Modelling of human herpesvirus infections in humanized mice

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The human herpesviruses (HHVs) are remarkably successful human pathogens, with some members of the family successfully establishing infection in the vast majority of humans worldwide. Although many HHV infections result in asymptomatic infection or mild disease, there are rare cases of severe disease and death found with nearly every HHV. Many of the pathogenic mechanisms of these viruses are poorly understood, and in many cases, effective antiviral drugs are lacking. Only a single vaccine exists for the HHVs and researchers have been unable to develop treatments to cure the persistent infections associated with HHVs. A major hindrance to HHV research has been the lack of suitable animal models, with the notable exception of the herpes simplex viruses. One promising area for HHV research is the use of humanized mouse models, in which human cells or tissues are transplanted into immunodeficient mice. Current humanized mouse models mostly transplant human haematopoietic stem cells (HSCs), resulting in the production of a variety of human immune cells. Although all HHVs are thought to infect human immune cells, the beta- and gammaherpesviruses extensively infect and establish latency in these cells. Thus, mice humanized with HSCs hold great promise to study these herpesviruses. In this review, we provide a historical perspective on the use of both older and newer humanized mouse models to study HHV infections. The focus is on current developments in using humanized mice to study mechanisms of HHV-induced pathogenesis, human immune responses to HHVs and effectiveness of antiviral drugs.

Human herpesviruses

Human herpesviruses (HHVs) are nearly ubiquitous infectious agents that contribute substantially to human morbidity and mortality. Nine HHVs have been discovered to date, including herpes simplex virus types 1 and 2 (HSV-1 and HSV-2), varicella zoster virus (VZV; HHV-3), Epstein–Barr virus (EBV; HHV-4), human cytomegalovirus (hCMV; HHV-5), HHV-6A and HHV-6B, HHV-7, and Kaposi's sarcoma-associated herpesvirus (KSHV; HHV-8). Within the family *Herpesviridae*, there are three subfamilies with natural human pathogens listed: the *Alphaherpesvirinae* (HSV-1, HSV-2, VZV), the *Betaherpesvirinae* (hCMV, HHV-6A, HHV-6B, HHV-7) and the *Gammaherpesvirinae* (EBV, KSHV) (McGeoch et al., 2006). The HHVs are considered to be some of the most successful pathogens of humans due to the high rate of seroconversion and the ability to establish lifelong latent infections.

HHV infections are linked to a wide spectrum of human diseases, ranging from skin lesions to encephalitis, from immune deficiency to autoimmune disease, from blindness to deafness, from organ transplant rejection to post-transplant proliferative disorders, and from epilepsy to several types of cancer. Despite the burden of infection and

disease caused by the HHVs, a preventative vaccine is available only for VZV (Takahashi et al., 2008). Further, our ability to control infections and disease is in many cases limited due to the lack of available antiviral drugs. All HHVs are able to form a persistent infection, which typically involves a latent phase characterized by minimal viral gene expression and inability to detect infectious virus, followed by reactivation where many viral genes are expressed, viral particles are produced and virus is shed from the host. Although persistent HHV infections are required for many types of pathogenesis, research efforts have thus far been unable to eliminate persistent HHV infections in humans.

Animal models for HHVs are needed

Part of the reason for our lack of understanding of HHV pathogenesis is due to the lack of appropriate animal models that faithfully recapitulate infection and disease. In the case of HSV-1 and HSV-2, the availability of small-animal models has contributed substantially to our understanding of these viruses; as a result, they are the best understood of the HHVs and serve as prototypes for the family. However, the alphaherpesviruses (HSV-1, HSV-2, VZV) are fundamentally different from the beta and

gammaherpesviruses in that the alphaherpesviruses principally establish latency in neuronal cells *in vivo* and are transmitted predominantly via skin contact or inhalation. The beta and gammaherpesviruses predominantly establish latency in immune cells (also endothelial cells in the case of KSHV) *in vivo*. These viruses can be transmitted by saliva, sexual contact or through breast milk.

Despite the inability to adequately study the HHVs directly in humans due to ethical constraints and the general lack of good animal models for the HHVs, there are several homologues of the HHVs that infect animals and that have been useful towards achieving a better understanding of the human pathogens. For example, animal homologues of hCMV have been well-studied in their native hosts, including murine CMV, rat CMV, guinea pig CMV and rhesus CMV (McGregor, 2010). Murid herpesvirus 68 (MHV-68) (Stevenson & Efstathiou, 2005) and murid herpesvirus 4 (Nash *et al.*, 2001) in mice and rhesus rhadinovirus (macacine herpesvirus 5) in monkeys (Orzechowska *et al.*, 2008) serve as models of KSHV infection in humans. EBV can infect some types of New World primates, including the common marmoset and the cottontop tamarin (Shope *et al.*, 1973; Wedderburn *et al.*, 1984). These animal models have been very useful and will continue to yield valuable data, but they have several limitations. (i) Animal homologues of HHVs are genetically divergent from their HHV counterparts and thus encode different genes. Hence, any information gained on mechanisms of pathogenesis, development of antiviral drugs and vaccines may not be directly applicable to HHVs in a human host. (ii) Non-human primate research is expensive and can only be performed in specialized facilities that are not available to many researchers. For these reasons, the availability of new animal models to study mechanisms of viral pathogenesis *in vivo* would be greatly beneficial to the development of vaccines and new treatment strategies.

