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Review Article

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Humanization of Laboratory Mice for HIV Research: Current Methods and Perspectives

Andrey Akinin*

Central Research Institute of Epidemiology, Moscow, Russia

ABSTRACT

Relevance of the Topic

Studying the pathogenesis of HIV infection and developing novel therapeutic strategies require adequate preclinical models that replicate key aspects of viral interaction with the human immune system. Traditional models, such as primates or transgenic animals, face significant limitations, including high costs, ethical concerns, and insufficient accuracy in mimicking human immune responses (Dash PK et al., 2021, Bennett MS, Akkina R, 2013). In this context, humanized mice, created by transplanting human hematopoietic stem cells (HSCs) or tissues into immunodeficient mice, have emerged as revolutionary tools. These models enable the study of HIV infection in vivo, including latent reservoirs, immune responses, and the efficacy of antiretroviral therapy (ART) (Zhang C et al., 2023, Baroncini L et al., 2023).

Article Objective

This review aims to systematize modern methods of humanizing mice, evaluate their applications in HIV research, and highlight key challenges and future prospects. Special emphasis is placed on the role of CRISPR-Cas systems in editing genes such as CCR5 and their contribution to developing functional cure strategies (Rothemejer FH, et al., 2023, Khamaikawin W, et al.. 2024).

Summary of Sections

The article is divided into four main chapters. The first chapter examines contemporary humanization methods, including immunodeficient mouse strains (e.g., NOD/SCID/γc^{null} and Rag2^{null}γc^{null}) and HSC transplantation. The second chapter analyzes the use of these models in HIV research, from evaluating latent reservoirs to testing gene therapies. The third chapter addresses challenges

Correspondence:

Dr. Andrey Akinin, Central Research Institute of Epidemiology, Novogireevskaya Str., 3a, 111123 Moscow, Russia, ORCID ID: 0009-0007-4256-0435

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such as incomplete immune system reconstruction and long-term study limitations, alongside prospects like CRISPR-Cas integration and improved humanization techniques. The concluding section underscores the importance of further research to overcome existing barriers.

Article Structure

This review follows a classical scientific structure, progressing from general context to technical and applied aspects. Each chapter includes critical analysis of sources and references to key studies, ensuring an evidence-based foundation for all claims.

Literature Search Strategy

This review synthesizes data from peer-reviewed articles, clinical studies, and preclinical reports indexed in PubMed, Web of Science, and Scopus between 2010 and 2024. The search utilized the following keywords and their combinations: "humanized mice", "HIV latency", "CRISPR-Cas", "CCR5 editing", and "antiretroviral therapy". Inclusion criteria prioritized studies that directly compared humanization methods (e.g., CD34⁺ vs. BLT models), evaluated CRISPR-based interventions in HIV-infected humanized mice, and addressed ethical or technical challenges in model optimization. Exclusion criteria removed non-English articles, non-peer-reviewed preprints, and studies lacking mechanistic insights. The final selection comprised 89 publications, including 12 clinical trials, 58 preclinical studies, and 19 review articles. Critical analysis of sources ensured a balanced representation of both pioneering and recent advancements.

Keywords: Humanized mice, HIV infection, CRISPR-Cas, latent reservoirs, gene therapy, CCR5, preclinical models.

MODERN METHODS OF MOUSE HUMANIZATION

Humanized mice represent unique preclinical models created by introducing elements of the human immune system into immunodeficient rodents. These models have become cornerstones in HIV research, enabling the study of pathogenesis, latent reservoirs, and therapeutic strategies *in vivo*. Below, we outline key approaches to humanization, their principles, advantages, and limitations.

Primary Immunodeficient Mouse Models

Modern humanization relies on immunodeficient mouse strains that do not reject human cells. The most common models include:

- **NOD/SCID/γc^{null} (NOG)**: These mice lack functional T, B, and NK cells due to mutations in *Prkdc* and *Il2rg*. This model supports high engraftment of human HSCs and multi-layered immune system development (Ibeh BO et al., 2016).
- **Rag2^{null}γc^{null}:** The absence of Rag2 recombinase and IL-2 receptor γ-chain makes these mice ideal for transplanting human thymus and liver (BLT model), facilitating studies of mucosal HIV transmission (Bennett MS, Akkina R., 2013).
- **NSG-SGM3 and NOG-EXL:** These strains express human cytokines (GM-CSF, IL-3, SCF), enhancing myeloid cell differentiation. However, cytokine hyperstimulation often triggers macrophage activation syndrome, limiting long-term studies (Willis E et al., 2024).

Advantages:

- High human cell engraftment rates (up to 80% in peripheral blood).
- Capacity to model systemic immune responses.

Limitations:

- Short lifespan (6–12 months).
- Incomplete innate immunity reconstruction (e.g., low dendritic cell levels)

Hematopoietic Stem Cell Transplantation

Injecting human HSCs into the liver of newborns or the bone marrow of adult immunodeficient mice remains the gold standard for humanization. This approach generates:

- **CD34⁺ Models:** HSCs differentiate into T/B lymphocytes, monocytes, and dendritic cells, forming functional lymphoid organs (Brehm MA et al., 2014).
- **BLT Models (Bone Marrow, Liver, Thymus)**: Combining fetal thymus and liver transplants with HSCs enables full mucosal barrier reconstruction, critical for studying sexual HIV transmission (Bennett MS, Akkina R, 2013).

CASE STUDY:

Dash (Dash PK et al., 2021) demonstrated that BLT models replicate latent HIV reservoirs in the gut and brain, which CD34⁺ models fail to achieve.

