Larisa Y. Poluektova · J. Victor Garcia
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Humanized Mice for HIV Research

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Foreword

As a clinician and researcher involved in human immunodeficiency virus (HIV) disease since the beginning of the epidemic, I have huge respect for the contributions of work with humanized mice. As one of the only animal models that facilitates working with HIV as opposed to other lenitviruses, work with humanized mice has encompassed the entire spectrum of HIV pathogenesis research from transmission to immune dysregulation and the impact of preventive and therapeutic interventions. Finding suitable animal models has been a major impediment to HIV pathogenesis work since the beginning, as naturally occurring rodent cells are completely refractory to HIV infection. Even animals closely related to humans, such as chimpanzees, that can be infected with HIV do not develop the same immunodeficiency and disease. In addition, HIV has very limited tropism and so any kind of in vivo modeling must use a very similar organism, the most common one being simian immunodeficiency virus (SIV). While extremely useful, there are important genetic and biological differences between HIV and SIV, just as there are between humans and chimpanzees.

Over the last few decades, enormous strides have been made to improve the "humanization" of mouse models, particularly in the area of HIV research. Humanized mice have evolved into an invaluable alternative to SIV-based nonhuman primate models, as they are simpler, less costly, and also highly susceptible to HIV infection. Mouse models have been employed in basic pathogenesis research, preclinical and clinical testing of compounds with potential antiretroviral activity, and more recently, HIV biomedical prevention. For example, a humanized mouse model demonstrated that human breast milk has antiretroviral properties and may protect infants against oral transmission, thus helping to inform the debate about breast feeding for infected mothers without access to safe alternatives. Humanized mouse models are also being used to provide efficacy data about protection against rectal and vaginal infections with an array of regimens that might be used for pre-exposure prophylaxis. The models have helped to define the limits of protection for various dosing schedules, and are increasingly being used to investigate key pharmacologic parameters.

Reports of at least two individuals being cured of HIV infection, and several more with apparent functional cures (defined as long-term health in the absence of antiretroviral therapy) have renewed interest and excitement in this area. An important challenge is the difficulty of quantifying virus at extremely low levels in

patients, but this will need to be overcome in future to be able to establish whether or not an infected individual has truly been cleared of any virus. Humanized mice have already been used in this context to demonstrate replication competent virus in the absence of any detectable plasma viremia, even using highly sensitive assays for HIV RNA and DNA. Mouse models are likely to play a key role in this scientific agenda, moving forward.

Dr. Larisa Poluektova has been working in this field for many years, and we have been working together since 2006. Originally focused on neuropathogenesis work, more recently our collaborative activities have been in the development of nanoformulated antiretroviral therapy (ART) under the direction of Dr Howard Gendelman [1–3]. Nanomedicines contain crystalline drug particles of small diameter, coated with low-molecular-weight excipients to produce specific sizes, charges, and shapes that optimize cell and tissue penetrance. We have been working on nanoformulations of existing antiretroviral agents, and humanized mouse work has been pivotal. Building on what we have learned from the mouse experiments, we have moved into studies in nonhuman primate and hope to advance to clinical trials in humans. This emerging area of discovery has potential to make enormous changes in the field and advance treatment. While highly successful if taken correctly by infected patients, current ART is limited by the need for lifelong daily therapy, by poor tissue penetration, and by adverse effects. Suboptimal adherence to therapy may promote the development of virologic resistance and treatment failure. Nanoformulated ART may be able to be administered intermittently, and thereby improve medication adherence, and also has potential for decreased adverse effects and improved tissue penetrance. Investigations of long-acting formulations are also underway for HIV prevention.

"Humanized Mice for HIV Research" covers all these topics, and more. From an in depth review of the genetic background of mice and tips for humanization through understanding of human immune cells, the book moves on to HIV biology and pathogenesis and how humanized mice can advance the field. With discussion of specific cellular and humoral immune responses, the book includes reviews of development of conventional and novel therapeutics for HIV treatment and prevention. Finally, other human-specific or selective pathogens are presented including dengue, tuberculosis, and malaria, all causes of enormous amounts of human disease. The last section moves to new horizons and exciting prospects for the future from experts in the field.

This is an essential book for scientists and their students and will provide them with comprehensive and up-to-date information about the role of humanized mice in HIV research. Despite a wealth of scholarly articles on this topic, including many from the authors in the book, there are very few comprehensive textbooks about humanized mice in HIV research—a gap that has now been filled very nicely.

