust human immune system (Figure 1C). The implantation of human fetal liver and thymic tissue fragments under the renal capsule on its own results in hematopoiesis within the thymic organoid, with human T cells comprising the bulk of human immune cells [46,47]. The injection of autologous CD34⁺ HSCs into mice previously receiving the subrenal implantation leads to reconstitution of the bone marrow with human hematopoietic progenitors, greatly improving the comprehensiveness of the immune system [48]. Unlike most huNSG and related models, adult mice are used as recipients for the thymic organoid and human HSCs. Thus, due to the thymic atrophy in adult immunodeficient mice, the HSC-derived human T cells mature only in the implanted organoid and experience human MHC restriction. The resulting robustly humanized mice are commonly referred to as bone marrow/liver/thymus, or “BLT” mice. Notably, as these mice also generate human mucosal immunity, more physiologically relevant routes of infection can be employed [48–51].

EBV expresses numerous oncoproteins during latency that confer the transforming potential of the virus. Using the BLT model researchers have demonstrated that lytic replication, as well as latent gene expression, contributes to the development of EBV⁺ lymphomas [52]. Interestingly, a “superlytic” EBV lacking BZLF1 promoter silencing motifs is able to promote lymphomagenesis in BLT mice with the same efficiency as wild type EBV [53].

Thus, the mechanisms by which EBV promotes oncogenesis may not be limited to the latent lifecycle.

While many groups have used humanized mice and longitudinal human studies to examine the events that occur in the first days and weeks following EBV infection, much less is known about primary KSHV infection. The first studies of KSHV infection of humanized mice took place prior to the advent of the BLT model, and utilized SCID-hu Thy/Liv mice [54]. The authors demonstrated efficient infection when KSHV was injected directly into the human thymic organoid, with lytic replication peaking around two weeks post infection then waning. Latent KSHV infection was demonstrated as long as 112 days post infection, and, despite T cells comprising the majority of human lymphocytes in this model, CD19⁺ B cells were shown to be the primary viral reservoir within the organoid. Furthermore, treatment with the antiviral drug ganciclovir dramatically reduced the lytic and latent viral burden.

A later study utilized a different humanized mouse model in which fetal bone and thymus fragments are surgically implanted subcutaneously into NOD/SCID mice [55]. Unlike SCID-hu Thy/Liv mice which exhibit relatively low B cell engraftment, these NOD/SCID-hu BM/Thy mice generate a greater number of peripheral B cells, and are suitable for studies of human antibody production. As such, this study identified human antibody production in the implanted mice. Further, when tested by immunofluorescence, the staining pattern resembled the punctate nuclear staining displayed by anti-LANA antibodies, however the functional quality of the antibody response was not examined. Remarkably, in addition to KSHV infection of NOD/SCID-hu BM/Thy mice, the authors demonstrated long-term infection of non-humanized NOD/SCID mice. Thus, in the presence of a defective immune system, KSHV may be able to establish chronic infection within a murine host. Furthermore, a separate group demonstrated KSHV infection of CD34⁺ HSCs, which persist in NOD/SCID mice and give rise to infected lymphoid and myeloid cells, suggesting that KSHV may utilize the HSC compartment for long-term persistence [56].

More recently, KSHV infection of BLT humanized NSG mice has been demonstrated [51]. In this study the authors report systemic KSHV infection in animals infected intraperitoneally, orally, and intravaginally, however multiple inoculations were used to establish infection. Interestingly, this study detected latent KSHV in multiple tissues not often examined in other studies, including the skin, liver, lung, kidney, and small intestine. In addition to B cells, the authors report robust infection of macrophages, which are one of the primary reservoirs of MHV68 [57–60], and could comprise some of the infected CD19⁺ cells observed in the SCID-hi Thy/Liv model [54].