Limitations:

- Dependency on donor HSC quality.
- High inter-animal variability (10–30% fail to fully humanize).

Role of CRISPR-Cas in Enhancing Humanization

CRISPR-Cas9 has revolutionized HIV research by enabling precise gene editing. For instance, *CCR5* knockout in human HSCs confers resistance to R5-tropic HIV (Rothemejer FH et al., 2023).

However, risks such as off-target effects and large unintended deletions in host DNA remain critical concerns (Liu Y et al., 2023) (Figure 1).

LONG-TERM RISKS OF CRISPR-BASED THERAPIES

While CRISPR systems offer unprecedented precision, their long-term safety in vivo remains a pivotal concern. Beyond immediate off-target effects, persistent genomic instability may arise from large deletions (>1 kb) near HIV-1 integration sites, as observed in humanized mice (Liu Y et al., 2023). Such structural variations could predispose cells to malignant transformation or functional impairment, particularly in long-lived hematopoietic stem cells (HSCs). Additionally, prolonged expression of bacterial-derived Cas proteins (e.g., Cas9, Cas13a) may elicit chronic immune responses, exacerbating inflammation or compromising engraftment efficiency (Baroncini L et al., 2023). Recent studies also highlight epigenetic dysregulation at edited loci, potentially altering differentiation pathways of immune cells (Lin J, Yang J, 2024). Mitigating these risks requires longitudinal monitoring in humanized models, coupled with advancements in high-fidelity editors (e.g., base editing) and transient delivery systems (e.g., mRNA-LNPs) to minimize residual nuclease activity (Stone D et al., 2021; Lin J, Yang J, 2024).

CASE STUDY

Khamaikawin et al. (Khamaikawin W et al., 2024) combined CRISPR-edited *CCR5* with antiretroviral therapy (ART), achieving proviral DNA elimination in 58% of humanized mice. This underscores the potential of combinatorial approaches but also highlights the need for improved delivery systems, such as lipid nanoparticles, which enhanced editing efficiency in hepatitis B models (Stone D et al., 2021) (Figure 2).

CHALLENGES:

- Off-target effects during editing (Liu Y et al., 2023).

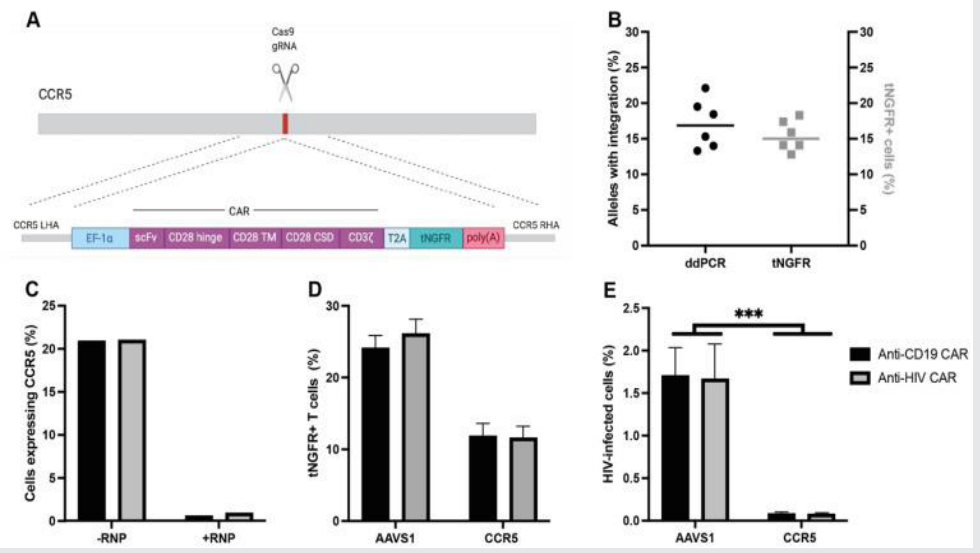

Figure 1: CRISPR/Cas-mediated CAR integration into the CCR5 locus (from Rothemejer et al. (Rothemejer FH et al., 2023)).

Humanization Method	Human Cell Engraftment Level	Immune System Reconstruction	Time to Full Humanization	Latent Reservoir Modeling Efficiency	Ethical Considerations
CD34+ Models	High (up to 80% in peripheral blood)	Partial (limited myeloid differentiation)	8-12 weeks	Limited (fails to model all reservoir types)	Low
BLT Models	High (complete mucosal barrier reconstruction)	Complete (including mucosal immune compartments)	12-16 weeks	High (models gut and neural reservoirs)	High (fetal tissue use)
CRISPR-Modified Models	Depends on editing efficiency	Targeted immune cell modification	6-8 weeks	Promising (potential for complete reservoir elimination)	Moderate

Figure 2: Comparison of Humanization Methods Efficiency.

- Low *in vivo* delivery efficiency of CRISPR components.

Method Comparison and Limitations

Key Limitations:

- Lack of human HLA complexes, distorting T-cell responses.
- Inability to model neuroimmune interactions in HIV-associated neurocognitive dysfunction (Zhang C et al., 2023).

CONCLUSION

Modern humanization methods offer unprecedented opportunities for HIV research but remain technically challenging. Integrating CRISPR-Cas and developing cytokine-expressing immunodeficient strains are promising avenues. However, full replication of human immune responses requires advances in genetic engineering and transplantology.