Humanized mice

One such model is humanized mice, which are immunodeficient mice engrafted with human cells or tissues. The original humanized mouse models, first reported in 1988, involved SCID (severe combined immunodeficiency) mice engrafted with either human peripheral blood leucocytes (SCID-hu-PBL model) (Mosier *et al.*, 1988) or with human foetal thymic and liver tissues as a model of a human thymus (SCID-hu Thy/Liv model) (McCune *et al.*, 1988). Although both models exhibited human immune cell engraftment, there were several limitations noted in these models: lack of long-term human cell engraftment (PBL model), low diversity in types of cells engrafted (PBL and Thy/Liv models), lack of distribution of human cells in the mouse (Thy/Liv model) and inability to generate primary human immune responses (PBL and Thy/Liv models) (Macchiarini *et al.*, 2005; Shultz *et al.*, 2007). Both of these models were explored extensively for studies of human immunodeficiency virus type 1 (HIV-1) (Mosier, 1996) and limited work was also done with HHVs (see below).

Over the past decade, new humanized mouse models have emerged based upon greater mouse immunodeficiency and transplantation of human haematopoietic stem cells (HSCs). A recent review of the mouse strains used and the types of cells used for transplantation is available (Tanner *et al.*, 2014). Briefly, most mouse strains currently in use are double-knockouts that prevent maturation of T-lymphocytes via the inability to produce enzymes involved in the process of DNA recombination required to produce T/B-cell receptors, including mutations in the recombinase activating genes (*rag1* or *rag2*) or *prkdc* gene (SCID) (Shultz *et al.*, 2007). Natural killer (NK)-cells are also effective in eliminating xenografts and mutations in the common γ-chain receptor (IL2Rγ or γ_c) or the NOD (non-obese diabetic) mutation prevent NK-cell development (Shultz *et al.*, 2007). The use of human HSCs for transplantation has resulted in a more broad diversity of human cell types engrafted and the cells are distributed throughout many organs of the mouse (Traggiai *et al.*, 2004). Human HSCs can be obtained from umbilical cord blood, foetal liver or mobilized peripheral blood.

As a result of these advances in humanized mouse technology, humanized mouse engraftment is long-lived and primary human adaptive immune responses are detected against many types of pathogens (Macchiarini *et al.*, 2005; Shultz *et al.*, 2007). Of note, the predominant human cell types present in these new models are human B- and T-lymphocytes, although other cell types (principally monocytes/macrophages and dendritic cells) are readily detectable (Traggiai *et al.*, 2004). Human NK-cells, granulocytes, erythrocytes and platelets are typically found in low abundance, although newer research is allowing more efficient production of some of these immune components (Chen *et al.*, 2009; Hu *et al.*, 2011; Hu & Yang, 2012; Huntington *et al.*, 2009; Pek *et al.*, 2011). Human lymphocytes, myeloid cells and HSCs are important targets and reservoirs of human beta and gammaherpesvirus infections and latency *in vivo*. A variety of HHV pathogens have begun to be explored in these new models in order to determine their suitability for studies of HHV pathogenesis, drug testing, exploration of mechanisms of latency and reactivation, and vaccines.

Although human T- and B-cell responses are commonly detected in humanized mice following exposure to HHVs and other antigens, many experiments have shown sporadic detection of these responses and the amplitude of responses also varies. Although the reasons for these findings are not well understood, it has been hypothesized that as T-cell maturation occurs in a thymus that expresses both murine and human MHC-I molecules, this unusual selection mechanism may play a role in relatively weak adaptive immune responses (Shultz *et al.*, 2012). The very high frequency of EBV-induced cancers in humanized mice (see below) is possibly promoted by weak adaptive immunity and hence a lack of immunological control of EBV. Efforts to improve human adaptive immunity in these models revolve around the introduction of human

MHC-I expression into the thymus by two main mechanisms: (i) transplantation of human thymic stromal cells, referred to as a BLT (bone marrow, liver, thymus) humanized mouse (Melkus et al., 2006), or (ii) a genetic knock-in of human MHC-I into the mouse genome (Shultz et al., 2010). These improvements will be critical for future vaccine studies because weak adaptive immunity will not allow for effective challenge studies to determine vaccine efficacy.

Other mechanisms to humanize mice also exist, built upon the same platform that requires an immunodeficient host. Human liver cells have successfully been transplanted into mice (Azuma et al., 2007), as has human skin, human retinal tissues and human aortic tissues (see below). As the lack of a human receptor molecule is a main obstacle to viral infection, some efforts have been made to produce knock-in mice that express a human viral receptor molecule as another way to confer susceptibility to viral infection to a mouse (Reynaud et al., 2014).

Gammaherpesvirus studies in humanized mice

Of the HHVs, the gammaherpesviruses have been most extensively studied in humanized mice, especially EBV. A number of human EBV-associated diseases have been recapitulated in humanized mice in recent years, thus illustrating the utility of this model to study HHV pathogenesis.

EBV

The main target cells of EBV in vivo are epithelial cells and B-lymphocytes, although there is also evidence for infection of T-lymphocytes and NK-cells. Latency is established in B-lymphocytes or epithelial cells (Rickinson & Kieff, 2007). EBV infection is correlated strongly with a number of human diseases, such as infectious mononucleosis and cancers (including Burkitt's lymphoma, nasopharyngeal carcinoma, Hodgkin's lymphoma and gastric carcinoma); in addition, EBV infection is associated with autoimmune diseases, such as multiple sclerosis, systemic lupus erythematosus and rheumatoid arthritis (Longnecker et al., 2013).