Omaha, NE
2013

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Preface

In 2012, our international editorial team, whose members are listed below, implemented work on a *comprehensive* textbook, or collection, entitled "Humanized Mice for HIV Research." In its current completed form, this detailed document is intended to serve as a scientific guide for graduate students, fellows, and investigators in bench science, academicians (e.g., hematologists, immunologists, virologists), clinicians (e.g., infectious disease specialists), and persons in the pharmaceutical industry (e.g., drug developers, vaccine developers, and pharmacologists/toxicologists) in the field of HIV and beyond. Importantly, humanized mice are the only animals, aside from chimpanzees, that are susceptible to HIV infection. Thus, humanized mice are an ideal platform for the study of HIV.

HIV has been, and still is, intensively investigated. However, the lack of robust small animal models has hindered progress in the basic understanding of HIV infection and pathogenesis. This lack also poses a considerable challenge for preclinical testing and the prioritization of new drug and vaccine candidates.

Stable, multilineage human hematopoietic engraftment can now be routinely achieved in immunodeficient mice. Surveillance of the development of human hematopoietic and lymphoid tissues in the mouse environment by researchers with different expertise provides valuable information. This book provides information on a wide range of different approaches, applications, ideas, observations, hypotheses, and insights. We expect this exchange of information to help facilitate exploration of HIV pathogenesis, and the development of new treatments and preventative approaches that will accelerate progress toward the eradication of this disease.

We sincerely appreciate the great efforts of all of our contributors, and apologize to anyone we may have left out with important new findings, observations, developments, or ideas to share. With the help of humanized mouse models, we hope to progress to an HIV/AIDS-free world. We expect that efforts to control other human-specific infections will also benefit from broadening the application of humanized mice to biomedical research.

Warm regards,

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Chapter 1
Mamoru Ito's Vision for the Future of Humanized Mouse Models

Mamoru Ito

Since development of NOD-*scid* IL-2Rg^{null} (NOG, NSG) and BALB/cA-Rag2^{null} IL-2Rg^{null} (BRG) at the start of the twenty-first century, given the high capacity of human cells and tissues to engraft and differentiate in these models, studies using humanized mice have universally attracted researchers' attention. This chapter describes past, present, and future xenotransplantation mouse models, with a particular focus on developments in Japan and the future technological progress needed for the use of humanized mouse models in translational research fields like HIV-1 infection.

1.1 History of the Development of Immunodeficient Mice in Japan

Centuries before xenotransplantation studies began in Japan and the USA, scientists in Europe were conducting cross-species transplantation studies using newborn animals dating as far back as the sixteenth century [1]. Importantly, failures and limitations associated with these transplants have helped improve understanding of basic immune mechanisms that control tissue compatibility. Finally, the discovery of immunodeficient nude mice dramatically increased the performance of xenotransplantation studies and opened a new door for performing xenotransplantation experiments on small laboratory animals [2]. While there are volumes of information regarding these developments for Europe and North America, little has been published regarding progress in this field of research in Asia. Thus, this chapter briefly describes the history of immunodeficient mice in Japan. Such developments

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began in Japan in 1973 when Dr. Tatsuji Nomura imported the nude mice from Dr. Friis in Denmark. Dr. Nomura was a Japanese pioneer in this field and helped found the International Nude Mice Workshop, referred to as the International Workshop of Immunodeficient Mice following the fifth workshop onward, with Drs. Rygaard and Povlsen of Denmark. This workshop created a foundation to study how nude mice could be used in biomedical fields which was held nine times between 1972 and 1997 at different locations around the world.

Dr. Nomura actively expanded the initial mouse colony from Dr. Friis, and 30,000 nude mice were produced over the following 3 years. In parallel, he formed a consortium with public institutes and pharmaceutical companies to perform cancer research using nude mice with support from the Japanese Ministry of Public Welfare. In conjunction with his work on expanding the initial colony of nude mice, Dr. Nomura continued the development of new immunodeficient mice as described later. After successful development of NOG mice as a results of his effort, in 2006, Dr. Nomura hosted the International Workshop of Humanized Mice in Tokyo, Japan, which has been attended by researchers every 3 years [3]. With these contributions, Dr. Nomura has helped to progress the field of immunodeficient mice in Japan and throughout the world.

Using Dr. Nomura's work as a foundation, in the early 1980s the Central Institute for Experimental Animals (CIEA) attempted to improve the recipient for xenotransplantation by crossing Dr. Nomura's nude mice with X-linked immunodeficient mice (XID) and beige mice, which were one of the few immunodeficient mouse models available in Japan at the time. Unfortunately, these initial attempts were unsuccessful. In 1985, the CIEA introduced a new immunodeficient mouse model, severe combined immunodeficiency (SCID). SCID mice lack T and B cells [4] and were discovered in the USA by Dr. Melvin Bosma in 1983.

