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CONFERENCE SUMMARY

Improvements and Limitations of Humanized Mouse Models for HIV Research: NIH/NIAID "Meet the Experts" 2015 Workshop Summary

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Abstract

The number of humanized mouse models for the human immunodeficiency virus (HIV)/acquired immunodeficiency syndrome (AIDS) and other infectious diseases has expanded rapidly over the past 8 years. Highly immunodeficient mouse strains, such as NOD/SCID/gamma chain^{null} (NSG, NOG), support better human hematopoietic cell engraftment. Another improvement is the derivation of highly immunodeficient mice, transgenic with human leukocyte antigens (HLAs) and cytokines that supported development of HLA-restricted human T cells and heightened human myeloid cell engraftment. Humanized mice are also used to study the HIV reservoir using new imaging techniques. Despite these advances, there are still limitations in HIV immune responses and deficits in lymphoid structures in these models in addition to xenogeneic graft-versus-host responses. To understand and disseminate the improvements and limitations of humanized mouse models to the scientific community, the NIH sponsored and convened a meeting on April 15, 2015 to discuss the state of knowledge concerning these questions and best practices for selecting a humanized mouse model for a particular scientific investigation. This report summarizes the findings of the NIH meeting.

Introduction

The plenary lecture, entitled "Of Next Generation Humanized Mice and Men," was presented by **Dr. Leonard D. Shultz**. Preclinical testing in humanized mice has been employed for studies of human immunodeficiency virus-1 (HIV-1) pathogenesis and latency, drug therapy, vaccine development, gene therapy, and mucosal immunity in preparation for clinical trials.[1] Infectious agents that have been investigated in humanized mice so far include HIV, human T lymphotropic

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virus 1, influenza, Rift Valley fever, dengue virus, Epstein Barr virus, Ebola virus, cytomegalovirus (CMV), measles virus, *Salmonella typhi*, *Mycobacterium tuberculosis*, *Neisseria gonorrhea*, and *Plasmodium falciparum*.[2]

The CB17 strain carrying the spontaneous severe combined immunodeficiency (*Prkdc*^{scid}, abbreviated as *scid*) mouse mutation was the first model in the chronological development path of humanized mouse models, followed by the NOD/Lt-*scid* mouse and the highly immunodeficient NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tm1Wjl}*/Sz* (NSG) mouse.[3–6] The NOD/Lt background confers defects in innate immunity, impaired macrophage activation, lack of hemolytic complement, and human-like polymorphism in the signal regulatory protein alpha (*Sirpa*) gene. The *scid* mutation prevents development of mature T and B cells. The interleukin 2 (IL-2) receptor common gamma chain-targeted mutation (*IL2rg*^{null}) inhibits the expression of receptors for IL-2, -4, -7, -9, -15, and -21 and prevents natural killer (NK) cell development.

Described hereunder are the hematolymphoid engraftment methods for various humanized mouse models (see also Table 1):

Human peripheral blood lymphocytes-SCID (Hu-PBL-SCID): Human peripheral blood leukocytes are injected intraperitoneally (IP) or intravenously (IV) into an immunodeficient mouse. This procedure leads to efficient T-cell engraftment, but the mice develop lethal xenogeneic graft-versus-host disease (GvHD).

Hu-SRC-SCID (SRC = ''*scid* repopulating cell''): Human hematopoietic stem cells (HSCs) from bone marrow, umbilical cord blood, mobilized peripheral blood, or fetal liver are injected IV into sublethally irradiated neonatal or adult immunodeficient mice. This leads to the development of multiple hematopoietic lineages. The human T cells are educated in the mouse thymus.

SCID-hu: Human fetal liver and thymus fragments are transplanted under the kidney capsule of CB17-*scid* mice. A functional human thymus develops; however, there is minimal peripheral immune system development.

Bone marrow, liver, thymus (BLT) mouse: Human fetal liver and thymus fragments are implanted under the kidney capsule of sublethally irradiated NSG or NOD-*scid* mice, and in addition autologous fetal HSCs are injected IV. The results are robust development of multiple hematopoietic lineages and T-cell education in the human thymus. One disadvantage is the development of lethal GvHD in some animals after more than 20 weeks of engraftment.

Table 1. Major Strain Platforms for Humanized Mice

Abbreviation	*Strain*	*Description*	*Source*	*Reference*
BALB/c-*Rag2*$^{-/-}$*gc*$^{-/-}$	C.129-*Rag2*^{tm1Fwa} *Il2rg*^{tm1Sug} (Rag2$^{-/-}\gamma$c$^{-/-}$)	Deletion of *Rag2* and IL2r common gamma chain (*IL2rg*)	Stanford University, Jackson Laboratory	9–15
NOD/SCID	NOD.CB17-*Prkdc*^{scid}	NOD mice homozygous for the severe combined immune deficiency (*scid*) spontaneous mutation	Jackson Laboratory	
NSG	NOD.Cg-*Prkdc*^{scid} *IL2rg*^{tm1Wjll}*/Sz*	NOD mice homozygous for the *scid* mutation and *IL2rg* KO	Jackson Laboratory	8,16,17,26,29,34, 38,40,42,44,54,56–60
NRG	NOD.129S7(B6)-*Rag1*^{tm1Mom} *IL2rg*^{tm1Wjll}/Sz	NOD mice with Rag1 and IL2rg KO	Jackson Laboratory	29,34,35
NOG	NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Sug}	NOD mice with *scid* mutation and truncated, IL2rg KO	Central Institute for Experimental Animals, Japan	75
BRG	C.129(Cg)*Rag2*^{tm1Fwa} *IL2rg*^{tm1Sug}*/Jic*	BALB/c background with Rag2 and IL2rg Kos	Yale, University Hospital Zürich	8
H2dRG	STOCK *Rag2*^{tm1Fwa} *H2*^{d} *IL2rg*^{tm1Krf}	STOCK background with Rag2 and IL2rg Kos	Pasteur Institute	76
TKO-C57BL/6	B6.129(Cg)-*Rag2*^{tm1Fwa} *Cd47*^{tm1Fpl} *Il2rg*^{tm1Wjl}/J	C57BL/6 mice with Rag2 KO, CD47 KO, and IL2rg KO	Jackson Laboratory	46,47
DRAG	NOD.Cg-*Rag1*^{tm1Mom} *Il2rg*^{tm1Wjl} Tg(HLA-DRA,HLA-DRB1*0401)39-2Kito/ScasJ	Cross between NOD-like mouse transgenic for HLA DR4 and Rag1 KO and NRG mouse	Naval Medical Research Center/ Walter Reed	49,53
DRAGA	NOD.Cg-*Rag1*^{tm1Mom} *Il2rg*^{tm1Wjl} Tg(HLA-DRA,HLA-DRB1*0401)39-2Kito Tg(HLA-A/H2-D/B2M)1Dvs/Scas	DRAG mouse crossed with HLA-A2.1.B2m$^{-/-}$ NOD mice	Naval Medical Research Center/ Walter Reed	Not published yet
NSG-Cmah$^{-/-}$	NOD.Cg-*Prkdc*^{scid} *Cmah*$^{-/-}$ *Il2rg*^{tm1Wjl}	NSG mice with cytidine monophospho-N-acetylneuramic acid hydrolase gene KO	University of Nebraska	Not published yet