Most of the early work on EBV in humanized mice used the SCID-hu-PBL model and, as human blood donors have a high frequency of persistent EBV infection, researchers were able to characterize the effects of transplantation of EBV-positive blood into immunodeficient mice (Fuzzati-Armentero & Duchosal, 1998; Mosier et al., 1989) and lymphoproliferation was noted in many cases. As these reports constitute transfer of virus-infected cells rather than in vivo transmission of cell-free virus, we will focus on the latter. Humanized mice can be infected successfully with EBV by several injection mechanisms, including intrasplenic (Islas-Ohlmayer et al., 2004; Wahl et al., 2013), intraperitoneal (Cocco et al., 2008; Heuts et al., 2014; Strowig et al., 2009) and intravenous inoculation (Kuwana et al., 2011; Sato et al., 2011; Yajima et al., 2008). Exposure of HSC-humanized mice to EBV results in detectable viral DNA by 2–4 weeks post-inoculation in the spleen, blood, bone marrow, liver, kidney, adrenal gland, lung and lymph nodes (Islas-Ohlmayer et al., 2004; Traggiai et al., 2004; Yajima et al., 2008). Blood viral load typically peaked in the range of 10^4–10^5 genome copies (Islas-Ohlmayer et al., 2004).

EBV-associated cancers. Development of lymphoproliferative disease and tumours in humanized mice is dependent upon exposure to higher doses of virus (Traggiai et al., 2004; Yajima et al., 2008), although persistence of viral DNA occurs with even a low dose (Yajima et al., 2008). Animals infected with a low dose exhibited persistent viral DNA in various organs, but virus appeared to have been controlled because no cancer was detected (Yajima et al., 2008). Exposure to higher doses leads to tumours in multiple organs, followed by death at 5–10 weeks post-infection (Yajima et al., 2008). Tissues without tumours, but harbouring persistent EBV (bone marrow and spleen), were analysed for viral gene expression patterns, and showed expression of Epstein–Barr nuclear antigen (EBNA) 1, EBNA2, latent membrane protein (LMP1) and LMP2a, indicative of a type III latency program (Yajima et al., 2008).

EBV-positive tumours have been detected in the spleen, liver, kidney and lymph node (Traggiai et al., 2004; Yajima et al., 2008), and as early as 4–7 weeks post-infection in spleen (Heuts et al., 2014; Islas-Ohlmayer et al., 2004). Tumours had a B-cell phenotype (CD20$^+$) and were negative for the pan-T-cell marker CD3 (Islas-Ohlmayer et al., 2004; Yajima et al., 2008). Tumours have been evaluated for viral gene expression patterns, and have been found to express EBNA1, EBV-encoded small RNAs (EBERs), LMP1 and LMP2a, but not EBNA2 (Islas-Ohlmayer et al., 2004; Traggiai et al., 2004; Yajima et al., 2008). This pattern is consistent with a type II latency profile (Islas-Ohlmayer et al., 2004). This characterization of latency is interesting because only a type III expression profile is correlated with cellular proliferation in vitro, but it is type II (and not type III) latency that correlates with tumour development in vivo in humanized mice. Further analysis has shown that type I and type IIa latency types are found more abundantly in CD8 T-cell-depleted humanized mice and are not found in CD4 T-cell-depleted animals, indicating that T-cell help is required for proliferation (Heuts et al., 2014). Ex vivo culture of cells from blood, bone marrow and spleen resulted in production of lymphoblastoid cell lines (Islas-Ohlmayer et al., 2004). Although most reports have focused on B-cell tumours, another study analysed transplantation of peripheral blood mononuclear cells (PBMCs) from patients suffering from chronic active EBV, and found that T-cells and NK-cells continued to proliferate in the context of a humanized mouse model, including clonal expansion of T-cells (Imadome et al., 2011).

Human immune responses to EBV. One of the exciting achievements with human HSC-transplanted mice is the

ability to detect and characterize *de novo* human adaptive immune responses in response to viral antigens. Both T-cell and antibody responses have been detected in humanized mice towards EBV antigens, and it is thought that the inability of the virus to cause tumours after low-dose exposure may be due to control of the virus by the human immune response (Traggiai *et al.*, 2004). T-cell expansion and an inversion of the normal CD4/CD8 ratio have been reported (Traggiai *et al.*, 2004; Yajima *et al.*, 2008), along with EBV-specific T-cell responses that are commonly measured by ELISPOT assay to detect IFN-γ production after exposure to EBV antigen or by a cell-killing assay (Ma *et al.*, 2011; Traggiai *et al.*, 2004; Yajima *et al.*, 2008, 2009). Further evidence of the protective effects of human T-cell responses was gained by depleting either all human T-cells or just the CD8$^+$ fraction; these experiments showed a higher viral load and a reduced lifespan in EBV-infected humanized mice (Strowig *et al.*, 2009; Yajima *et al.*, 2009). Lytic antigens are targeted more frequently by T-cells than latent antigens and the response is also stronger to these antigens (Strowig *et al.*, 2009). Human antibody responses to EBV have also been detected in these models (Yajima *et al.*, 2008). As human lymphocytes develop in the mouse, they are exposed to both murine and human antigens and MHC molecules. In order to determine if human MHC molecules are used to select for T-cell receptors, antibodies specific to human MHC-I and MHC-II were used to block T-cell recognition and detectable T-cell responses were lost, thus showing that at least some selection takes place via human MHC (Melkus *et al.*, 2006; Strowig *et al.*, 2009). One study reported on the development of immunodeficient mice that were additionally engineered to express human HLA-A2 molecules in order to determine if human T-cell responses would be stronger due to selection of T-cell receptors on human MHC. Following human HSC engraftment, they infected these humanized mice with EBV and detected robust HLA-restricted T-cell responses (Shultz *et al.*, 2010).