In subsequent work by Dr. Joseph McCune and colleagues in the USA using SCID mice, human T and B cells were successfully generated following transplantation of human fetal liver and thymus into these mutant mice in 1988. These humanized mice, termed *SCID-hu*, are able to maintain human T cells and have been of major interest to researchers, particularly in the study of HIV-1 infection [5]. However, *SCID-hu* mice cannot be used in Japan due to bioethical concerns about the use of human fetal organs. Thus, nonhuman SCID mice have been mainly used for basic immunology and cancer studies in Japan.

Human peripheral blood mononucleated cell (PBMC), as well as fetal organs, can be engrafted into SCID mice [6]. In turn, such mice have been used in Japan for studies involving HIV-1 infection. The *scid* gene (formally, $Prkdc^{scid}$) was introduced into the NOD mouse inbred strain to generate NOD-*scid* mice showing ability to support high levels of HIV-1 viremia after transplantation of human cells [7–9]. Then, international groups of collaborating scientists reported that human hematopoietic stem cells (HSC) differentiate when transplanted into NOD-*scid* mice [10, 11]. Therefore, these mice have been used extensively in stem cell biology for more than a decade, until the development of NOG and BRG mice.

Until the early 1990s, immunodeficient mice had only been obtained accidentally, following a spontaneous mutation. Targeting technology using embryonic stem

(ES) cells, established in 1989 by Italian-born American molecular geneticist and Nobel prize recipient Dr. Mario Capecchi, helped to pave the way for artificially developing numerous immunodeficient mouse models [12]. In 2002, artificially generated IL-2Rg knockout mice were crossed with NOD-*scid* mice to create NOG mice, which can inactivate the gene encoding IL-2Rg [13]. Around this same time, our lab also developed BRG mice by inactivating the gene encoding IL-2Rg from BALB/cA-Rag2^{null} mice. In 2004, Dr. Marcus Manz and colleagues at the University Hospital Zurich reported a humanized mouse model using these BRG mice [14]. In 2005, Dr. Leonard Shultz of the Jackson Laboratory in the USA generated NSG mice, which are similar to NOG mice [15]. In 2010, Dr. Takiguchi and colleagues at Kumamoto University in Japan generated NOD-*scid*-*Jak3*^{null} mice, which have the same immunodeficiency as NOG and NSG mice [16]. These immunodeficient mouse models have been critical in the recent progress in normal and diseased human cell/tissue transplantation for regenerative medicine, cancer and therapeutics development.

1.2 Currently Available Humanized Mouse Models Generated Using NOG, NSG, and BRG Mice and Their Limitations

In general, the following three strains of immunodeficient mice are currently used to generate humanized mouse models: NOG [13], NSG [15], and BRG [14]. The common characteristics of immunodeficient mice are that they are deficient in T, B and NK cells, due to SCID/RAG2^{null} and inactivation of IL-2Rg. Inactivation of IL-2Rg allows for a high level of engraftment and differentiation of human cells into NOD-*scid* and BALB/cA-Rag2^{null} mice. Still, the reason why inactivation of IL-2Rg supports engraftment and differentiation of xenografts is unclear. It is quite possible that inactivation of IL-2Rg is linked to the dysfunction of cytokines responsible for T, B, and natural killer (NK) cell proliferation and differentiation. Our team recently demonstrated a crucial role of interferon gamma (IFNg)-producing CD11c+B220+CD122+ cells in xenograft rejection. IFNg-producing cells constitute a subpopulation of plasmacytoid dendritic cells and are absent in NOG mice [17]. Production of IFNg is impaired in IL-2Rg-deficient mice [18], which suggests that IFNg has an important role in xenograft rejection. The genetic backgrounds of NOG/NSG and BRG mice are the NOD and BALB/cA inbred strains, respectively. The engraftment rate of human cells is generally considered to increase in NOG/NSG mice compared with BRG mice. This is thought to be because the NOD strain has SIRPa polymorphism similar to human and reduced innate immunity, whereas the BALB/cA strain does not [9, 19].

Engraftment of xenografts, including human cells and tissues, is extremely effective in NOG, NSG, and BRG mice compared with conventional immunodeficient mice like NOD-*scid* and C.B-17-*scid*. The high engraftment capacity of these mice enables improved humanized mouse models to be generated. In general, two tech-

niques are used to generate humanized mice. One involves the transfer of mature human PBMC, and the other involves the transfer of HSCs isolated from human cord blood, bone marrow, or fetal liver (e.g., BLT). In the PBMC technique, transferred mature lymphocytes, CD3+ cells in particular, essentially attack the mouse, resulting in early death due to severe graft versus host disease (GVHD). For instance, NOG mice die at 2 weeks after intravenous transfer of 1×10^7 PBMC. In contrast, severe GVHD does not occur in NOD mice as seen in NOG mice. NOD mice survive more than 2 months, and the GVHD occurs only by intraperitoneal transfer of 1×10^7 PBMC [20].