Due to the remarkable prevalence of the human gammaherpesviruses, millions of people are co-infected with both EBV and KSHV. Furthermore, the KSHV-driven malignancy, primary effusion lymphoma (PEL), also harbors EBV in ~80% of cases. The interplay between these two viruses within the same host is a longstanding topic of interest, but has been difficult to model prior to the advent of robust humanized mouse models. A recent study used huNSG mice to examine the biology of EBV/KSHV co-infection [61]. Fascinatingly, co-infection correlated with greater EBV and KSHV burden in the spleen compared to singly infected animals. Additionally, while animals infected with KSHV alone did not develop any grossly

observable pathology, co-infected animals exhibited a significantly reduced survival and greater tumor incidence than counterparts infected only with EBV.

Of particular note from this study was the development of PEL-like lymphomas in dually infected huNSG mice. The lymphomas from EBV/KSHV infected mice showed enrichment of genes expressed in PEL cell lines compared to lymphomas from EBV infected mice. Further, the EBV/KSHV infected animals exhibited greater EBV lytic gene expression relative to singly infected mice. This increased activity of the lytic program was associated with enhanced tumorigenesis, as mice dually infected with KSHV and an EBV lacking the major lytic transactivator, BZLF1, showed reduced tumor incidence and greater survival than mice dually infected with KSHV and wild-type EBV. Interestingly, this study found no effect of EBV lytic gene expression on KSHV copy number. Lastly, the authors were able to correlate these findings to analysis of human B cell lymphomas, in which they observed greater EBV lytic antigen expression in malignancies that also harbored KSHV. Thus, this study demonstrates a new animal model for the co-infection of EBV and KSHV. Initial findings from this model suggest that EBV and KSHV may interact in a mutualistic fashion, resulting in greater persistence of each virus and enhanced tumorigenesis.

Concluding remarks

Here we have discussed some of the most commonly used humanized mouse models, and their usefulness for the study of gammaherpesvirus infection and oncogenesis. Each model confers specific benefits and potential drawbacks, so careful consideration must be made when deciding upon the best model to address a particular research question. Gammaherpesviruses are exquisitely species-specific, and as such, small animal models of gammaherpesvirus infection and pathogenesis are limited. Thus, humanized mouse models represent a powerful tool with which to address longstanding questions in the gammaherpesvirus field. Importantly, as relatively little is known about primary KSHV infection, humanized mice represent an opportunity to explore previously unanswerable questions regarding the biology of KSHV.

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Figure 1. Humanized mouse models for the study of gammaherpesvirus infection.

The diagram above illustrates the three most common humanized mouse models for the study of EBV and/or KSHV infection, and highlights key benefits and drawbacks of each model. **A)** The cord blood humanized mouse model utilizes CD34⁺ mononuclear cells (MNCs) isolated from human cord blood. These cells are infected with EBV *ex vivo*, then intraperitoneally injected into immunodeficient NSG mice. The injected mice maintain human B and T cells for >4 weeks, and nearly all mice will succumb to DLBCL-like malignancies between 4–6 weeks post injection. **B)** HSC humanized mice are created by transplanting CD34⁺ human HSCs into sub-lethally irradiated newborn mice. The injected stem cells support multilineage hematopoiesis resulting in long-term reconstitution with human immune cells. Animals are monitored to determine the frequency of human immune cells in circulating blood, and are typically used for infection studies ~12 weeks post-transplant. Due to their selection in the murine thymus, the human T cells in this model are H2 restricted. This model has been used for EBV, KSHV, and EBV/KSHV co-infection studies. **C)** To generate BLT mice, small fragments of human fetal liver and thymus are implanted under the renal capsule of sub-lethally irradiated adult mice. Autologous CD34⁺ HSCs are then injected, resulting in mice with the most comprehensive human immune system of the models discussed here. Like HSC humanized mice, BLT mice are bled to determine the extent of human immune cell reconstitution before use in experiments. As

these mice harbor human thymic tissue, the human T cells will exhibit HLA restriction. This model has been used for EBV and KSHV infection studies.

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