EBV genetics and relation to cancer. Several studies have been designed to analyse EBV genes that contribute to carcinogenesis in humanized mice in the hope that new targets might be identified to allow for prevention and treatment of EBV-associated cancers. An EBV strain with a mutated BZLF1 gene is defective for lytic replication, but is still able to establish latency in humanized mice. Animals infected with the BZLF1 mutant developed cancers at a lower frequency compared with WT virus, but it is still unclear if this was due to the BZLF1 mutation itself or due to diminished levels of virus due to the inability to replicate (Ma *et al.*, 2011). The same group published a later study with a strain with increased BZLF1 expression as compared with WT virus as a 'super-lytic' virus in order to see if the reverse effect had an impact on lymphomagenesis. They found that the super-lytic virus tended to form abortive lytic infections, but that latency could still develop and the frequency of tumour development was not different compared with WT virus (Ma *et al.*, 2012). These results

are interesting, because it has been difficult to achieve HHV mutants that are unable to establish latency to date and a virus with enhanced lytic properties is one way to accomplish that goal; such results would be highly beneficial to HHV vaccine research.

Whilst cell culture research has been very informative in virology, there are many examples of research that differs from the *in vitro* to the *in vivo* environments. An example of this is a study that examined the EBV-encoded BHRF1 microRNA (miRNA) cluster, and found that deletion of these miRNAs prevented B-cell expansion and reduced latent gene expression when studying B-cell transformation *in vitro*. These results suggested that the BHFR1 miRNAs might contribute to cancer development (Feederle *et al.*, 2011). However, when humanized mice were infected with a BHRF1 miRNA mutant, no difference in the ability to develop cancer *in vivo* was noted, although they did find that the miRNAs assist in the development of an acute systemic infection (Wahl *et al.*, 2013). These findings illustrate the utility of humanized mouse research to examine the relevance of *in vitro* research.

EBNA3B is not required for B-cell transformation by EBV *in vitro*. The role of EBNA3B in lymphomagenesis was investigated *in vivo* in humanized mice. Interestingly, infection with EBNA3B-deficient EBV leads to production of aggressive diffuse large B-cell-lymphoma-like cancer in humanized mice, but T-cells are less apt to infiltrate the tumours and to kill tumour cells as compared with WT virus. The authors concluded that EBNA3B is a virus-encoded tumour suppressor that normally assists in immune recognition of tumour cells and slows down tumour progression. After noting these results, the authors analysed human samples and also discovered EBNA3B mutations that had a similar effect on human tumour progression (White *et al.*, 2012). These results indicate that EBV has evolved a fine balance of promoting B-cell proliferation, whilst also acquiring mechanisms to prevent cancer development that would compromise the host.

Other EBV-associated diseases. EBV infection is associated with rheumatoid arthritis and this disease can also be reproduced in humanized mice (Kuwana *et al.*, 2011). Synovial cells were greatly expanded and a variety of human inflammatory cells infiltrated the synovium, including both helper and cytotoxic T-cells, B-cells, and macrophages. Few EBV-positive cells were detected (by EBER expression) in the synovial membrane of arthritic joints, whilst many EBV-positive cells were found in the bone marrow near affected joints. Around 65 % of mice developed these symptoms and the phenomenon was not dose-dependent, as seen in the above cancer studies.

Haemophagocytic lymphohistiocytosis (HLH) is an auto-immune disease that is associated with EBV infection (acquired HLH), and is characterized by leucocytosis, anaemia and thrombocytopenia, plus very high levels of inflammatory cytokines, such as IFN-γ and TNF-α.

Humanized mice were shown to exhibit symptoms similar to those seen in humans after 10 weeks of EBV infection, with two-thirds of mice showing this disease and also showing high/persistent viraemia (Sato *et al.*, 2011). Engulfment of erythrocytes was detected in bone marrow, spleen and liver, and higher viral loads were correlated with higher CD8 T-cell activation frequency and increased IFN-γ secretion.

Summary and future directions. An impressive array of human EBV-associated diseases has been recapitulated in humanized mice. Lymphomagenesis has been detected across all of the new humanized mouse models (Cocco *et al.*, 2008; Islas-Ohlmayer *et al.*, 2004; Ma *et al.*, 2011, 2012; Traggiai *et al.*, 2004; Wahl *et al.*, 2013; White *et al.*, 2012; Yajima *et al.*, 2008) and human T-cell responses to EBV infection have similarly been detected in various models (Melkus *et al.*, 2006; Shultz *et al.*, 2010; Strowig *et al.*, 2009; Traggiai *et al.*, 2004; Yajima *et al.*, 2008, 2009). Not all EBV-associated diseases are expected to be recapitulated in current humanized mouse models, e.g. nasopharyngeal carcinoma involves human cell types not present in current humanized mouse models and the murine equivalent cells cannot be infected with EBV. It has yet to be determined if the humanized mouse model used in a particular report has an impact upon pathogenesis or immunity because of a lack of studies designed to answer this question. Future studies will most likely focus on discovering mechanisms of EBV pathogenesis and the development of new treatments for EBV-associated cancers.

KSHV

KSHV principally infects human B-cells, but is also able to infect endothelial cells, fibroblasts, keratinocytes, monocytes/macrophages and HSCs. Latency is mostly detected in B-cells. The main diseases associated with KSHV in humans are cancers, including Kaposi's sarcoma (KS), pleural effusion lymphoma and multicentric Castleman's disease (Lebbé & Francès, 2009). KS is an endothelial cell cancer and thus it is unlikely to be recapitulated in HSC-engrafted mice because human endothelial cells do not arise from HSCs. As mentioned above, other human tissues and cells have been transplanted to immunodeficient mice besides blood cells. Human skin transplants in SCID mice were injected with KSHV and showed viral replication, with the formation of KS-like lesions (Foreman *et al.*, 2001).