Having examined the diverse methodologies for mouse humanization - from immunodeficient strains to CRISPR-enhanced models - it becomes evident that each approach offers unique advantages while presenting specific limitations for HIV research applications. The evolution from basic humanization techniques to sophisticated gene editing strategies reflects the field's progression toward more precise and therapeutically relevant models. Understanding these methodological foundations provides the necessary context for exploring how these models are applied in practical HIV research scenarios, where they serve as critical bridges between in vitro discoveries and clinical translation. The following chapter will demonstrate how these theoretical capabilities translate into concrete research applications, from basic pathogenesis studies to cutting - edge therapeutic interventions.

APPLICATIONS IN HIV RESEARCH

Humanized mice have become indispensable for

studying HIV pathogenesis, testing therapies, and analyzing immune responses. These models replicate key stages of the viral lifecycle, including transmission, acute/chronic phases, and latent reservoirs. Below, we discuss major research directions, results, and limitations.

Limited Analysis of Immune Responses: While humanized mice have advanced HIV research, their utility in evaluating vaccine or immunotherapy efficacy is constrained by incomplete innate immunity reconstruction. Key deficiencies, such as low dendritic cell and natural killer (NK) cell levels (Terahara K et al., 2021); Willis E et al., 2024), hinder the modeling of critical antiviral mechanisms, including antigen presentation and cytotoxic clearance of infected cells. For instance, the absence of functional dendritic cells impairs the priming of adaptive immune responses,

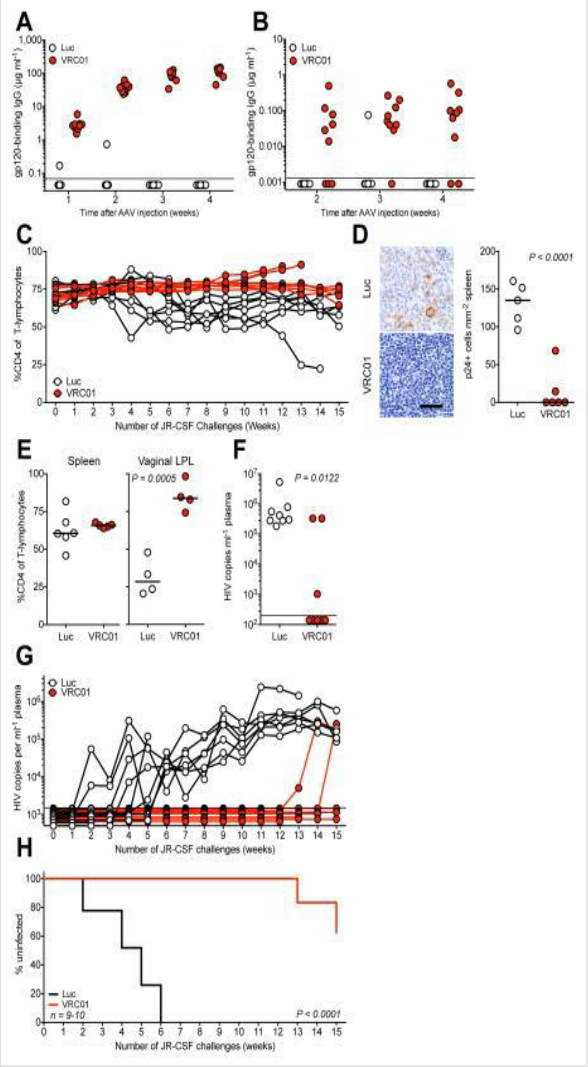

Figure 3: Intravaginal inoculation model for studying mucosal HIV transmission in BLT mice. (From Balazs et al., (Balazs AB et al., 2014).

which is essential for assessing vaccine-induced T-cell activation or antibody neutralization (Zhang C et al., 2023); Baroncini L et al., 2023). Similarly, diminished NK cell activity limits the evaluation of antibody-dependent cellular cytotoxicity (ADCC), a cornerstone of many immunotherapies targeting HIV reservoirs (Baroncini L et al., 2023). These shortcomings are particularly problematic for studies relying on humanized mice to predict clinical outcomes, as innate immune defects may overestimate therapeutic efficacy or obscure off-target effects. For example, Balazs et al. **(Balazs AB et al., 2014)** demonstrated robust protection against mucosal HIV transmission using vectored immunoprophylaxis in BLT mice, yet the lack of intact innate immune components (e.g., interferon-α-secreting plasmacytoid dendritic cells) may underestimate viral escape mechanisms observed in humans (Zhang C et al., 2023; Terahara K et al., 2021). Addressing these gaps requires integrating cytokine-enhanced models (e.g., NSG-SGM3) to improve myeloid differentiation or combining humanized mice with ex vivo systems to validate findings against human innate immune benchmarks (Willis E et al., 2024).

Modeling Transmission and Early Infection

BLT models are widely used to study mucosal HIV transmission. For example, intravaginal or intrarectal inoculation mimics natural infection routes, critical for developing microbicides or vaccines (Bennett MS, Akkina R, 2013).

CASE STUDY:

Balazs et al. (Balazs AB et al., 2014) demonstrated that vectored immunoprophylaxis (VIP) using broadly neutralizing antibodies (bNAbs) fully protected mice from repeated vaginal challenges with R5-tropic HIV (Figure 3).

Limitations:

- Lower transmission efficiency compared to humans (requires high viral doses).

Lack of intact epithelial barriers in some models (Zhang C et al., 2023).

Investigating Latent Reservoirs

Latent HIV reservoirs are the primary barrier to a cure. Humanized mice under ART sustain proviral

DNA in CD4⁺ T cells, macrophages, and tissues (e.g., gut, brain), enabling studies of persistence mechanisms (Dash PK et al., 2021).

CASE STUDY:

Dash et al. (Dash PK et al., 2021) showed that combining CRISPR-edited *CCR5* with ART eliminated proviral DNA in 58% of mice.