The main limitations of current humanized mouse model are as follows[7]:

1. Engraftment with mature human T cells leads to xenogeneic GvHD.
2. Human leukocyte antigen (HLA) molecules are required for appropriate T-cell selection following human HSC engraftment.
3. Many human cytokines and other factors are species specific.
4. Remaining innate immunity impairs engraftment.
5. Impaired humoral immune responses, low level of immunoglobulin production, and impaired immunoglobulin class switching.
6. Impaired lymph node development, poorly developed germinal centers.

Next generation humanized mice

Mouse major histocompatibility complex (MHC) class I (NSG-*B2m*^{tm1Unc}) and (*H2D1*^{tm1Bpe} *H2K1*^{tm1Bpe}*(KD)*^{null} or class II (NSG-*H2-Ab1*^{tm1Gru}) (abbreviated as *I-A*^{null}) and *H2*$^{dlAb1\text{-}Ea}$ (abbreviated as *I-A/I-E*)^{null}) knockout (KO) mice have been developed to reduce xenogeneic GvHD. Progress has also been made in generating a panel of human HLA class I and II transgenic NSG mice. NSG class I transgenic mice available include expression of HLA-A2, A11, A24, B7, B27, and Cw3 alleles, and NSG class II transgenic NSG mice available include HLA-DR1, DR2, DR3, DR4, and DQ8 alleles. For example, NSG-HLA-A2 transgenic mice engrafted with HLA-A2$^+$ HSCs develop HLA-A2-restricted human cytotoxic T cells.

Another new approach is the transgenic expression of human cytokines in immunodeficient mouse strains and targeting of mouse genes.[8] The new NSG-*Kit*$^{W\text{-}41}$ mice support human HSC engraftment without X-ray preconditioning. NSG-*Ifnar1*^{tm1Agt} [type I interferon (IFN) receptor KO] mice show increased sensitivity to dengue virus infection and increased sensitivity for testing neutralizing activity of anti-dengue human IgM antibodies. Another emerging model for humanized mice is the implantation of human-induced pluripotent stem cells-derived thymic epithelial cells into NSG-*Foxn1*^{nu} (nude) mice.

Preexposure Prophylaxis and Novel Treatment Strategies for HIV/Acquired Immunodeficiency Syndrome

Dr. Ramesh Akkina's presentation highlighted the utility of humanized mice in multiple areas of HIV/acquired immunodeficiency syndrome (AIDS) research in generating important preclinical data. Hu-HSC mice derived by injecting human CD34$^+$ HSCs into neonatal C.129-*Rag2*^{tm1Fwa} *Il2rg*^{tm1Sug} (Rag2$^{-/-}\gamma c^{-/-}$) mice (Rag-hu mice) were primarily used in these studies.[9] Multilineage human hematopoiesis is seen in these mice, which permits efficient HIV infection. Long-term viremia lasting more than 1 year could be established with both CXCR4- and CCR5-tropic viruses.[9] With the presence of HIV-susceptible cells in mucosal tissues, this model also permits HIV transmission through both vaginal and rectal routes; thus it is suitable for the evaluation of HIV preexposure prophylactic (PrEP) strategies.[9]

The integrase inhibitor raltegravir (RAL) and CCR5 inhibitor maraviroc (MVC) were tested as oral PrEP in humanized mice and were shown to protect 100% of the mice from intravaginal challenge.[10] In the context of topical microbicide application, protection was also achieved with MVC and a broadly neutralizing antibody (bNAb) VRC01 against vaginal HIV-1 challenge.[11,12] A major question for PrEP is the optimal concentration of combination antiretroviral therapy (cART) (which is designed for therapeutic purposes) that needs to be reached in mucosal tissues for conferring full protection against viral exposure. In this context, the hu-mice were found to be suitable for conducting pharmacokinetics-pharmacodynamics (PK-PD) studies.[13] ART drugs tenofovir (TFV), MVC, and RAL both individually and in combination (TFV+RAL, TFV+MVC, RAL+MVC) were tested. Higher concentrations were found in mucosal tissues than in plasma, and the drug interactions and synergistic effects are currently being evaluated.

In the second part of the talk, anti-HIV small interfering RNAs (siRNAs) were shown to be highly effective in suppressing viral replication *in vitro*, but specific delivery to HIV-infected cells *in vivo* has been difficult. To overcome this hurdle, siRNAs conjugated with an HIV-1 gp120-specific aptamer were tested in HIV-infected hu-mice.[14] Marked suppression of viral loads was seen with concomitant protection against viral-mediated CD4 T-cell loss.