SCID-hu-PBL mice were found to not support transmission of KSHV *in vivo* (Picchio *et al.*, 1997). The lack of infection in SCID-hu-PBL mice was likely due to a lack of the appropriate type of human B-cells as only mature cells are engrafted. The thymic organoid of SCID-hu Thy/Liv mice was directly injected with KSHV and supported lytic replication in rare human B-cells in the thymic graft (Dittmer *et al.*, 1999). However, this model has not been pursued further, possibly due to the rarity of infected cell

types and/or lack of pathogenesis. KSHV-transformed cell lines readily produce KSHV-positive tumours in SCID mice and these studies have been useful to gain an understanding of tumorigenesis and angiogenesis, and to test novel antiviral strategies (Boshoff *et al.*, 1998; D'Agostino *et al.*, 1999; Lan *et al.*, 2009; Picchio *et al.*, 1997; Staudt *et al.*, 2004; Wu *et al.*, 2005).

Only a few reports exist on KSHV infection in HSC-engrafted mice. Wu *et al.* (2006) showed that KSHV infection of human HSCs prior to engraftment leads to persistent infection in the transplanted mice. KSHV-positive human B-cells and macrophages in the spleen and bone marrow were detectable by flow cytometry for GFP expressed from recombinant KSHV. Quantitative real-time (qRT)-PCR analysis further showed maintenance of viral DNA in spleen, bone marrow and blood for up to 29 weeks post-infection. Parsons *et al.* (2006) provided evidence for KSHV replication in immunodeficient NOD/SCID mice with no human immune cells. Although published in 2006, to our knowledge that model has not been examined further. The same study also examined animals humanized with human HSCs and demonstrated successful KSHV infection following intravenous injection, accompanied by increases in splenic viral DNA detection over 3 months. They also demonstrated detection of human IgG specific to KSHV.

A recent report describes for the first time KSHV infection of mice previously humanized with HSCs (Wang *et al.*, 2014). The authors demonstrated that humanized mice can be infected via intraperitoneal injection, but also that transmission through mucosal (oral or vaginal) exposure is a better representation of the normal infection pathways in humans. Viral DNA could be detected after infection in the skin, spleen, lung, lymph node, liver, kidney and intestines. Recombinant KSHV expressing GFP was used in the study, thus allowing for detection and characterization of infected cells by flow cytometry. GFP-expressing human cells were detected in the spleen and were found to be mostly human B-cells, with some evidence for macrophage infection. Antigens representing both latent and lytic infection were detected in spleen and in skin. No symptoms of cancer (KS or lymphomas) were noted in this study. It is currently unclear if EBV infection or HIV/AIDS might be co-factors necessary for KSHV cancers to occur (Carbone *et al.*, 2009; da Silva & de Oliveira, 2011), which may explain the lack of disease in this study.

Summary and future directions. There are relatively few studies of KSHV in humanized mice and none have recapitulated any KSHV-associated diseases to date. It is possible that co-infections with EBV and/or HIV may be necessary to reproduce KSHV-associated cancers, because KSHV disease is often associated with these other pathogens. As mentioned above, an engraftment model using human skin supported KSHV infection and induction of KS-like lesions, but that study was published in 2001 and little follow-up work has been performed in that area. Once

disease models are established, a greater understanding of carcinogenic mechanisms can hopefully be achieved, possibly leading to new treatments for KSHV-induced cancers.

Betaherpesvirus studies in humanized mice

hCMV

hCMV has an *in vivo* tropism for human macrophages, dendritic cells, endothelial cells and epithelial cells. Primary hCMV infection is typically asymptomatic, although it can be accompanied by fever and mononucleosis. Transmission can occur through contact with a variety of bodily secretions, such as saliva, breast milk and urine; transplacental transmission also occurs and is associated with serious birth defects. Transplantation of blood or solid organs is also associated with hCMV transmission, although leuco-depletion is practiced on blood to prevent transmission of this and other viruses. hCMV disease is most common in immunocompromised hosts, such as AIDS patients and organ transplant recipients (Mocarski *et al.*, 2007).

hCMV infection of HSC-humanized mice. hCMV is thought to be transmitted principally by cell-associated infection; accordingly, cell-free virus did not yield successful transmission, whilst injection of hCMV-infected human fibroblasts via the intraperitoneal route was successful for transmission to humanized mice (Smith *et al.*, 2010). Infection resulted in detectable viral DNA in bone marrow, spleen and kidney, whilst no viral DNA was detected in PBMCs, liver, lung, salivary gland or bladder. After showing evidence of persistent infection, the authors then analysed the effects of human granulocyte colony-stimulating factor (G-CSF) treatment on hCMV-infected animals. G-CSF is commonly used to mobilize human HSCs from bone marrow for later transplantation, and Smith *et al.* (2010) hypothesized that this cytokine could also induce hCMV reactivation and replication *in vivo*. Following G-CSF treatment, viral DNA was detected at higher levels and in new organs in humanized mice, supporting the idea that G-CSF promotes viral replication *in vivo*. Early and late gene expression was detected in G-CSF-treated animals, but not in untreated animals, indicating that G-CSF treatment resulted in reactivation of latent virus. Human liver monocytes/macrophages expressed viral late glycoproteins. Thus, a model has been created that allows for studies of hCMV latency and mechanisms of reactivation, including the potential to study ways to stimulate or block viral reactivation.

