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The use of humanized mice for studies of viral pathogenesis and immunitys

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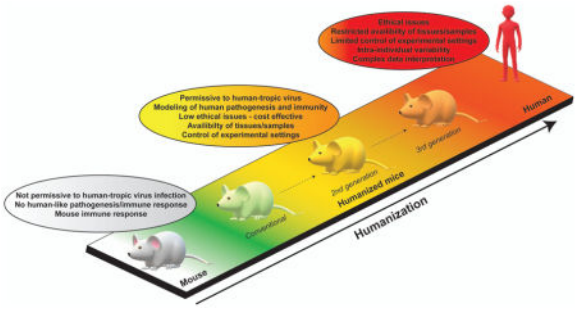
Abstract

Humanized mice, i.e. animals engrafted with human tissues and/or expressing human genes, have been instrumental in improving our understanding of the pathogenesis and immunological processes that define some of the most challenging human-tropic viruses. In particular, mice engrafted with components of a human immune system (HIS) offer unprecedented opportunities for mechanistic studies of human immune responses to infection. Here, we provide a brief overview of the current panel of HIS mouse models available and cite recent examples of how such humanized animals have been used to study immune responses and pathogenesis elicited by human-tropic viruses. Finally, we will outline some of the challenges that lay ahead and strategies to improve and refine humanized mice with the goal of more accurately recapitulating human immune responses to viral infection.

Graphical Abstract



Keywords

Humanized mice; virus infection; immunity; pathogenesis; host tropism

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Introduction

Human-tropic viruses, such as human immunodeficiency virus (HIV), hepatitis B virus (HBV) and Dengue virus (DENV), are causes of major health and economic concerns worldwide [1]. However, the restricted human tropism of these viruses, along with the scarcity of permissive animal models, has significantly hampered our ability to study the life cycles of these viruses and, ultimately, to develop anti-viral therapies and vaccines [2]. Characterizing the pathogenesis of these viruses and the immune response they invoke in human patients has also proven challenging. With the exception of the peripheral blood, human tissues are not readily accessible, and comprehensive data analysis is complicated by a high number of variables, including, but not limited to, intra-human genetic variation, age, gender, co-morbidities, nutritional and microbiome status. Over the past three decades, immunodeficient mice engrafted with components of a human immune system (HIS) have been an effective tool for studying the life cycle of a broad range of human-tropic pathogens in controlled experimental settings. Among these pathogens are HIV [3], human cytomegalovirus [4], Epstein Barr virus (EBV) [5,6], DENV [7–12], human T-cell leukemia virus (HTLV) [13], Karposi sarcoma-associated herpes virus (KSHV) [14], yellow fever virus vaccine strain 17D (YFV-17D) [15], *Salmonella typhi* [16], *Borrelia hermsii* [17] and *Mycobacterium tuberculosis* [18,19]. By modeling critical aspects of pathogenesis or immunological features observed in infected human patients, humanized mice have allowed major breakthroughs in our understanding of the interactions between viruses of strong clinical relevance and their human host.

Human-hemato lymphoid system mice models for studying viral pathogenesis and immunity

The accurate modeling of virus-human interactions in humanized mice is heavily dependent on the generation of mouse models able to support human hematopoietic stem cell (HSC) engraftment, recapitulate the complexity of human hematopoiesis and mount human-like immune responses, both in terms of function and spatial organization. To meet these requirements, suitable xenorecipient strains need to have 1) limited human graft rejection and 2) a favorable environment for human hematopoiesis.

Key mutations or genetic deficiencies that strongly attenuate rejection of transplanted human HSCs by the murine immune system have been identified, such as *Scid* or *Rag1*/*Rag2* mutations that prevent mouse B/T cell maturation [20–28] and an IL-2 receptor γ mutation that disrupts IL-15 mediated signaling and natural killer (NK) cell development [27,29–31]. A polymorphism discovered in the signal regulatory protein α (SIRPα) in the non-obese diabetic (NOD) genetic background can promote mouse macrophage tolerance to human cells upon HSC engraftment of HIS mice [23,24,32,33]. Up till now, the most commonly used xenorecipient strains for generating HIS mice utilized different combinations of these three mutations, such as *NOD-Scid* [23,24,34], *NOD-Scid IL2rγ*^{null} (NSG or NOG) [29–31], *BALB-c-Rag2*$^{-/-}$*IL2rγ*^{null} (BRG) [27] or *NOD- Rag2*$^{-/-}$*IL2rγ*^{null} (NRG) [35,36]. These conventional HIS mice (Figure 1) all support efficient human hematolymphoid engraftment, including a broad variety of lymphoid cells and some myeloid cells.