The third part of the talk focused on potentiating antiviral immune responses through PD-1 blockade.[15] Mice treated with human anti-PD-L1 monoclonal antibody (mAb) showed decreased viral loads and improved T-cell function, suggesting its potential application for immuno-enhancement therapy. The advantages of the Hu-HSC model over BLT mice for some studies are summarized as follows[9]:

- Easy to prepare and lower cost
- More animals can be generated per cohort
- Negligible GvHD and longer life span
- Chronic HIV infection lasting more than 1 year
- Long-term safety and toxicity assessments possible

Dr. Alejandro B. Balazs presented a novel approach for delivery of bNAbs, called vectored immunoprophylaxis (VIP). Adeno-associated virus (AAV; serotype 8) is used as a vector, because it is nonpathogenic in humans, nonintegrating, and has excellent expression characteristics *in vivo*. Despite the limited capacity to carry foreign genes (4.8 kb), a transgene for the expression of antibody from muscle cells was presented.[16] In the first experiment, BLT mice were administered a vector resulting in expression of 100 μg/ml of VRC07G54W antibody and challenged intravaginally 21 times with the transmitted founder strain of HIV REJO.c. VIP protected against CD4 T-cell loss in splenic lymphocytes, gut intraepithelial lymphocytes (IEL), gut lamina propria lymphocytes (LPL), and vaginal LPL. All treated mice were entirely resistant to mucosal challenge with the REJO.c founder strain by sensitive viral load assays.[17]

The second experiment addressed vectored immunotherapy for established HIV infections. BLT mice were infected with REJO.c, given a suboptimal highly active antiretroviral therapy (HAART) regimen, which transiently suppressed viremia. These animals were administered AAV-expressing VRC07 antibody. After the antibody concentration reached a stable plateau, HAART was withdrawn. VRC07 in this model resulted in durable suppression (7 weeks below 10^3 RNA copies/ml) of REJO.c despite the lack of effective control of

virus. Dr. Balazs described a third experiment, in which AAV vectors expressing either PG9, PGT121, or VRC07 were prophylactically administered, and the mice challenged with 10^7 human CD4 cells from an infected patient suppressed with ART drugs. The blood was monitored for signs of virus emerging from infected cells. High concentrations were achieved for PGT121 (219 μg/ml) and PG9 (58 μg/ml) and lower concentrations for VRC07 (1.6 μg/ml), but only PGT121 protected the mice from HIV infection and CD4 cell loss. In contrast to PG9 and VRC07, the PGT121-transfused mice had undetectable viral load (below 10^3 RNA copies/ml), but the PG9- and VRC01-treated animals became viremic to similar levels as the control mice. In conclusion, VIP enables the long-lived expression of any desired antibody after a single intramuscular injection.

Dr. Fatah Kashanchi's talk discussed the use of irradiation to activate viral transcription and induce apoptosis in HIV-1-infected cells. It has previously been shown that HIV-1 long terminal repeat (LTR) can be induced with various transcription activators including irradiation,[18] which can be reversed by specific peptide or cyclin-dependent kinase inhibitors in humanized mouse models.[19–22] Here, total body irradiation was used to better define the latent tissue reservoirs in animals and also potentially use low-level irradiation as part of a ''kick and kill'' strategy activating HIV-1 cells under ART. Total body irradiations are frequently used to treat lymphomas, including solid epithelial tumors, lymphomas, breast, neck, and head cancers, and Hodgkin and non-Hodgkin lymphomas. Total body radiotherapy was also applied for the ''Berlin'' patient before CCR5Δ32/Δ32 transplantation.

Using an NSG ''latent HIV mouse model'' under a cocktail of cART, Dr. Kashanchi's laboratory was able to show that latent viruses could be activated using low-level whole body irradiation.[23] These low doses were not lethal or cancerous to animals at least up to 6 months. The question is whether T cells are equally affected by low-level irradiation compared to myeloid cells. It was shown that X-ray irradiation activates HIV-1 transcription by removing negative inhibitors from HIV-1 DNA and promotes transcriptional elongation. The effect may be further enhanced by proteasome inhibitors (to improve Tat stability *in vivo*). Latently infected T cells are more sensitive to cell death (as compared with uninfected T cells) by low-level irradiation than myeloid cells, possibly by activation of the wild-type p53 pathway in cells. The effect of cell death was not observed in myeloid-infected (or uninfected) cells with low-level irradiation, pointing to a different mechanism of apoptosis in T cells versus myeloid cells. Importantly, most tissues (blood, liver, lung, brain, and spleen) from NSG-humanized mice contain latent HIV-1 that can be activated with low-level irradiation alone. Reverse phase protein microarray on few infected cells can show the status of latently infected cells (i.e., senescence vs. exhaustion) in the presence or absence of activators. Irradiation plus ALLN (proteasome inhibitor) or bryostatin enhances transcription activity more than low-level irradiation alone. Collectively, the data indicate that latent HIV-1 can be transcriptionally activated with low-level irradiation, which is not toxic to the host, and potentially ''purge'' the virus from various secluded reservoirs including brain.

Dr. Scott G. Kitchen reported on the characterization of chronic immune activation and type I IFN signaling during HIV infection in humanized mice. The progression from naive to exhausted T cells involves chronic antigen stimulation during chronic infection, triggering the expression of exhaustion markers PD-1, LAG-3, CD244 (2B4), and Tim-3.[24,25] Similar to what is observed in humans, HIV-infected BLT mice have elevated expression of cellular activation and exhaustion markers. In particular, the expression of exhaustion (PD-1, Tim-3) and activation markers (HLA-DR) is increased in chronically infected BLT mice. T cells from infected mice have higher Tim-3 expression and an impaired ability to produce IL-2 and IFN-γ upon stimulation. After HIV infection of humanized mice containing human cells genetically modified with a molecularly cloned anti-HIV T-cell receptor (TCR),[26] Tim-3$^+$ HIV antigen-specific cells produce less IFN-γ and IL-2 in response to immunodominant HIV Gag p17 (SL9) peptide stimulation. It was also found that Tim-3 is elevated on HIV nonspecific T cells in chronically infected mice.