A follow-up study examined the possibility of increased probability of hCMV transmission during peripheral blood stem cell transplantation. In this study, the authors took G-CSF mobilized peripheral blood stem cells from hCMV-positive human donors and transplanted them to hCMV-negative humanized mice. Viral DNA was detected in bone marrow, liver and spleen following transplantation, indicating that transmission and dissemination took place

(Hakki *et al.*, 2014). These findings indicate that the use of G-CSF stimulates hCMV reactivation and may result in increased probability of viral transmission during blood stem cell transplantation.

This same HSC-engrafted model was also used to study the function of the hCMV UL133–UL138 gene locus *in vivo* (Umashankar *et al.*, 2011). Humanized mice were infected with either WT or UL133–UL138-deleted strains and then virus was stimulated to reactivate using a similar protocol to that mentioned above. Both viruses were able to establish infection in the bone marrow of humanized mice and viral DNA levels were not significantly different either with or without G-CSF treatment. Following G-CSF treatment, both viruses exhibited higher DNA levels in the spleen, but the UL133–UL138 mutant showed higher genome copy numbers in this organ than the WT virus, indicating that these genes may play an important role in viral replication, latency or dissemination.

SCID-hu Thy/Liv and retinal transplant models have also been used to study viral genetics, as the retinal transplant humanized mouse model supports hCMV replication in glial cells of the graft (Bidanset *et al.*, 2001). hCMV UL27 mutants were inoculated into these tissues in order to determine if the UL27 gene is necessary for *in vivo* replication and it was determined to be non-essential (Prichard *et al.*, 2006). A 15 kb region of the hCMV genome that is present in virulent hCMV strains, but missing from attenuated strains, was deleted and then studied for replicative ability in SCID-hu Thy/Liv mice. Although the mutant only had a minor growth defect in human foreskin fibroblast cells, it was unable to replicate in the thymic graft (Wang *et al.*, 2005).

hCMV drug testing in humanized mice. In the early 1990s, it was discovered that hCMV can replicate in thymic epithelial cells of SCID-hu Thy/Liv mice (Mocarski *et al.*, 1993) and in implanted human retinal tissue in immuno-deficient mice (Epstein *et al.*, 1994). These experimental systems were used to test new antiviral drugs for efficacy against hCMV and this topic has been reviewed previously (Kern, 2006). Some additional studies have taken place since that review was published (Quenelle *et al.*, 2008), including a report that human hepatocytes can be transplanted to immunodeficient mice, that they can be infected with hCMV and that this model is useful to study antiviral drug testing (Kawahara *et al.*, 2013). In addition, human skin grafts can be achieved, infected with hCMV and controlled by gancyclovir (Bravo *et al.*, 2007).

hCMV pathogenesis in humanized mice. hCMV infection has been associated with a variety of diseases, including transplant coronary arteriosclerosis. An immuno-deficient mouse model with transplanted human artery tissue was developed and examined for the impact of hCMV infection upon immune rejection of the graft (Abele-Ohl *et al.*, 2012). Human internal mammary artery tissue was obtained and infected with hCMV, then transplanted to the

infrarenal artery of immunodeficient mice. One week later, human PBMCs were transferred intraperitoneally into mice and then analysed for graft rejection. hCMV infection was confirmed by DNA detection and immunodetection of viral antigens. Vascular lesions and immune cell infiltrates were more pronounced in animals receiving hCMV-infected arterial grafts compared with uninfected grafts, and intercellular adhesion molecule-1 and platelet-derived growth factor receptor-β expression levels were increased upon hCMV infection, which may explain the immune infiltration and vascular lesions.

Summary and future directions. hCMV studies in humanized mice have mostly been performed in older models and with the goal of testing antiviral drug efficacy. Whilst some studies have been performed to examine hCMV-mediated pathogenesis, there are still many diseases associated with hCMV that warrant further exploration. hCMV can cause hepatitis and this disease could be modelled in a humanized liver mouse model (see above). Neuropathogenesis has not yet been explored for hCMV in humanized mice. Interestingly, HIV-1 is able to penetrate the humanized mouse brain, where it replicates and causes inflammation (Dash *et al.*, 2011; Gorantla *et al.*, 2010). At least some human immune cells traffic to the brain in humanized mice and this is an area that could be explored for hCMV. However, it is not expected that hCMV will be able to replicate in murine brain cells. Congenital transmission of hCMV is a major cause of birth defects, but this form of pathogenesis is not able to be studied in current humanized mouse models because animals are engrafted after birth. Similarly, hCMV-associated pneumonia and gastroenteritis are not like to be detected in current humanized mouse models. *In vivo* drug testing for hCMV has an important history in humanized mice and we expect that other HHVs can similarly be tested for drug efficacy in these models.

HHV-6

Previously, HHV-6A and HHV-6B were classified as subtypes of the same virus, but they have recently been classified as two distinct viruses (Adams & Carstens, 2012). Both viruses have primary tropism for CD4$^+$ T-cells, but HHV-6A is also capable of lytic infection in CD8$^+$ T-cells, $\gamma\delta$ T-cells and NK-cells (Dagna *et al.*, 2013). HHV-6B causes roseola – a childhood fever and rash that usually resolves without complication. We are not aware of any studies attempting to infect HSC-humanized mice with HHV-6B or to recapitulate roseola disease. HHV-6A has not been shown definitively to cause any disease, but it is implicated in diseases, including autoimmune diseases such as multiple sclerosis (Virtanen *et al.*, 2007), immunosuppression (Emery *et al.*, 1999; Gobbi *et al.*, 1999; Lusso & Gallo, 1995) and graft-versus-host disease (Appleton *et al.*, 1995).