However, although there is evidence that the engrafted HIS becomes activated upon microbial challenge in such strains, the immune response is generally weak [5,19,37–39]. This limited immune functionality is due to numerous factors, including aberrant organization of secondary lymphoid structures, the lack of human MHC molecules on non-hematopoietic tissues, and the underrepresentation of critical human immune cell lineages, which are key in activating the adaptive immune response.

In particular, the scarcity of human dendritic cells (DCs) – a central cell type bridging innate and adaptive immune responses [40] – and other myeloid lineages and NK cells decreases the functionality of the engrafted HIS. The low frequency of these cell populations can be explained by the limited biological cross-reactivity of non-redundant cytokines that promote lineage differentiation [41,42].

To address such caveats, there have been continuous efforts to further refine xenorecipient strains and humanization protocols, hence leading to improved, second-generation HIS mouse models (Figure 1). Recent work has particularly focused on combining an immunodeficient genetic background with additional mutations to promote a more favorable environment for human hematopoiesis and immune response. Mutations in the mouse gene encoding c-kit improved HIS reconstitution in non-irradiated BRG or NSG mice [43,44]. Additionally, the transgenic or knock-in expression of one or several human cytokines (such as IL-3, TPO, GM-CSF or Flt3L) [45–53] enhanced engraftment and differentiation of multiple myeloid subsets and NK cells, which have been poorly represented in conventional humanized NSG and BRG mice (Figure 1). HIS mice expressing human MHC molecules have also been used to facilitate tracking of human antigen-specific T cells [5,6,38,54].

Additionally, the reconstitution of non-immune human microenvironments in HIS mice can also promote more physiological hematopoiesis (Figure 1). For instance, the generation of a bone marrow microenvironment by co-engraftment of human HSCs with ossicles created *ex vivo* from bone marrow-derived mesenchymal stromal cells (MSCs) enhanced human-like hematopoiesis [55–59]. Furthermore, HIS mice engrafted with small fragments of fetal liver and thymus, namely bone marrow-liver-thymus (BLT) mice [10,12,60–62], or with embryonic stem cell-derived thymic epithelial progenitors [63], can stimulate human lymphopoiesis and enhance T cell education.

Recent findings in viral pathogenesis and immune responses to infection using humanized mice

Some of the most challenging human viruses display a very narrow tropism restricted to humans and a few primate species [2]. Hence, HIS mice often represent the only and most appropriate alternative to characterize the life cycle of these viruses *in vivo*. Here, we present recent selected findings that highlight the utility of HIS mice for uncovering virus-host interactions that regulate viral pathogenesis and immune responses in humans. Specifically, we will discuss three distinct groups of human-tropic viruses that are of great significance to human health and for which *in vivo* investigation has been considerably challenging: lymphotropic viruses, flaviviruses and hepatotropic viruses.

Lymphotropic viruses

Many lymphotropic viruses, such as HIV, HTLV and EBV, cause significant mortality and morbidity worldwide [64–66]. HIS mice models have been instrumental in unveiling molecular interactions between these viruses and their target cells *in vivo*. In the case of HIV, humanized mice have a long-standing track record as the only small animal model for testing preclinically the efficacy of anti-retroviral therapies and vaccination approaches [67,68]. While HIV infection can now be controlled in patients through administration of highly active anti-retroviral therapy (HAART) [69], a cure for HIV and a prophylactic vaccine protecting against HIV remain elusive. To develop more effective, possibly curative, therapies, a better understanding is needed of the dynamic interactions between HIV and the human immune system *in vivo.* Towards this goal, HIS mice have provided mechanistic insights into HIV latency and anti-viral immunity following viral reactivation. Work with humanized NOD/SCID mice demonstrated that tissue macrophages constitute a latent HIV reservoir *in vivo,* highlighting that cell types besides CD4+ T cells allow HIV persistence after long-term suppressive antiretroviral therapy [70]. Reactivated latent viruses have also been found that harbor mutations which promote escape of the CD8+ T cell response. In HIS mice, stimulation of specific CD8+ T cell clonal populations able to recognize latent HIV viruses induced the elimination of the reactivated viruses [71].