Analytical Methods:

- Real-time PCR for proviral DNA quantification.
- Cell phenotyping with activation markers (CD69, HLA-DR).

Challenges:

- Failure to replicate human reservoir diversity (e.g., brain microglia) (Terahara K et al., 2021).

Antiretroviral Therapy Testing

Humanized mice evaluate the efficacy and toxicity of novel antiretrovirals, including long-acting (LA-ART) and nanoformulated drugs.

CASE STUDIES

LA-ART: Weekly TMC278 (rilpivirine) and TMC181 (protease inhibitor) suppressed viremia in 100% of mice (Nischang M et al., 2012).

Nanoformulations: Nanoformulated dolutegravir penetrated the brain, reducing CNS viral load (Zhang C et al., 2023).

Advantages:

- Testing combination therapies.
- Monitoring viral resistance (e.g., *pol* gene mutations).

Limitations:

- Short drug half-lives in mice.
- Absence of human metabolic features (e.g., cytochrome P450 activity).

Gene Therapy and CRISPR-Cas

CRISPR-Cas systems have emerged as transformative tools for targeting both host and viral factors in HIV cure strategies. Beyond the well-established *CCR5* knockout, novel approaches focus on disrupting viral persistence mechanisms. For instance, integrating chimeric antigen receptor (CAR) genes into the *CCR5* locus via CRISPR/Cas9 has been shown to confer dual resistance to HIV-1 while preserving T-cell functionality (Rothemejer FH et al., 2023). This strategy not only blocks viral entry but also enhances CD4⁺ T-cell survival in humanized mice, offering a multi-layered defense against infection.

Recent advancements highlight the potential of combinatorial CRISPR therapies. Khamaikawin et al. (Khamaikawin W et al., 2024) demonstrated that coupling *CCR5* editing with the HIV-1 fusion inhibitor C46 achieves resistance to both R5- and X4-tropic strains, addressing viral tropism diversity. Similarly, CRISPR-mediated excision of conserved HIV-1 long terminal repeat (LTR) regions disrupts proviral transcription, effectively silencing latent reservoirs (Khamaikawin W et al., 2024). However, challenges

Parameter	CRISPR-Cas9	CRISPR-Cas13a
Target	DNA	RNA
Proviral DNA Elimination	Yes	No
Delivery Vehicles	AAV, LNPs, electroporation	Limited (size constraints)
Immune Response Risk	Moderate (humanized variants)	High (bacterial origin)
Clinical Trials	Phase I/II (e.g., NCT05144386)	Preclinical only
Key Advantage	DNA reservoir eradication	No genomic damage

Figure 4: Comparative Analysis of CRISPR Systems for HIV Therapy.

persist in ensuring specificity. Liu et al. (Liu Y et al., 2023) reported unintended large deletions in cellular DNA adjacent to HIV-1 integration sites during CRISPR editing, underscoring the need for improved precision tools like base editors or high-fidelity Cas9 variants.

The translational potential of CRISPR is further exemplified in studies beyond HIV. Stone et al. (Stone D et al., 2021) achieved sustained hepatitis B virus (HBV) suppression in humanized mice using lipid nanoparticle-delivered CRISPR-Cas9, a delivery method now being adapted for HIV therapies. Meanwhile, CRISPR-Cas13a, which targets viral RNA, presents a safer alternative by avoiding genomic integration risks. Yin et al. (Yin L et al., 2020) showed that Cas13a effectively degrades HIV-1 RNA *in vitro*, though its *in vivo* efficacy remains under investigation.

CRISPR-Cas13a in HIV Research: Bridging the Gap between in *Vitro* Success and *In Vivo* Challenges

Despite promising *in vitro* results demonstrating CRISPR-Cas13a's ability to degrade HIV-1 RNA without genomic integration (Yin L et al., 2020), its *in vivo* application remains limited. Key barriers include:

Delivery Efficiency: Cas13a's large size (~160 kDa) complicates packaging into viral vectors (e.g., AAV), unlike smaller Cas9 variants (e.g., saCas9, ~105 kDa) (Lin J, Yang J, 2024).

RNA Targeting Limitations: While Cas13a cleaves viral RNA, it cannot address integrated proviral DNA, a critical reservoir for HIV persistence (Liu Y et al., 2023).

Immune Recognition: Cas13a, derived from *Leptotrichia wadei*, may trigger stronger host immune responses compared to Cas9 orthologs optimized for human cells (Baroncini L et al., 2023).

Off-Target RNA Binding: Cas13a exhibits collateral activity, degrading non-target RNAs in close proximity, raising toxicity concerns in complex *in vivo* environments (Yin L et al., 2020).

To advance Cas13a toward *in vivo* use, future efforts should prioritize: engineering compact Cas13a variants for efficient delivery, combining RNA-targeting Cas13a with DNA-editing Cas9 to address both viral RNA and latent DNA, and developing immunosuppressive regimens to mitigate immune activation (Yin L et al., 2020; (Lin J, Yang J, 2024).

CRISPR Delivery Barriers: Technical Challenges and Emerging Solutions

The clinical translation of CRISPR-based HIV therapies faces multiple delivery obstacles that compromise therapeutic efficacy in humanized mice. **Cellular Uptake Barriers**: CRISPR-Cas components encounter significant challenges crossing cellular membranes due to their large size (Cas9: ~160 kDa) and negative charge. While viral vectors like AAV demonstrate tropism for hematopoietic stem cells, they face packaging limitations (~4.7 kb) and trigger immune responses against capsid proteins. **In Vivo Distribution Challenges**: Systemic delivery of CRISPR components results in rapid renal clearance and non-specific tissue distribution, with only 5-15% reaching target cells in bone marrow niches. Recent advances in lipid nanoparticle (LNP) formulations have shown promise, with Stone et al. (Stone D et al., 2021) achieving 30% editing efficiency in humanized mice using ionizable lipids optimized for HSC targeting.