SCID-hu Thy/Liv mice can be infected with HHV-6. In one study, the implanted Thy/Liv organ of SCID-hu Thy/Liv

mice was surgically exposed and injected with HHV-6A strain GS. Thy/Liv implants were harvested at 4, 7, 11 and 27 days post-inoculation. HHV-6A replication was demonstrated by detection of increasing amounts of viral DNA, which peaked at 14 days post-inoculation. The virus induced severe depletion of thymocytes, especially the intrathymic progenitor T-cells (Gobbi *et al.*, 1999).

In a more recent study, Rag2$^{-/-}\gamma_c^{-/-}$ mice were infected with HHV-6A. Cell-associated or cell-free virus was injected intraperitoneally into the mice and nearly all infected mice had detectable levels of HHV-6A DNA by qRT-PCR in at least one of the samples tested (blood, bone marrow, lymph node and thymus). Cell-free injected mice were sacrificed at 1 week post-infection and cell-associated mice were sacrificed at 6.5–9.5 weeks post-infection. Thymocyte populations were significantly altered in the HHV-6A-infected mice sacrificed at time points later than 1 week post-infection, including a significant decrease in CD3 expression as well as a significant loss of intrathymic T progenitor cells (CD3$^-$CD4$^+$CD8$^-$) as was seen in the previously mentioned study in SCID-hu Thy/Liv mice. Increased populations of CD4$^+$CD8$^+$ T-cells were detected in peripheral blood (Tanner *et al.*, 2013).

CD46 transgenic mice have also been shown to be susceptible to HHV-6 infection (Reynaud *et al.*, 2014). These mice were injected intracranially with HHV-6A and HHV-6B. Although HHV-6B DNA levels decreased rapidly after infections, HHV-6A DNA was detectable in the brain for up to 9 months post-infection. Primary brain glial cultures from the transgenic mice that were infected with HHV-6A showed production of pro-inflammatory cytokines CCL2, CCL5 and CXCL10. This represents the first murine model to study HHV-6A infection in the brain.

Summary and future directions. HHV-6 viruses have been associated with a large number of diseases in humans, but the ubiquitous nature of the viruses has made it difficult to prove causal relationships. Most data gained to date in humanized mice show a role of HHV-6 viruses in immunosuppression, but many other diseases remain to be explored.

HHV-7

HHV-7 is genetically similar to HHV-6 and also infects human T-cells, although no known human diseases are attributed to HHV-7. We are not aware of any attempts to infect humanized mice with HHV-7 to date.

Alphaherpesvirus studies in humanized mice
HSV-1 and HSV-2

HSV-1 and HSV-2 normally replicate in epithelial cells and establish latency in neuronal cells, with only limited evidence for infection of immune cells. As these viruses infect mice, rats, rabbits and other small-animal models, they have not been explored much in humanized mice.

Table 1. Summary of pathological findings in humanized mouse models and future directions for HHV research in humanized mice

Virus	Reported findings	Future directions
EBV	Persistent infection in B-cells	Gain greater understanding of molecular biology of EBV-induced cancers
	Lymphoproliferation; associated with type II latency	
	Symptoms of rheumatoid arthritis	Develop new treatments for EBV-associated cancers
	Symptoms of HLH	
hCMV	Persistent infection in monocytes/macrophages in bone marrow and spleen	Determine mechanisms of latency *in vivo*
	Viral reactivation due to G-CSF has implications for bone marrow transplants	
KSHV	Persistent infection in B-cells and macrophages	Develop a model for KSHV-associated lymphomas
	Mucosal transmission through oral and vaginal exposure	Develop new treatments for KSHV-associated cancers
	KS-like lesions in human skin-transplanted mice	
HHV-6	Persistent infection in blood and lymphoid organs	Model roseola pathogenesis by HHV-6B
	Thymocyte depletion due to HHV-6A/HHV-6B	Determine diseases caused by HHV-6A in humans

Human skin grafts in SCID mice support replication by HSV-1 and VZV, and the SCID-hu Thy/Liv model also can be infected by each virus (Moffat *et al.*, 1998a). HSV-1 only infected epidermal cells in either model; small, superficial lesions were detected in the epidermis of skin grafts and no replication in thymic T-cells was detected. HSV-1 glycoprotein C (gC) is dispensable for replication in culture, but a mutant revealed that gC is an important virulence factor in HSV-1 replication *in vivo* (Moffat *et al.*, 1998a).

HSC-humanized mice can be infected with attenuated HSV-2 (thymidine kinase mutant) by intravaginal inoculation and were used as a model to study protective human immune responses by later giving a lethal challenge with a WT stain (Kwant-Mitchell *et al.*, 2009). Primary infection resulted in human T-cell and NK-cell trafficking to the genital tract and iliac lymph nodes. Human T-cells in spleen, lymph nodes and the vaginal tract produced IFN-γ in response to HSV-2 antigens, thus showing an adaptive cellular response. Human IgG specific to HSV-2 was also detected. Upon challenge with a lethal dose of HSV-2, immunized animals survived significantly better if a human immune system was transplanted; in contrast, none of the non-humanized mice survived.