The role of type I interferons (IFNs) in HIV replication and pathogenesis has also been explored in HIS mice. Although HIV does not induce type I IFN production in infected CD4+ T cells, a recent report showed that TREX1 (TRanscription-Export complex 1) downregulation in CD4+ T cells of the genital mucosa increases type I IFN *in vivo* and inhibits early HIV replication in BLT-NSG mice [72]. Importantly, intravaginal injection of recombinant type I IFN had similar effects, but systemic injection of recombinant type I IFN enhanced viral replication and dissemination. However, this is still a matter of debate. Other groups have reported that type I IFN signaling and type I IFN produced by plasmacytoid DCs inhibit viral replication but promote HIV-induced cell death in HIS mice [73,74], suggesting a differential effect of type I IFNs depending on the concentration and/or spatio-temporal dynamics of induction.

Furthermore, the anti-HIV activity of specific host proteins has also been investigated in HIS mice. Two host factors, APOBEC3 and BST2, strongly inhibit HIV replication, evolution and dissemination [75,76], thereby highlighting the importance of HIV's ability to antagonize these restriction factors *in vivo* for the establishment of chronic infection and pathogenesis. Additionally, IL-21-mediated upregulation of micro-RNA129 in HIS mice inhibited early HIV infection [77].

Another lymphotropic virus is EBV, a γ-herpesvirus widely prevalent in the human population that replicates in B cells and can cause B cell lymphomas [78]. The scarcity of animal models for EBV has not only impeded the development of treatments but has also hampered studies of EBV-associated disease [79]. HIS mice have emerged as a versatile experimental platform for not only analyzing immune responses and EBV-associated pathogenesis but also for defining the role of specific EBV viral proteins *in vivo*. For example, it was recently shown that the EBV large tegument protein BPLF1 is an important promoter of B-cell transformation and subsequent tumor formation in HIS mice [80].

Humanized mice used to model a clinically relevant co-infection of EBV and KSHV demonstrated that persistent KSHV infection can enhance EBV-associated tumor formation *in vivo* [81], highlighting the synergy between the pathogenesis processes of these two tumor viruses. Additionally, with their utility for modeling EBV-associated tumorigenesis, HIS mice also represent a pre-clinical platform for therapeutic strategies against EBV. Recently, a group demonstrated that T-cell responses against EBV can be enhanced via a PD-1/CTLA-4 pathway blockade, leading to a reduction of EBV-infected cells and better control of EBV-induced lymphoma in HIS-NSG mice [82].

Flaviviruses

Arthropod-borne flaviviruses, such as DENV, Zika virus (ZIKV) and YFV, pose significant clinical health concerns [83–87]. Although flaviviruses causing disease in humans usually display a broad cellular tropism and can replicate in a variety of non-human primate tissues [88–90], the pathogenic processes associated with these viruses mostly result from specific interactions with human specific-components [88,91–93].

With no treatment available, DENV is responsible for 50 to 100 million infections with clinical manifestations every year [83]. Dengue fever, caused by DENV infection, is usually non-lethal, but re-infection with heterologous DENV serotypes can lead to severe hemorrhagic disease [94] as a result of antibody-dependent enhancement (ADE). Inbred immunocompetent mice are not permissive to DENV infection, presumably due to the inability of DENV to evade murine antiviral responses [95]. When specific murine innate immune signaling pathways are blunted, infection with mouse-adapted DENV strains can be established [89]. However, the utility of such immunocompromised mouse models is limited by the partial functionality of the immune system and having to study replication of a human virus in the murine cellular environment [89]. Consequently, HIS mice have proven useful for exploring the *in vivo* replication of not only lab-adapted but also patient-derived viruses and the intricate role of the immune response in controlling or promoting DENV-pathogenesis [12,89].