Type I IFNs (IFNα and β) have both antiviral and immunomodulatory effects and could contribute to chronic immune activation and exhaustion.[27] HIV-infected mice have chronically elevated levels of type I IFN signature gene expression than uninfected animals. In addition, HIV infection upregulates immunoregulatory dendritic cells,[28] as characterized by high levels of expression of both PD-L1 and CD95. Type I IFN receptor blockade significantly reduced type I IFN signaling, led to a reduction of activation and exhaustion markers on T cells, and improved their cytokine production upon stimulation. The BLT mouse model can be used to examine the mechanisms of immune defects during HIV infection and for testing therapies aimed at reversing immune suppression.

Dr. Florian Klein presented data from monocloncal antibody therapy studies in HIV-1_{YU2}-infected humanized mice. NOD.129S7(B6)-*Rag1*^{tm1Mom}/Sz (NRG) mice were irradiated with 1.0–3.6 Gray (Gy) and 4–6 h later injected intrahepatically with human HSCs. HIV-1-infected hu-mice demonstrated stable HIV-1 infection for more than 100 days, had a decreasing CD4$^+$/CD8$^+$ T-cell ratio, and the virus diversified within the host (2.2×10^{-3} mutations/bp 21 days after infection).

Antibody-mediated therapy with a single bNAb (e.g., 45–46^{G54W}) led to a transient decrease viremia, but escape occurred rapidly mediated by HIV envelope mutations (e.g., N279H and N280Y for antibody 45–46^{G54W}).[29] In contrast, antibody combinations (e.g., 3BNC117 + 10-1074+PG16) led to a sustained decrease of HIV-1 RNA.[29,30]

In contrast to the experiments in hu-mice, monotherapy appeared to be more effective in nonhuman primates: A single infusion with 3BNC117 or 10-1074 resulted in a transient suppression and although classical escape mutations were detected after 10-1074 monotherapy (N332K, S334N), no apparent escape was seen after 3BNC117 infusion.[31] In conclusion, antibody monotherapy can transiently suppress viremia in HIV-1-infected hu-mice, but escape and rebound occur rapidly. However, escape can be prevented by combining bNAbs that target different epitopes. Single bNAbs can effectively decrease viremia in nonhuman primates and in some cases maintain suppression until antibody serum levels decay.[31,32]

In a recent clinical trial, 30 mg/kg of 3BNC117[33] was given as a single infusion to uninfected or HIV-1-infected individuals.[34] The antibody was well tolerated and had preferable pharmacokinetics. Interestingly, the average drop in viremia at nadir was 1.48 $\log_{10}$ and a significant reduction

of the viral load was detected up to 28 days after antibody infusion.[34]

Finally, experiments in humanized mice demonstrated that bNAbs can synergize with non- or weakly neutralizing antibodies by inducing HIV escape variants. As shown *in vitro*, escape variants can be more susceptible to commonly generated HIV antibodies (e.g., V3-loop antibodies). When the bNAb 10-1074 was injected with one of the V3-loop antibodies 10-188 or 1-79 into HIV-1_{YU2}-infected humanized mice, viremia was suppressed more effectively.[35] Therefore, HIV-1 infection is, in part, more readily controlled during immunotherapy because escape from bNAbs can create holes in the glycan shield that render the virus susceptible to otherwise ineffective antibodies that are present in nearly all HIV-infected individuals.

Dr. Jerome A. Zack presented modeling of cell-based therapeutics in BLT mice. His laboratory has found that anti-MART-1 HLA class I-restricted TCR (F5) introduced into human stem cells results in the generation of mature CD8 T cells, which can eliminate human melanomas in BLT mice.[36,37] In addition, an anti-HIV Gag class I-restricted TCR introduced into human stem cells results in the generation of mature CD8 T cells that can greatly reduce HIV replication in BLT mice.[26,38] However, HLA restriction renders TCR-based approaches difficult to use clinically because multiple anti-HIV TCRs would be required. In addition, rapid resistance of HIV would be likely because of the rapid mutation rate.

A previously established approach created a CD4-zeta chimeric antigen receptor (CAR), which contained the extracellular and transmembrane domains of the human CD4 molecule, and the CD3-zeta signaling domain. The CAR recognizes HIVgp120 independent of HLA restriction. Ligation induces TCR signaling and activation. This construct demonstrated stable, safe engraftment with a persistence of greater than 10 years and has been used in peripheral T cells in multiple clinical trials.[39] It has modest clinical efficacy because of functional defects in modified peripheral T cells.

The new CD4-zeta CAR Triple Vector expresses a CCR5sh1005 gene for CCR5 knock down, a sh516 gene, which targets the LTR R region of HIV-1 and a CD4-zeta gene.[40] NSG mice were implanted with the thy-liv ''organoid,'' irradiated with 270 cGy, and $CD34^+$ cells transduced either with a control vector or with the Triple CD4CAR vector, and then infected with HIV. CD4-zeta CAR-modified stem cells develop into multiple hematopoietic lineages, such as T cells, macrophages, and NK cells. CD4 CAR-expressing cells have decreased expression of CD3, TCR$\alpha\beta$, and TCR excision circles in thymocytes,[40] suggesting that the CAR leads to allelic exclusion of endogenous TCRs in some cells. CAR-expressing T cells developed into effector cells and were activated in response to HIV. CD4-zeta CAR-modified mice have reduced HIV viral loads and preserved $CD4^+$ T-cell counts than control mice. Engineered immunity is potentially a feasible approach in eliminating chronic viral infections.

Finally, human $CD34^+$ cells can be engineered with TCRs plus a nonimmunogenic positron emission tomography (PET) reporter gene through lentiviral vectoring. Addition of the proper PET tracer allows localization of antigen-specific cells *in vivo* in BLT mice.[41] This approach may be useful in tracking engineered immune cells during clinical trials.