Bioavailability and Stability Issues: Naked CRISPR RNAs degrade rapidly in biological fluids (half-life: <30 minutes), necessitating chemical modifications (2'-O-methyl, phosphorothioate) that paradoxically reduce editing efficiency. The blood-brain barrier poses additional challenges for targeting CNS viral reservoirs, with current delivery systems achieving <1% brain penetration. **Immune Recognition and Clearance**: Pre-existing anti-Cas9 antibodies in human populations reduce therapeutic potential, while repeated dosing triggers adaptive immune responses that clear CRISPR components within hours. These barriers collectively contribute to the inconsistent outcomes observed in HIV eradication studies, where only 58% of treated mice achieved sustained viral suppression (Khamaikawin W et al., 2024).

To address these challenges, emerging strategies focus on **Cell-Type-Specific Delivery**: Conjugating Cas9 proteins with HSC-specific ligands (e.g., anti-CD133 antibodies) enhances targeting precision while reducing off-target effects. **Tissue-Responsive Carriers**: pH-sensitive liposomes that release CRISPR cargo specifically in inflammatory microenvironments show promise for targeting activated immune cells

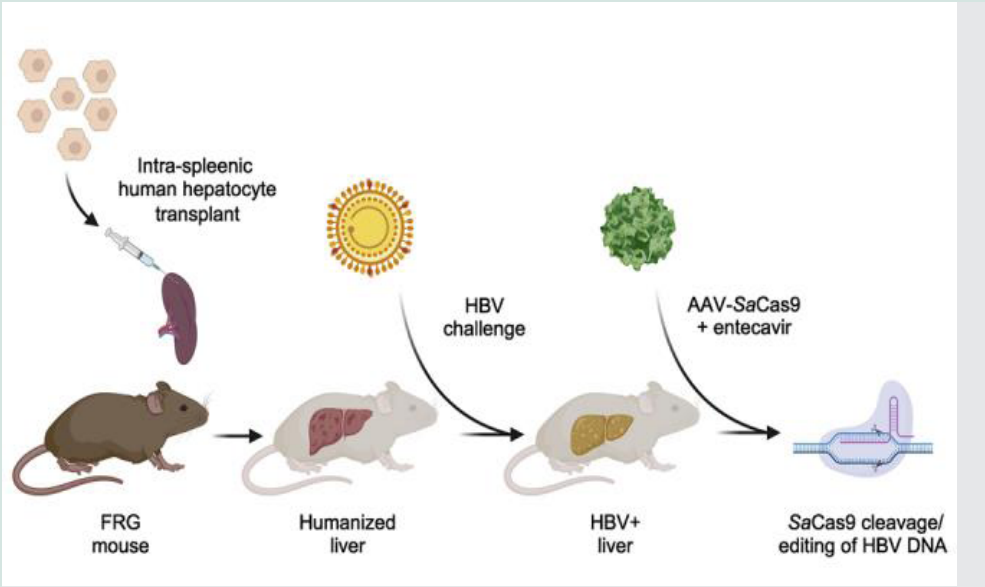

Figure 5: Lipid nanoparticle-mediated CRISPR delivery system for enhancing in vivo editing efficiency. (From Stone et al., (Stone D et al., 2021)).

harboring latent HIV. **Biodegradable Encapsulation**: Natural polymer systems (e.g., chitosan-based nanoparticles) provide sustained release profiles while minimizing immune recognition. These technological advances are essential for translating CRISPR-based HIV cure strategies from humanized mouse models to clinical applications.

KEY ADVANCES:

CAR-T Cell Engineering: CRISPR-edited CAR T cells targeting HIV envelope proteins exhibit prolonged antiviral activity in humanized models (Rothemejer FH et al., 2023).

Dual-Targeting Strategies: Combining *CCR5* knockout with fusion inhibitors broadens resistance to diverse HIV strains (Khamaikawin W et al., 2024).

Non-Viral Delivery: Lipid nanoparticles enhance CRISPR component delivery to hematopoietic stem cells, improving editing efficiency (Stone D et al., 2021).

REMAINING CHALLENGES:

- **Off-Target Effects**: Unintended genomic alterations necessitate rigorous screening protocols (Liu Y et al., 2023).
- **Immune Responses**: Anti-Cas9 antibodies may limit repeated dosing *in vivo* (Baroncini L et al., 2023).
- **Latency Reactivation**: CRISPR alone insufficiently activates latent proviruses; synergy with latency-reversing agents is critical (Dash PK et al., 2021).

Immune Response Analysis

Humanized mice enable study of humoral and cellular immunity:

- **bNAbs**: Test broad-spectrum antibodies (e.g., 10-1074) for neutralization and ADCC activation (Baroncini L et al., 2023).
- **T-cell Responses**: HLA-restricted T cells in BLT models assess cytotoxicity (Bennett MS, Akkina R, 2013).

CASE STUDY:

Baroncini L et al. (Baroncini L et al., 2023) showed that bNAb 10-1074 delayed viral rebound by depleting latent reservoirs, confirmed via phylogenetic analysis.

Limitations:

- Limited HLA allele diversity.
- Weak innate immune responses (e.g., low interferon-α).

Model Limitations

Despite progress, humanized mice face critical constraints:

Incomplete Immunity: Lack of functional dendritic cells and NK cells (Terahara K et al., 2021).