In humanized NSG mice, human NK cells were shown to be critical for controlling DENV infection *in vivo*, limiting viral replication, thrombocytopenia and liver damage [96]. Additionally, contacts between NK cells and DENV-infected monocyte-derived DCs were crucial for NK cell activation and control of infection. As DENV is a mosquito-borne virus, early skin-specific inflammatory events at the site of infection are particularly important for the DENV life cycle and development of immunopathogenesis. Although DENV infections in advanced HIS mouse models dually engrafted with allogenic skin explants have yet to be performed, previous studies have reported that DENV disease and replication are more severe in conventionally humanized NSG mice after mosquito-mediated infection than after intra-dermal injection [97]. HIS mice have also been used to test the efficacy of several anti-viral strategies, such as antibody-based therapies [98].

Beyond conventional humanized mice, BLT-NSG mice have also been a valuable platform for studying DENV infection, mounting a more extensive adaptive immune response to infection. BLT-NSG mice can support sustained DENV replication, mount an HLA-A2-restricted human T-cell response and produce DENV-neutralizing IgM antibodies upon

infection [10,12,99]. Nevertheless, with limited evidence for class-switching and affinity maturation, the quality of the humoral response and, ultimately, the neutralizing capacity of virus-specific antibodies in BLT mice is still not vastly different from those in conventional HIS mouse models. These aspects limit proper characterization of e.g. DENV-induced ADE *in vivo* and highlight the need for further immunological improvements in HIS mice to better understand key flavivirus-mediated immunopathogenesis processes. More recently, humanized BLT mice have also been employed as a challenge model for ZIKV infection and were shown to support long-term replication of the virus [100].

With a significantly higher mortality rate than DENV, YFV is responsible for around 200,000 new infections and 30,000 deaths each year [86]. Although YFV was one of the major infectious diseases in the 18th and 19th centuries, the generation of a potent, live-attenuated vaccine in the 1930s [101], YFV-17D, significantly constrained the spread of the virus, which is endemic in only South America and Africa. However, the host mechanisms governing YFV pathogenesis and the potent immunogenicity of YFV-17D remain poorly understood. Additionally, recent vaccine shortages and low vaccination coverage in at-risk areas create ideal conditions for YFV re-emergence and outbreaks of significant health concern [102–104]. Altogether, this situation highlights the urgent need for improving our understanding of the pathogenesis and immunological processes that govern YFV infection *in vivo*.

Similar to DENV, YFV replication is inhibited by the murine innate immune response in immunocompetent mouse models [36,105], and *in vivo* infection is restricted to a few primate species. Recently, our group provided proof-of-concept that HIS mice are permissive to YFV-17D infection [36]. Effective viral replication in the blood and in lymphoid tissues was dependent on the presence of a HIS, and we identified a set of human-specific, spatio-temporal interactions between particular immune cell subsets and YFV-17D. Hence, HIS mice represent ideal platforms for understanding how virulent YFV strains and YFV-17D differentially interact with the HIS, which could ultimately open important avenues for the design of novel vaccine or immunotherapy strategies against flavivirus infections.

Hepatotropic viruses

An estimated 330–350 million people are persistently infected with HBV, hepatitis delta (HDV) and/or hepatitis C virus (HCV) [106,107]. Chronic carriers are at risk of developing severe liver disease including fibrosis, cirrhosis and hepatocellular carcinoma. Despite significant advances in prevention and treatment, hepatitis viruses remain a medical problem. There is no vaccine protecting against HCV, but direct-acting antivirals can now cure chronic hepatitis C in a majority of patients. Conversely, an effective prophylactic vaccine for HBV and HDV does exist, but there are no approved treatments for treating chronic hepatitis delta and current HBV therapies that can suppress viremia are rarely curative. The unique human-tropism and liver-specific replication cycle of these viruses [108,109] have posed challenges for creating small animal models suitable for studying immune responses and pathogenesis of these viruses and ultimately develop drug and vaccine candidates. Mice engrafted with human hepatocytes, so-called human liver chimeric

mice, remain the gold standard and have proven suitable model for chronic HBV, HCV, HDV and, most recently, hepatitis E virus infection [110–119]. However, the highly immunocompromised status of these engrafted mice precludes the study of immune-mediated pathogenesis by human hepatitis viruses.