Mouse Viral Outgrowth Assay

Dr. Joel N. Blankson reported that NSG mice have a high level of human T-cell engraftment and a high degree of immune activation because of GvHD. For the mouse viral outgrowth assay (MVOA), 25–55 million peripheral blood mononuclear cells (PBMCs) from patients on suppressive cART regimens (1–6 years) were IP injected into NSG mice. Seven days later, anti-CD8 mAb was administered. The mice were bled weekly to evaluate viral load and CD4 T-cell count. All mice became viremic after PBMC transfer from patients on suppressive cART regimens.[42]

Next, elite suppressors were studied to answer the question whether MVOA is more sensitive than the quantitative viral outgrowth assay (QVOA). Elite suppressors have much lower frequencies of latently infected cells than patients on cART.[43] However, HIV isolates from HLA-B57 elite suppressors replicate vigorously in BLT-humanized mice.[44] Elite suppressors were selected who had a frequency of latently infected cells ranging from 1 in 15 million to 1 in 25 million $CD4^+$ T cells. Sixty-six million PBMCs from one elite suppressor or 20–26 million purified CD4 T cells from four other elite suppressors were injected IP into NSG mice. On day 7 anti-CD8 mAb was administered IP to the mice engrafted with PBMCs, and for the mice engrafted with purified CD4s, anti-CD8 mAb was given as needed. Two mice were treated with anti-CD3 IP. The mice were bled weekly to evaluate viral load and CD4 T-cell count. The NSG mice became viremic after PBMC or CD4 transfer from elite controllers. Elite suppressor CD8 T cells have potent antiviral activity,[45] and the expansion of these cells may potentially explain why virus does not continue to replicate *in vivo*.

The advantages of the MVOA are as follows:

- Recapitulates what happens *in vivo* when cART is interrupted.
- Continuous stimulation of cells for up to 6 weeks because of xenogeneic response.
- Can assay very large number of cells.
- Can be used for nonhuman primate (simian immunodeficiency virus) and human (HIV) studies.
- At least as sensitive as the QVOA; sensitivity may increase with anti-CD3 and/or anti-CD28 mAbs treatment *in vivo*.

Future directions are to validate the sensitivity with a larger number of patients to optimize *in vivo* stimulation with mAbs, to determine whether low-level viremia represents replication-competent virus, and to determine whether NSG mice can be engrafted with $CD4^+$ T cells from lymphoid tissue [gut-associated lymphoid tissue (GALT), lymph nodes].

Novel Mouse Models

Dr. Kim J. Hasenkrug presented the TKO-BLT model (B6.129S-*Rag2*^{tm1Fwa}*Cd47*^{tm1Fpl} *Il2rg*^{tm1Wjl}/J) (TKOs).[46,47] CD47 is the ''don't eat me'' signal, and ''TKO'' means triple knock out. The animals develop T cells, B cells, NK cells, dendritic cells, and other myeloid lineages, but no red blood cells and platelets. Mouse splenocytes were transfused 5 days post-transplantation to prevent radiation-induced anemia. The TKO-BLT model has human lymphoid reconstitution in the gut, such that the GALT reconstitution supports mucosal

HIV infection with pathogenic loss of $CD4^+$ T cells. HLA class II-expressing cells and both $CD4^+$ and $CD8^+$ human cells are present in lymphoid tissues. Spleens and mesenteric lymph nodes form B-cell follicles with interspersed T cells.

TKO-BLT mice have functional immune systems. HIV-specific IFNγ Enzyme-Linked ImmunoSpot responses from splenocytes could be detected 7 weeks postinfection (p.i.) and could be mapped to different Gag, Pol, and Env peptides. Antibodies (IgG) to gp120JR-CSF were present 8 weeks p.i. although at levels lower than that seen in HIV-infected patients. In vaccine experiments, no antibody responses were detectable after inoculation of Ad5 HIV vaccine vectors or DTP (diphtheria, tetanus, pertussis) vaccination. Experiments to improve B-cell responses by transfusing mesenchymal stem cells isolated from human fetal lung to provide human stromal cells in lymphoid tissues showed trafficking to multiple tissues. However, there was no improvement to vaccine-induced antibody responses. As seen with other models, there is an age-dependent loss of B cells with a concomitant increase in T cells and stable monocytes and dendritic cells.

Evidence of possible GvHD was seen in only 2 of 55 cohorts (more than 2,000 mice). Humanized mice in the TKO background are healthy for more than 35 weeks with no signs of GvHD. Thus, this model is an excellent platform for long-term studies, such as HIV latency and cure.

Dr. Sofia Casares discussed the generation of NOD-*Rag1*^{tm1Mom} *Il2rg*^{tm1Wjl} Tg(HLA-DRA,HLA-DRB1*0401) 39-2Kito/ScasJ (DRAG) mice.[48] HLA-DR4 Tg RagKO (C57BL/6) mice were crossed with NOD-*Rag1*^{tm1Mom} (RagKO mice). The hybrid mouse was backcrossed into the NOD background for 12 generations. The resulting HLA DR4 Tg Rag1 KO mouse was crossed with the NRG mouse, resulting in the DRAG mouse. Compared with NRG mice, DRAG mice express human HLA-DR4 molecules in cells from spleen, thymus, and bone marrow. For humanization, the DRAG mice were irradiated with 3.5 Gy and then infused with human HSCs from umbilical cord blood that was enriched for $CD34^+$ cells. The expression of HLA-DR4 molecules favors engraftment of human pro-T cells in the mouse thymus. The rate of human T-cell reconstitution is much higher in the DRAG mice than in the NRG mice at 25 weeks post-transfusion. In addition, all four human IgG subclasses (1–4), human IgA, and human IgE can be found in a number of the DRAG mice. The DRAG mice also elicit specific IgG antibodies upon immunization with tetanus toxoid vaccine. In addition, antibody responses to malaria parasites were found (IgM and IgG).