Short Lifespan: Cytokine-overexpressing models (e.g.,

NSG-SGM3) develop macrophage activation syndrome (Willis E et al., 2024).

Ethical Issues: BLT models require fetal tissues (Bennett MS et al., 2013).

CONCLUSION:

Humanized mice have transformed HIV research but require optimization. Developing cytokine/HLA-expressing strains and refining CRISPR delivery are key priorities.

The applications of humanized mice in HIV research demonstrate remarkable versatility, from modeling viral transmission to evaluating novel therapeutic approaches. However, this extensive research utility also reveals fundamental limitations that constrain the full potential of these models. While the studies reviewed in this chapter have provided invaluable insights into HIV pathogenesis and treatment efficacy, they simultaneously expose critical gaps in our current understanding - particularly regarding long-term safety profiles, complete immune system reconstruction, and sustainable therapeutic interventions. These challenges necessitate a comprehensive examination of the obstacles that must be overcome to realize the full promise of humanized mouse models. The subsequent chapter will address these challenges while outlining future directions that could transform humanized mouse research into an even more powerful platform for HIV cure development.

CHALLENGES AND FUTURE DIRECTIONS

Humanized mice are powerful tools for HIV research but face technical, biological, and ethical hurdles. This chapter analyzes limitations, proposes solutions, and explores emerging trends.

INSUFFICIENT MITIGATION STRATEGIES FOR CRISPR-CAS RISKS: While the risks of CRISPR-Cas systems—such as genomic instability due to large unintended deletions (Liu Y et al., 2023) and immune responses to bacterial-derived Cas proteins (Baroncini L et al., 2023)—are acknowledged, current proposals to address these limitations lack mechanistic depth. For example, base editing and prime editing, cited as high-fidelity alternatives (Lin J, Yang J, 2024), are not critically evaluated for their applicability in HIV-infected humanized models. Although base editors theoretically reduce off-target effects by avoiding double-strand breaks, their efficiency in editing quiescent HIV reservoirs (e.g., latently infected CD4⁺ T cells) remains unproven in vivo (Lin J, Yang J, 2024). Similarly, lipid nanoparticle (LNP)-mediated delivery, validated for hepatitis B virus (HBV) CRISPR editing (Stone D et al., 2021), has not been systematically tested in humanized mice for HIV-specific challenges, such as crossing the blood-brain barrier to target CNS reservoirs (Zhang C et al., 2023). Khamaikawin et al. (Khamaikawin W et al., 2024) demonstrated partial HIV eradication using CRISPR-edited CCR5 and ART, yet the study did not address how nanoparticle formulations could enhance HSC-targeted delivery to improve editing consistency. Furthermore, transient delivery systems (e.g., mRNA-LNPs) proposed to minimize Cas9 persistence (Lin J, et al., 2024) require validation in longitudinal studies to assess whether reduced nuclease activity compromises long-term proviral DNA suppression. To bridge these gaps, future work should prioritize comparative studies of editing platforms (e.g., Cas9 vs. base editors) in HIV latency models and optimize LNP formulations for tissue-specific delivery, leveraging lessons from HBV research (Stone D et al., 2021).

Key Challenges

Incomplete Immune Reconstitution

- **Myeloid Deficiencies**: Low dendritic cell/ macrophage levels impair innate immunity (Terahara K et al., 2021).
- **HLA-Mismatch**: Murine MHC incompatibility distorts T-cell responses (Bennett MS, Akkina R, 2013).
- **Cytokine Deficits**: Absence of human IL-7, IL-15, and GM-CSF reduces T-cell survival (Kim YS, Ko JH, 2018).

CASE STUDY:

NSG-SGM3 mice exhibit macrophage activation syndrome due to GM-CSF overexpression (Willis E et al., 2024).

CRISPR-Cas Technical Hurdles

Low *in vivo* delivery efficiency (~20–30%) remains a bottleneck. Nanoparticle-based delivery systems,

validated in hepatitis B studies (Stone D et al., 2021), and adeno-associated viral vectors show promise for improving HSC transduction. Additionally, machine learning algorithms are being trained to predict optimal guide RNA sequences, minimizing off-target effects (Deng B, Xue J, 2023). Khamaikawin et al. (Khamaikawin W et al., 2024) achieved HIV eradication in only 58% of mice using CRISPR + ART, highlighting precision needs.

Ethical and Practical Issues

- **Fetal Tissue Use**: BLT models raise ethical debates (Bennett MS, Akkina R, 2013).
- **High Costs**: Maintaining cytokine-expressing strains is expensive (Hosur V et al., 2017).

Ethical Considerations: Current Challenges and Practical Solutions in Humanized Mouse Research

The utilization of humanized mice in HIV research raises complex ethical considerations that must be carefully balanced against their scientific value. Current Ethical Challenges: The primary ethical dilemma centers on the use of fetal human tissues in BLT (Bone Marrow, Liver, Thymus) models, which requires donated fetal liver and thymus tissue obtained through legally sanctioned medical procedures (Bennett MS, Akkina R, 2013). This practice raises concerns about informed consent, tissue sourcing transparency, and the potential commodification of human biological materials. Additionally, the creation of immunodeficient humanized animals creates unique welfare concerns, as these models often experience shortened lifespans, increased susceptibility to opportunistic infections, and potential autoimmune complications (Willis E et al., 2024).