Ongoing efforts are thus aimed at engrafting suitable xenorecipients with both human hepatocytes and components of a HIS (HIS-huHEP mice) [120–122]. Such dually humanized mice initially gained traction for studying immune responses and liver disease progression in the context of HCV infection [123]. More recently, HIS-huHEP mice were shown to support persistent HBV infection over several months [124–126]. Liver inflammation and fibrosis were observed in infected HIS-huHEP mice, and increased M2 macrophage infiltration of the liver potentially promoted liver disease [124]. Unlike immunodeficient human liver only-engrafted mice, HIS-huHEP mice exhibit partial immune control of HBV infection with detectable antigen-specific IgGs and liver-infiltrating Kupffer cells, NK cells and CD4+ T cells [126]. Nevertheless, the immune response to HBV remains low in HIS-huHEP mice [124,126] as most of the mouse strains employed thus far to generate HIS-huHEP mice are conventional strains with low myeloid and NK cell engraftment. In addition to HSC transfer, a recent modified humanization protocol enhanced donor-matched fetal hepatoblast engraftment by human oncostatin-M administration [125]. Interestingly, the more robust hepatic reconstitution also led to higher frequencies of human monocytes and NK cells model in comparison to conventional HIS mice, underscoring the important crosstalk between the liver and immune system even in steady state. Upon HBV infection, NK cell frequencies increased significantly, and NK cells acquired an activated phenotype.

HIS mice models for studying virus infection: Promises and upcoming challenges

Understanding the pathogenesis and immune responses elicited by human-tropic pathogens presents considerable challenges. Over the past decades, humanized mice have proven susceptible to a large number of human-tropic pathogens and have thus emerged as valuable platforms to model human-specific infectious processes *in vivo*. However, despite the ability of HIS mice to support the complete replication cycle of multiple human-tropic viruses [127], recapitulating the full panel of interactions between these viruses and the HIS as well as the complex immunological mechanisms that result from them remains challenging. Such limitations hamper our understanding of the molecular mechanisms governing the frontier between virus-induced protective immunity and viral pathogenicity/chronicity.

Protocols have been developed that can yield humanized mice with high human hematopoietic chimerism. However, in conventional models, important human subsets, in particular those of the erythro-myeloid lineage and NK cells, are not generated at physiological levels. This can be attributed to the absence or limited biological cross-reactivity of cytokines crucial for the development of these subsets. Consequently, immune functionality is impaired, and there is limited evidence that even the most advanced HIS mice models can mount a potent human-like immune response. Despite recent

accomplishments in promoting superior engraftment of key myeloid/NK cell subsets and/or superior hematopoiesis [45–53,128], more refined HIS mice models displaying robust memory B- and T-cell responses, human-like neutralizing titers and long-term protective immunity remain a major challenge.

In the future, overcoming these caveats would open unique opportunities for i.) a better understanding of how the immune response is evaded and exhausted during chronic and latent viral infection; ii.) exploring human-specific immunopathogenesis processes (such as DENV-induced ADE) during hemorrhagic fever virus infection; iii.) establishing correlates of protection by in-depth study of effective human vaccines, such as the YFV vaccine, and test novel vaccine strategies; and iv.) understanding the impact of pre-existing heterologous immunity on human immune function to other human pathogens.

For the generation of third-generation HIS mice, one promising approach could co-engraft key human tissues that would work in synergy to enhance human hematopoiesis and immune response into a mouse genetic background modeling a favorable human cytokine environment by expression of one or more human cytokines (Figure 1). For instance, the co-engraftment of human HSCs along with human lymph nodes in a mouse genetic background promoting human myeloid and NK cell subset expansion likely represents a valuable strategy to enhance B-cell effector and memory response. Additionally, the co-engraftment of HSCs and human hepatocytes in a similar such genetic background would open novel avenues for better understanding the immune response to human hepatotropic viruses *in vivo* (Figure 1). Humanized mice have been continually refined over the last three decades and have matured from simple challenge models for human-tropic viruses to experimental platforms for studying aspects of human immunity *in vivo*. The future refinements of these models undoubtedly hold great promise for uncovering novel molecular mechanisms regulating viral pathogenicity and immunity.