DRAG mice can also be used as a resource to generate mAbs. The DRAG mice are vaccinated with a protein, toxin, or infectious agent. Upon euthanasia of the DRAG mice, human B cells are recovered from the spleen, and B cell hybridomas generated producing human mAbs. For example, the 8F1-2C9 human IgG2 antibody inhibits malaria parasite growth *in vitro* and oocyst development on *Anopheles stephensi* mosquitos.[49]

Another new transgenic-humanized mouse is the DRAGA mouse (NOD.HLA-A2.HLA-DR4.RagKO.IL2RgcKO). The DRAGA mice are generated by crossing HLA-DR4 Tg/RagKO.IL2RgcKO (DRAG) mice with HLA-A2 transgenic mice. The resulting F1 generation is intercrossed, and the F2 generation is selected for the HLA-A2.HLA-DR4.RagKO.IL2RgcKO phenotype. DRAG and DRAGA mice reconstitute similar number of human CD4 and CD8 cells. Human CD8 T cells from DRAGA mice are HLA-A2 restricted and functional, as shown by MHC dextramer staining. Immunization with radiation-attenuated sporozoites or live sporozoites under chloroquine prophylaxis confers >80% malaria protection in humans, which can be experimentally reproduced in the DRAGA mouse model. Therefore, DRAGA mice may be useful for testing malaria vaccines.

Dr. Mangala Rao discussed the immune response in mucosal tissues of humanized DRAG mice. Mucosal memory CD4 T cells are of effector memory phenotype and enriched for CCR5 expression.[50–52] Human cells reconstitute the female reproductive tract (FRT) of humanized DRAG mice. There is a distinct distribution of B and T cells in the gut of humanized DRAG mice. $CD4^+$ and $CD4^+CD8^+$ T cells are highly enriched for CCR5 and $\alpha4\beta7$ expression.

Concentrated stocks of primary HIV-1 were prepared for mouse inoculation by ultracentrifugation and removal of microsomes and exosomes by antiacetylcholinesterase beads and anti-CD45 beads. The stocks were titrated on P4R5 HeLa-CD4-LTR-β-gal (MAGI) cells and had a $TCID_{50}$ of more than 10^5/ml. As little as 500 infectious units of primary HIV-1 BaL were sufficient to infect a DRAG mouse intravaginally with HIV-1 BaL.

In conclusion, humanized DRAG mice show a high level of reconstitution of human T and B cells in the gut, FRT, and spleen. The majority of $CD4^+$ T cells (79%–96%) exhibited a memory phenotype. The majority of $CD4^+$ T cells in the FRT, IEL, LPL, and Peyer's Patches (PP) expressed CCR5 (50%–80%). Mucosal tissues, which are the primary sites of HIV-1 transmission, had $CD4^+\alpha4\beta7^+$ T cells in varying frequencies. The proportion of $\alpha4\beta7^+$ $CD4^+CD8^+$ T cells in IEL and LPL was higher than that of $\alpha4\beta7^+$ $CD4^+$ T cells. Finally, a single low-dose intravaginal challenge with primary HIV-1 BaL resulted in 100% infectivity of humanized DRAG mice. Future studies include (1) imaging humanized mice infected with luciferase-expressing HIV-1 to determine the localization of the virus in the different organs/tissues and (2) immunization studies with subtype C gp145 adsorbed to $AL(OH)_3$ gel and added to unilamellar liposomes containing monophosphoryl lipid A.

Dr. Atef Allam discussed the distribution of follicular helper T (T_{FH}) cells in gut tissues PP, LPL, and IEL) and in the FRT of humanized DRAG mice before and after HIV-1 infection.[53] T_{FH} cells ($CXCR5^+PD\text{-}1^{++}$ $CD4^+$ T cells) are characterized by high expression of PD-1, CXCR5, ICOS, and BCL-6. Another subset of $CD4^+$ T cells found in gut tissues and FRT express intermediate levels of PD-1 and are named pre-T_{FH} cells ($CXCR5^+PD\text{-}1^+$ $CD4^+$ T or pT_{FH}^+ cells). Their frequency was higher in mucosal tissues of the gut and FRT than in the lymphoid tissues of humanized DRAG mice. A high proportion of T_{FH} cells in mucosal tissues express CXCR3, and there is a strong correlation between the expression of CXCR3, PD-1, and CCR5 and permissiveness to HIV-1 infection. Since the finding of T_{FH} cells in the FRT of humanized DRAG mice was unexpected, their presence was also confirmed in endocervical and ectocervical tissues obtained from routine hysterectomies; the majority of $CXCR5^+$ $PD\text{-}1^{++}CD4^+$ T cells expressed BCL-6, confirming the presence of human T_{FH} cells in the human FRT.

In conclusion, T_{FH} cells accumulate in the PP and FRT during the chronic phase of HIV-1 infection. T_{FH} cells are present in the endo- and ectocervix of humans and in the FRT of humanized DRAG mice and are highly permissive to HIV-1 with impaired IL-21 production over the course of infection. Owing to the high frequency of T_{FH} cells in gut and FRT, DRAG mice are a suitable model for testing HIV-1 vaccines.

Latency and Viral Dynamics

Dr. Andrew D. Luster presented data about the cellular and viral dynamics of intravaginal HIV-1 transmission in humanized NOD-*scid* BLT (BLT-NS) and NSG BLT (BLT-NSG).[54,55] One week before intravaginal infection, BLT mice were pretreated with progesterone, followed by an atraumatic intravaginal application of 10^5 $TCID_{50}$ HIV-1 JR-CSF. The viral dissemination and cytokine/chemokine expression in the BLT mice were characterized as follows: at day 2 p.i., local amplification occurred in the cervico-vaginal tract; hCXCL9, hCXCL10, and hTNFα were expressed. Between days 2 and 6 p.i., the virus spread to the draining and nondraining lymph nodes. Between days 8 and 15 p.i., hCXCL9, hCXCL10, and hIFNβ were expressed in the draining lymph nodes. Between days 10 and 15 p.i., the virus could be detected in the mesenteric lymph nodes, the gut, and plasma. At day 15 p.i., 85 of 90 (95%) BLT-NS and BLT-NSG mice from 13 different cohorts were clearly positive for plasma HIV RNA. Lymphocytes and pDCs accumulated in the cervicovaginal tissue following HIV infection. In conclusion, the paradigm described for SIV infection of macaques appears to be mirrored during HIV infection of humanized BLT mice.[56]

The next study investigated the various stages of GvHD. The clinical phenotype is divided into grade I (conjunctivitis and/or blepharitis only), grade II (thinning hair and/or alopecia at their surgical incision sites), and grade III (generalized alopecia and/or dermatitis). The pathologic phenotype involves the following tissues:

- Skin (grade I: minimum infiltrates in epithelium and/or hair follicles, grade II: important inflammation with thicker epithelium, but preserved hair follicles, grade III: complete loss of hair follicles).
- Cervico-vaginal tract (infiltration of $CD45^+$ and $CD3^+$ cells).
- Large intestine (infiltrates in the epithelium and abnormal cell death).