Practical Solutions and Ethical Alternatives: The field has made significant progress in developing ethical alternatives that reduce reliance on fetal tissues. iPSC-Derived Models: Recent breakthroughs in induced pluripotent stem cell technology offer promising alternatives, as demonstrated by Leidy-Davis et al. (Leidy-Davis T et al., 2018), who achieved 25-kbp gene humanization using embryonic stem cell approaches that bypass fetal tissue requirements. While current iPSC-derived models face functional maturity limitations, ongoing research in cytokine optimization and three-dimensional tissue engineering continues to improve their immunological relevance (Zhang C et al., 2023).

Tissue Engineering Solutions: Advanced organoid technologies combined with humanized mouse models represent another ethical alternative. These systems can model tissue-specific HIV reservoirs in gut and brain compartments without requiring fetal material (Terahara K et al., 2021). Standardized Ethical Protocols: International collaborations, such as the International Society for Humanized Mice (ISHM), have established standardized protocols that emphasize the three Rs principle (replacement, reduction, refinement) while maintaining scientific rigor (Brehm MA et al., 2014). These frameworks ensure that research progresses within acceptable ethical boundaries.

Regulatory Compliance and Transparency: Modern research practices require comprehensive ethical oversight through institutional review boards and animal welfare committees. Researchers must demonstrate that the scientific value of humanized mouse studies justifies the ethical costs, particularly when fetal tissues are involved. This includes rigorous cost-benefit analyses and exploration of alternative methodologies before proceeding with ethically sensitive approaches.

Solutions

Enhancing Immune Reconstitution

- **Human Cytokine Expression**: IL-7, FLT3L, and M-CSF improve cell survival (Kim YS, Ko JH, 2018).
- **HLA-Transgenic Models**: Mice expressing HLA alleles (e.g., HLA-A2) enable T-cell studies (Bennett MS, Akkina R, 2013).

CASE STUDY:

NOG-EXL mice with human GM-CSF/IL-3 show improved myeloid reconstitution without hyperinflammation (Willis E et al., 2024).

Refining CRISPR Technologies

- **Precision Editors**: Base/prime editing reduces off-target risks (Lin J, Yang J, 2024).
- **Nanoparticle Delivery**: Lipid nanoparticles enhance *in vivo* editing (Dash PK et al., 2021).

CASE STUDY:

Zhang et al. (Zhang C et al., 2023) reported that smaller Cas9 variants (saCas9) improve *CCR5* editing specificity.

Alternative Approaches

- **Immune Organoids**: Combining humanized mice with gut/brain organoids models tissue-specific reservoirs (Terahara K et al., 2021).
- **Personalized Models**: iPSC-derived HSCs bypass ethical concerns associated with fetal tissue. Leidy-Davis et al. (Leidy-Davis T et al., 2018) demonstrated extensive gene humanization (25 kbp) in mice via iPSCs, offering a scalable platform for patient-specific therapies.
- **Proposals for Advancing Humanized Models**

The continued refinement of humanized mouse models represents a critical frontier in HIV research. Recent proposals suggest several promising directions for development. First, the integration of multi-omics approaches could enhance our understanding of humanized immune system development and HIV pathogenesis at single-cell resolution. Second, advances in genetic engineering, particularly with base editors and prime editors, offer opportunities to create more sophisticated models with precise control over human immune component expression. Third, the development of dual humanized models incorporating both immune cells and relevant tissue architecture would better recapitulate human HIV reservoir dynamics. Fourth, implementation of standardized protocols for humanized model creation and evaluation would improve reproducibility across research groups. Finally, leveraging artificial intelligence for predictive modeling of engraftment success and immune reconstitution patterns could minimize inter-animal variability and optimize experimental design. These developments, if pursued systematically, could transform humanized mouse models into even more powerful tools for HIV research, bridging critical gaps between basic science and clinical application.

Future Directions

Combinatorial Therapies: CRISPR-edited cells + bNAbs + immunomodulators (Rothemejer FH et al., 2023; Khamaikawin W et al., 2024).

AI-Driven Optimization: Predictive models for therapy design (Deng B et al., 2023).

Organoid Integration: Humanized mice coupled with brain/gut organoids to study tissue-specific reservoirs (Terahara K et al., 2021).

CONCLUSION:

Addressing challenges requires interdisciplinary efforts in genetic engineering, nanotechnology, and ethics. Global collaboration and model standardization will accelerate progress toward an HIV cure.

DISCUSSION

Humanized mice bridge the gap between *in vitro* experiments and clinical trials, replicating mucosal transmission, latent reservoirs, and immune responses (Bennett MS, Akkina R, 2013; Zhang C et al., 2023). Despite technical and ethical challenges, they remain the gold standard for preclinical HIV research.

Scientific Impact

Humanized mice enable:

Pathogenesis Studies: Modeling neurocognitive disorders and CNS persistence (Terahara K et al., 2021).

Therapeutic Testing: From LA-ART to CRISPR-edited cells (Khamaikawin W et al., 2024; Nischang M et al., 2012).

Cure Strategies: Identifying bNAb roles in reservoir depletion (Baroncini L et al., 2023).

Overcoming Limitations

The development of HLA-transgenic models (e.g., HLA-A2 NOG-EXL) improves T-cell functionality, enabling studies on immune correlates of protection (Willis E et al., 2024). Meanwhile, advances in base editing reduce risks of genomic instability, as shown in *CCR5* editing trials (Lin J, Yang J, 2024).

Genetic Engineering: HLA/cytokine-expressing models improve T-cell functionality (Kim YS, Ko JH, 2018).

Nanotechnology: Lipid nanoparticles enhance CRISPR delivery (Dash PK et al., 2021).

AI Integration: Predictive modeling optimizes therapies (Deng B, Xue J, 2023).