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Highlights

- Studying human-tropic virus has been challenging due to their often-narrow host range.
- HIS mice have been instrumental for characterizing human-tropic virus infection *in vivo*.
- Key virus-induced pathogenesis and immunological processes have been uncovered.
- However, HIS mice still exhibit only a partial human immune response.
- Future refinements of HIS mice models will be critical for further discoveries.

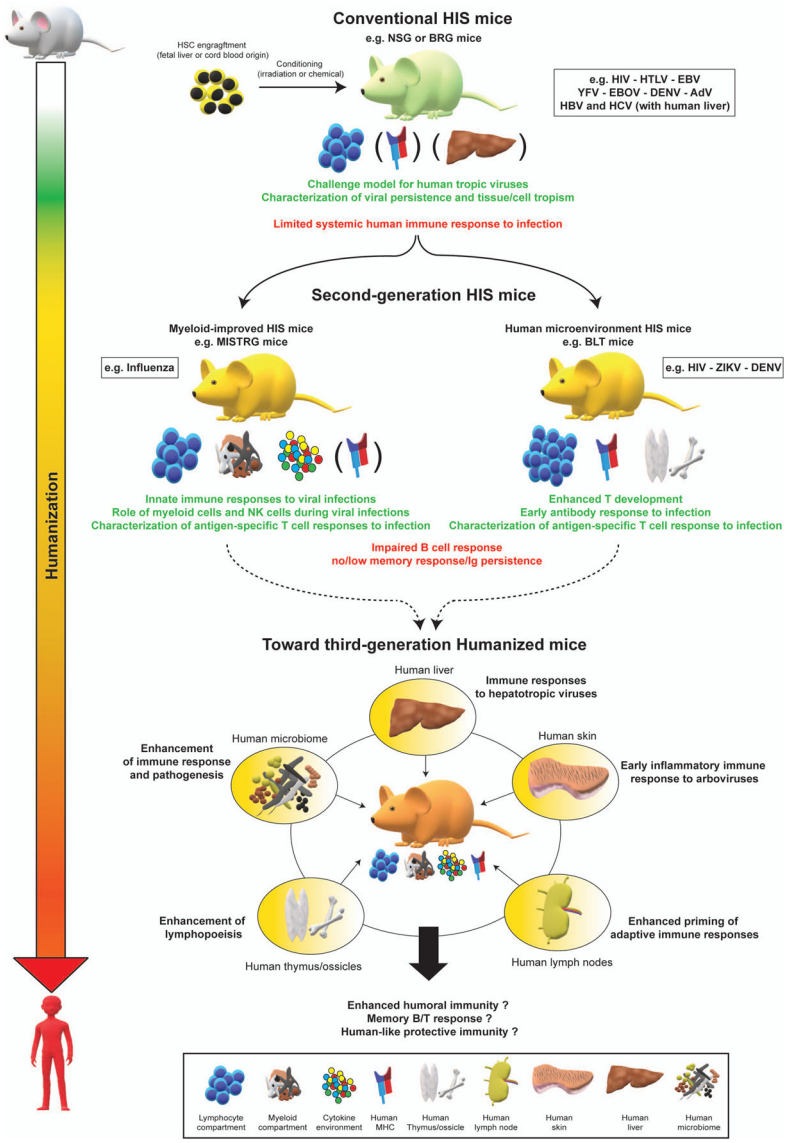

Figure 1. Current and future humanized mouse models for the study of viral infection and immunity

Advantages (green text) and limitations (red text) of conventional HIS mice and second-generation HIS mice (myeloid-improved HIS mice and human-microenvironment HIS mice) are shown. Across the entire figure, humanization levels are symbolized by a color gradient from white (unaltered/wildtype mice) to red (humans). Examples of viral pathogens are listed in a box beside the respective model in which they have been studied. Pictograms representing the specific characteristics and/or improvements of each category are shown (legend at bottom of figure). Characteristics/improvements not ubiquitously present across all HIS mice models of a given category (conventional or second-generation) are indicated in parentheses. Putative improvements of second-generation HIS mice toward the generation of third-generation HIS mice are depicted in yellow circles. HSC, human hematopoietic cells.