The number of histological changes in skin and cervico-vaginal tract correlated directly with the clinical severity and grade (I to III) of GvHD. The number of $CD45^+$ and $CD3^+$ cells increased with the clinical grade of GvHD, and may render humanized mice more susceptible to HIV infection.

Dr. J. Victor Garcia presented work to develop the BLT-humanized mouse as a flexible platform for the study of HIV latency and persistence.[57,58] The first step was to establish an efficient ART regimen that results in consistent reduction of plasma viral RNA (vRNA) levels in BLT mice. After 50 days of infection, BLT mice were administered a combination of RAL, emtricitabine, and TFV that reduced viral loads to below detection levels ($\sim$670 copies/ml).[59] To determine whether bona fide HIV latency had been established, a QVOA was performed using resting $CD4^+$ T cells isolated from multiple pooled organs from suppressed BLT mice. Using this assay, an average of eight infectious units per million cells (IUPM) were found in this model.[59] Like in humans, HIV plasma viral load rebounded after analytical treatment interruption. Further analysis of the number of $vRNA^+$ cells that occur during ART demonstrated a durable reduction in the levels of HIV-infected cells after treatment in all tissues analyzed, including thymic organoid, spleen, lymph nodes, liver, and lungs. But these results also demonstrated the presence of a significant number of $vRNA^+$ cells in the tissues of suppressed mice.

The presence of $vRNA^+$ cells despite therapy represents a significant problem for eradication studies. Therefore, an immunotherapeutic approach for the destruction of the ''active'' reservoir of HIV-infected cells was evaluated. The 3B3-PE38 immunotoxin consisting of the *Pseudomonas aeruginosa* exotoxin A translocation and cytotoxic domains linked to an Env-targeting moiety was tested *in vivo* for its ability to kill HIV-infected cells expressing vRNA that remain despite ART.[60] The results demonstrated effective *in vivo* killing of residual HIV^+ cells in tissues of BLT-humanized mice. Based on these results, the BLT mouse model was presented as providing an experimental platform for *in vivo* evaluation of ''Kick and Kill'' HIV eradication strategies. Dr. Garcia also presented data regarding another humanized animal model developed in his laboratory, the T-cell only mouse (ToM).[61] ToM mice are similar to BLT mice, but they only receive the Thy-Liv implant and not the $CD34^+$ HSC transplant. In this model, his laboratory demonstrated the efficient establishment of HIV latency and reactivation that occurs in the complete absence of human monocytes, macrophages, and dendritic cells. Progress was also mentioned in the development of another model reconstituted with human B cells and myeloid cells referred to as myeloid-only mice, because these are the only HIV targets in this model.

Gene Therapy

Dr. Priti Kumar gave an overview of the current approaches and clinical trials for HIV gene therapy. One approach to target human T cells and monocytes is through CD7, which is a 40 kDa cell surface glycoprotein from the immunoglobulin superfamily. It is expressed on the majority of human thymocytes and peripheral blood T cells. A key characteristic of CD7 is its rapid internalization after antibody binding, even with monovalent antibody fragments. A humanized single chain antibody (scFv) targeting human CD7 may be used for functional delivery of siRNA when attached to a cell-penetrating peptide 9R. scFVCD7 targets siRNA to naive/resting human T cells in Hu-HSC mice. Treatment with a combination of siRNAs targeting viral gene products and the CCR5 coreceptor prevents HIV infection in Hu-HSC mice. scFvCD7-9R:siRNA treatment prevents viral rebound in patient PBL-derived NSG mice. As shown by microscopy, human T cells in the FRT of Hu-BLT mice can also be specifically targeted with scFvCD7-9R:siRNA complexes. The resulting CCR5 knockdown confers protection against multiple exposures to the founder virus Rejo-1. A more permanent gene therapy approach can be established using lentiviral vectors pseudotyped with a modified Sindbis

virus envelope protein (SIN), in which the IgG-binding domain of protein A (ZZ) was inserted into the E2 region for binding to CD7. Human T cells of Hu-PBL mice are selectively transduced with the ZZ-SIN:αCD7 construct. Mice treated with the ZZ-SIN:αCD7 construct in combination with shCCR5 resist HIV challenge and selectively expand shCCR5-transduced cells.

An alternative gene-editing approach for mutating the CCR5 gene uses triplex-forming peptide nucleic acids (PNAs) that stimulate site-specific genome modification by forming triplex structures that induce cellular pathways of nucleotide excision repair and homology-dependent recombination. Importantly, PNAs are very safe as they are not themselves associated with nuclease activity and display highly specific binding that does not tolerate even a 2 bp mismatch obliterating off-target activity. PNAs can stimulate the recombination of short 50–60 bp donor DNA fragments for containing a stop codon into the human CCR5 gene. PLGA nanoparticles have been created for codelivery of PNA and donor DNA. These nanoparticles, upon injection through the tail vein, can render hematopoietic cells HIV resistant. HIV-1 can, therefore, be controlled in Hu-PBL mice by PNA-mediated gene editing. Furthermore, PNA-edited $CD34^+$ HSCs from Hu-HSC mice can be transplanted into secondary recipient mice to confer HIV resistance.