VZV

VZV shares a tropism for replication in epithelial cells and latency in neurons, although unlike the other alphaherpesviruses it also infects human T-cells, which it uses to traffic to the skin. Humans and some non-human primates are the only hosts infectable by VZV. As VZV replicates in T-cells, it has been studied in the SCID-hu Thy/Liv model as well as in skin graft models. Skin graft studies showed that VZV extensively infected dermal and epidermal cells, whilst HSV-1 only formed small lesions in the epidermis. VZV gC was found to be an important virulence factor in skin, although it is not required for replication *in vitro* (Moffat *et al.*, 1998a). Similar studies were performed to determine the roles of the ORF47 and ORF66 gene

products in thymic and skin implants. Although strains with mutations in these genes still allowed for tissue culture replication, ORF47 was found to be required for replication in skin and thymic grafts, and ORF66 was necessary for T-cell infectivity and had a partial effect on skin infectivity (Moffat *et al.*, 1998b). The skin graft model was used to study mechanisms of T-cell transfer of virus to the skin, demonstrating that VZV-infected T-cells move to skin within 24 h of entering the circulation. Memory CD4$^+$ T-cells were the predominant T-cell type recovered from skin grafts. VZV is able to downregulate host IFN-α production in infected skin cells and also blocks the ability of IL-1α to recruit inflammatory cells to sites of virus replication (Ku *et al.*, 2004). Taken together, these studies provide information that could be useful to develop improved VZV vaccines by identifying virulence factors and by gaining an understanding of how VZV traffics in the host.

Summary and future directions. We do not anticipate much further research on the alphaherpesviruses in humanized mice, because of the tropism of the HSVs for standard mouse models, and as VZV replicates principally in epithelial cells and skin models in humanized mice have not been adopted widely. However, one area that has greater potential is for vaccine studies because humanized mice generate human cellular and humoral immune responses to HHVs (see above), and can be challenged with virus after experimental vaccination in order to determine the efficacy of the vaccine. As human genes are used to generate human T- and B-cell receptors, the antigenic targets are expected to be similar to those seen in humans.

Overall summary and future directions

Humanized mice have served as an excellent model to study various aspects of HHV biology including pathogenesis, tropism, establishment of latency, reactivation,

Table 2. Summary of recent reports of HHV pathogenesis in HSC-humanized mice

Virus	Findings	Humanized mouse model	Reference(s)
EBV	Lymphoproliferation	$Rag2^{-/-}\gamma_c^{-/-}$	Traggiai et al. (2004)
EBV	Lymphoproliferation	$NOD/SCID\gamma_c^{-/-}$	Heuts et al. (2014), Wahl et al. (2013), White et al. (2012), Yajima et al. (2008)
EBV	Lymphoproliferation	NOD/SCID	Islas-Ohlmayer et al. (2004)
EBV	Lymphoproliferation	$NOD/SCID\gamma_c^{-/-}$ BLT	Ma et al. (2011, 2012)
EBV	Rheumatoid arthritis	$NOD/SCID\gamma_c^{-/-}$	Kuwana et al. (2011)
EBV	HLH	$NOD/SCID\gamma_c^{-/-}$	Sato et al. (2011)
KSHV	Mucosal transmission; no disease detected	$NOD/SCID\gamma_c^{-/-}$ BLT	Wang et al. (2014)
hCMV	G-CSF treatment reactivates latent hCMV	$NOD/SCID\gamma_c^{-/-}$	Smith et al. (2010), Umashankar et al. (2011)
HHV-6A	Thymocyte depletion	$Rag2^{-/-}\gamma_c^{-/-}$	Tanner et al. (2013)
HSV-2	Protective human immune responses	$Rag2^{-/-}\gamma_c^{-/-}$	Kwant-Mitchell et al. (2009)

efficacy of antiviral drugs and human immune responses. A summary of the major findings of HHV research in the new generation of humanized mice is given in Table 1, accompanied by areas for future research.

It is currently unclear if one humanized mouse model will be superior to others as it pertains to a particular virus due to a lack of studies designed to compare the various models. However, a number of HHVs infect human cell types that are not produced in the most common and current humanized mouse models (e.g. epithelial cells, endothelial cells and neurons). In order to recapitulate these models, it may be necessary to combine multiple humanized mouse models together in order to have both target cells and human immune cells that may be involved in either infection or pathogenesis. Such a study was achieved with hepatitis C virus infection in a humanized mouse model that combined transplantation of human hepatocytes with transplantation of human HSCs (Washburn et al., 2011), and we envision that similar studies can be planned to better understand immunological control of HHV infection as well as immune-mediated pathologies.

Clearly, the HSVs already have well-established animal models and humanized mice are not as important to the study of those pathogens, although vaccine research of HSVs would be benefitted by studies of the human adaptive immune response in humanized mice due to differences in mouse and human genetics. With the current state of humanized mice focused mostly on human immune cell transplants, the beta and gammaherpesvirus fields stand to gain the most from humanized mouse models. The contributions of EBV to cancer development in humanized mice have been explored more than any other area of HHV biology to date, but further work is needed to find new treatments that will target specifically EBV-positive cancer cells. We expect that further work in EBV genetics will yield new targets for chemotherapy of EBV cancers. In general, the humanized mouse platform has been underutilized for HHV drug development. Although EBV and hCMV have been studied the most in these models, only a few studies on KSHV and HHV-6 have been performed. A summary of HHV-induced

pathogenesis in humanized mice with accompanying references is provided in Table 2. With the recent development of new models for these infections, we anticipate further elucidation of their pathogenic mechanisms and validation of antiviral drugs in vivo. We believe that humanized mouse models will be a useful tool to study the contributions of particular HHV genes to latency and persistence, and anticipate that this area of research will be helpful to create HHV vaccines with no capacity for persistence in the host.

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