CASE STUDY:

Dash et al. (Dash PK et al., 2021) eliminated HIV in 58% of mice via CRISPR + ART, demonstrating combinatorial potential.

ETHICAL AND PRACTICAL CONSIDERATIONS

The use of humanized mice in HIV research presents a complex interplay of ethical dilemmas and logistical challenges that demand rigorous scrutiny. Below, we critically evaluate these issues through contrasting viewpoints, supported by evidence from open-access peer-reviewed studies.

INTERNATIONAL RECOMMENDATIONS AND LEGISLATION FOR HUMANE ANIMAL TREATMENT IN RESEARCH

The humane treatment of laboratory animals, including mice, is governed by a comprehensive framework of international recommendations and national legislation. The three Rs principle—replacement, reduction, and refinement—forms the ethical foundation of animal research regulations worldwide (MacArthur Clark JA, Sun D, 2020). This principle, first proposed by Russell and Burch in 1959, has been incorporated into legislation and guidelines across multiple jurisdictions.

In the European Union, Directive 2010/63/EU requires systematic application of the three Rs, emphasizing alternative methods development and refinement of animal housing and experimental procedures. The United States Animal Welfare Act (AWA), established in 1966, sets legal standards for laboratory animal care and use, while the Guide for the Care and Use of Laboratory Animals provides additional guidelines (Cardon AD et al., 2012).

Japan's Law of Humane Treatment and Management of Animals endorses the three Rs principles, and China has developed its own Guidelines on the Humane Treatment of Laboratory Animals. These frameworks typically require institutional animal care and use committees (IACUCs) or animal welfare bodies (AWBs) to oversee research protocols, ensure compliance with ethical standards, and promote animal welfare throughout all research activities

ETHICAL CHALLENGES

Fetal Tissue Use in BLT Models

BLT models rely on fetal liver and thymus tissues, raising ethical concerns over tissue sourcing and informed consent. Critics argue that fetal tissue procurement conflicts with principles of human dignity (Bennett MS, Akkina R, 2013). Conversely, proponents emphasize the irreplaceable role of fetal tissues in replicating human mucosal immunity (Bennett MS, Akkina R, 2013). To address this, alternatives like iPSCs are highlighted. Leidy-Davis et al. (Leidy-Davis T et al., 2018) demonstrated that iPSC-derived HSCs achieve 25-kbp gene humanization, though functional maturity remains limited (Zhang C et al., 2023).

Animal Welfare and Compliance

Humanized mice often exhibit shortened lifespans and immune pathologies (Willis E et al., 2024). Innovations like non-invasive imaging and machine learning reduce animal numbers (Deng B, Xue J, 2023). Global initiatives, such as the International Society for Humanized Mice (ISHM), advocate standardized protocols (Brehm MA et al., 2014).

PRACTICAL LIMITATIONS

Financial and Resource Burdens

Maintaining humanized colonies incurs costs exceeding $10,000 per mouse annually (Hosur V et al., 2017). Open-access repositories like HuMoRe lower costs (Kim YS, Ko JH, 2018), but disparities persist between regions.

Engraftment Variability

Engraftment efficiency ranges from 10% to 80%, influenced by donor HSC heterogeneity (Terahara K et al., 2021). CRISPR preconditioning of HSCs enhances reproducibility (Khamaikawin W et al., 2024), but neurotropic HIV reservoir modeling remains inconsistent (Terahara K et al., 2021).

BALANCING INNOVATION AND ACCOUNTABILITY

CRISPR-Cas9 editing risks unintended genomic deletions (Liu Y et al., 2023). The NIH's Somatic Cell Genome Editing program emphasizes transparent screening (Lin J, Yang J, 2024). Organoid-integrated models reduce animal use (Dash PK et al., 2021).

CONCLUSION

Ethical and practical challenges in humanized mouse research are deeply interconnected. While fetal tissue alternatives and cost-sharing initiatives show promise,

global collaboration and regulatory harmonization are essential to advance HIV cure strategies equitably.

Hybrid Humanization Models: Combining CD34⁺ HSC transplantation with CRISPR-edited iPSC-derived microglia could better replicate neuroimmune interactions, addressing gaps in modeling HIV-associated neurocognitive disorders (Zhang C et al., 2023; (Terahara K et al., 2021).

Ethical Frameworks for BLT Alternatives: Establish international consortia to standardize iPSC-based humanization protocols, reducing reliance on fetal tissues while ensuring functional maturity through cytokine cocktails (e.g., FLT3L + IL-7) (Leidy-Davis T et al., 2018; Kim YS, Ko JH, 2018).

CRISPR Delivery Optimization: Prioritize lipid nanoparticle (LNP) formulations conjugated with HLA-specific targeting ligands to improve in vivo editing efficiency in HSCs, as demonstrated in HBV studies (Stone D et al., 2021; Lin J, Yang J, 2024).

AI-Driven Model Validation: Develop machine learning algorithms trained on multi-omics data (transcriptomics, proteomics) to predict engraftment success and immune reconstitution patterns, minimizing inter-animal variability (Deng B, Xue J, 2023).

FUTURE GOALS

Enhanced Models: Full myeloid/HLA reconstruction (Willis E et al., 2024).

Combo Therapies: CRISPR + vaccines + immunomodulators (Rothemejer FH et al., 2023).

Global Collaboration: Open-access biobanks for HSCs (Brehm MA et al., 2014).

CONCLUSION

Humanized mice revolutionized HIV research, but their potential is untapped. Integrating CRISPR-Cas, AI, and ethical practices will unlock new horizons. Success hinges on interdisciplinary collaboration among scientists, clinicians, and bioethicists.

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