Dr. John C. Burnett presented combinatorial anti-HIV RNA-based therapeutics in the NSG-humanized mouse model. Two-day-old pups were irradiated with 100cGy and intrahepatically injected with 5×10^5 $CD34^+$ HSC from fetal liver tissues. The distribution of CD45 cells was 33% human origin and 67% mouse origin. Of the human CD45 cells, 38% were hCD19, 17% hCD14, 29% hCD4, 9% hCD8, and 34% hCD3 (more information on engraftment data is included in[62,63]). Hu-NSG mice have central memory $CD4^+$ T cells (CD27, CD45RO) that are known to support latent infection. Three groups of humanized mice were challenged with HIV-1 BaL or NL4-3 together with a control group. The highest plasma viremia was detected after 2 weeks with a challenge dose of 200 ng BaL p24, followed by 100 ng BaL p24, and then 200 ng NL4-3 p24. The infected mice were placed on an ART regimen of 300 mg TFV disoproxil fumarate, 200 mg Emtricitabine, and 400 mg RAL in drinking water mixed with MediDrop Sucralose. HIV RNA levels were reduced nearly 100-fold after 2 weeks of ART and were undetectable after 5 weeks, as measured by qRT-PCR. ARV-treated animals also exhibited concurrent increases in the percentage of $CD4^+$ T cells in peripheral blood.

Next, he gave an overview of anti-HIV RNA-based therapeutics. He evaluated a combinatorial RNA-based gene therapy for HIV using lentiviral vector delivery with anti-HIV siRNAs to silence CCR5 in an attempt to create an HIV-resistant immune system.[64]

Specifically, the four constructs delivered individually were a combination of small RNAs targeting *tat*, *rev*, CCR5, and a conserved U5 region within the HIV-1 LTR and transactivation response element decoys in an MCM7 (DNA replication licensing factor) platform. After HIV-1 JRFL challenge, the combinatorial lentivectors suppressed viral replication and protected $CD4^+$ T lymphocytes and $CD4^+$ monocytes.[64]

The next part of the talk focused on aptamers, which are *in vitro* evolved nucleic acids that bind to selected ligands (proteins, carbohydrates, and other nucleic acids) with high affinities similar to antibodies. They are composed of single-stranded DNA or RNA molecules, often with chemically modified nucleotides. The discovery process involves the selection of high-affinity binding aptamers out of as many as 10^{14} molecules from random sequence libraries. Six novel CCR5 aptamer candidates were selected after eight rounds of whole cell-based systematic evolution of ligands by exponential enrichment. Aptamers can be used for targeted delivery of anti-HIV siRNAs (HIV gp120 envelope, CD4, CD7, CCR5). An *ex vivo* HIV-1 challenge to test a CCR5-specific aptamer (G-3) in humanized mouse spleen cells was performed.[65]

Brain Models

The first neuroAIDS mouse model was developed by Drs. William Tyor, Howard E. Gendelman, and Yuri Persidsky, who transplanted infected human macrophages into the SCID mouse brain to induce HIV encephalitis.[66,67] When NOD/SCID mice became available, HIV-infected macrophages were injected intracranially and simultaneously Hu-PBL IP. The model was useful for 2–4-week-long experiments.[68] Later, central nervous system involvement was studied in the $CD34^+$ HSC-reconstituted NOD/*scid*-IL-2rγ^{null} (CD34-NSG model) during natural HIV-1 progression of disease.[69] The CD34-NSG model became a valuable tool for long-acting antiretroviral drug(s) development (nanomedicine program led by Dr. Gendelman).[70,71]

Another investigator at the University of Nebraska Medical Center, Dr. Mike Boska, conducted magnetic resonance imaging (MRI), proton magnetic resonance spectroscopy, and microstructural diffusion tensor MRI evaluations of the humanized mouse brain. These models also provided the possibility to observe longitudinal behavior, brain imaging, and metabolite changes.[72] The manganese (Mn)-enhanced MRI strategy to see changes in neuronal activities by Mn^{2+} uptake was also tested on HIV-1-infected CD34-NSG mice.[73]

Only a small number of human cells can be found in perivascular spaces and meninges in humanized NSG mice. Moreover, the cells very rarely show microglial morphology. With HIV infection, trafficking of human cells in the brain significantly increased; some cells had activated microglia morphology and were HIV-1 p24 positive. Alternatively, transplantation of dissociated fetal brain cell cultures led to the inclusion of human cells with human microglia and macrophages in NSG mice.[74] Neurospheres prepared from a human brain can be dissociated to single-cell suspension and transplanted intracranially, whereas HSCs are transplanted intrahepatically. Changes of supplemental growth factors *in vitro* favored development of human astrocytes *in vivo.* Simultaneous transplantation of $CD34^+$ HSCs and human astrocyte progenitor cells into newborn NSG mice is feasible. The development of the humanized blood and brain (named as ''huBB'' or ''2B'') in mice will be important for neuroAIDS studies. The amount of human astrocytes in selected brain regions can reach 60%–90%, for example, in periventricular spaces or the corpus callosum. Some animals were able to establish a rostral migratory stream. These huBB mice had usual levels of peripheral human cell engraftment and HIV-1 infection. Mice engrafted with human neuroglial cells can be used for studying immune responses, peripheral and

brain pathology of HIV-associated opportunistic infections, like JC virus, CMV, and other human-specific members of the *Herpesviridae* family.

The University of Nebraska Medical Center was awarded a Center for Humanized Mice grant (R24 OD 018546). The Center is creating five new mouse strains with improved background for human immune system function (NSG-Cmah$^{-/-}$), human macrophages distribution (CD11b- and CD11c-DRT-NSG, CD18^{mt}-NSG, i.e., *Joker*), and human-type liver metabolism (PXR-CAR-CYP3A4/3A7-NOG). The new Center will evaluate the new strains for their suitability for human infections, immunity, drug interactions, and vaccine studies.

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Author Disclosure Statement

Dr. Leonard D. Shultz is a faculty member and Professor at The Jackson Laboratory, an independent, nonprofit organization focusing on mammalian genetics research to advance human health. The Jackson Laboratory also manages a repository of mouse models and distributes mouse strains to researchers at academic institutions and companies. No other competing financial interests exist.

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