Human CD3+ cells infiltrate the organs of the NOD mice. These proliferating cells are considered to be xenoreactive and can secrete various cytokines in response to the mouse cells. This secretion of cytokines results in further proliferation and activation of the human cells in a paracrine manner. Severe GVHD does not occur when human PBMC are transferred into NOG mice that have been depleted of major histocompatibility complex. Instead, human T cells proliferate less in these mice than in NOG mice with normal levels of the major histocompatibility complex (unpublished data). It is speculated that humanized mice can be generated by the transfer of particular cells, such as NK cells, that are purified from human PBMC. On the other hand, the long-term maintenance of these cells in NOG mice is expected to be difficult. For example, when human NK cells isolated from PBMC are transferred, the cells only survive in mouse peripheral blood for approximately a week (unpublished data). However, depending on the study, such humanized mice could be used for short-term experiments [21, 22].

In contrast to PBMC transfer, various hematopoietic cells differentiate from HSC in NOG/NSG/BRG mice, and such humanized mice have been of particular interest to researchers. When HSC are transferred into NOG mice, myeloid cells typically develop after 3–4 weeks; B cells typically develop after 6–8 weeks, and T cells typically develop after 10–12 weeks. T cells differentiate into CD4+ or CD8+ cells in NOG mice, whereas T-cell differentiation rarely occurs in NOD-*scid* mice. Conversely, certain cell lineages, such as erythrocytes and granulocytes, rarely develop at all, even in NOG, NSG, and BRG mice. The reasons for this phenomenon are beginning to be understood, and it appears that mouse factors are unable to compensate for the absence of human factors responsible for the differentiation of these cells.

T and B cells that differentiate from HSC in NOG mice can be maintained for as long as 1 year without GVHD. At one time, it was expected that such mice could be used to develop hematolymphoid humanized mice with a complete immune system. However, humanized NOG mice do not produce antigen-specific human immunoglobulin (Ig) G antibodies, even when they are challenged with antigens. Antigen-specific cytotoxic T lymphocytes (CTL) were not also induced in NOG mice. This lack of responsiveness may be because human T/B cells and antigen-presenting cells do not interact in humanized NOG mice when human T cells are educated in the mouse thymus. By using NOG/NSG mice that express class I or II human leukocyte antigens (HLA), antigen-specific human IgG antibodies and CTL can be induced following transfer of HLA-matched HSC [23–27]. Thus, human immune responses can be partially elicited in immunodeficient mice that express HLA.

Humanized mice with engrafted human T cells following transfer of PBMC and HSC can be used to evaluate anti-HIV-1 drugs. Still, such mouse models cannot be used to research immunological responses to HIV-1 infection or the development of an HIV-1 vaccine due to functional deficiency of human T and B cells resulting in the lack of robust adaptive immune responses. NOD-*scid* mice that have received human fetal bone marrow, liver, and thymus (i.e., BLT mice), have a working human immune system and can be used as a HIV-1 infection model [28]. However, there are bioethical concerns about the use of such mice, and consequently, they cannot be used in Japan.

1.3 Novel Humanized Mouse Models Generated Using Improved Immunodeficient Mice

Recently, to overcome the disadvantages of conventional NOG, NSG, and BRG mice, several improved immunodeficient mouse models have been developed primarily through the introduction of various human genes [23–34]. Our team has developed and improved several immunodeficient mouse models (http://www.ciea.or.jp/kiban-s/index.html). Our new models were primarily established through the introduction of human cytokine genes and mutated mouse genes (unpublished data). Mice that have been modified to have genes encoding HLA are of interest because they exhibit human immune response following transfer of human haplotype-matched HSC.

Here, we briefly describe the characteristics of the humanized mouse models of particular interest developed by CIEA. In NOG mice expressing human interleukin (IL)-2 following HSC transfer, human NK cells generally developed 4 weeks before T and B cells. These NK cells accounted for 80–90 % of human cells in NOG-hIL-2 mice, and the NK cells effectively suppressed the growth of NK-sensitive K562 leukemia cells in vivo (paper submitted). Myeloid lineage cells, including granulocytes and monocytes, successfully formed in NOG mice that expressed human granulocyte macrophage colony-stimulating factor (GM-CSF) and IL-3, whereas they rarely form in conventional NOG mice. Additionally, a passive cutaneous anaphylactic reaction was successfully elicited in these humanized NOG mice following intracutaneous inoculation of sera from pollenosis patients followed by intravenous inoculation of pollen antigen and Evans Blue dye [35]. HSC did not differentiate when transferred into NOG mice that express human IL-4, but the reason for this remains unclear. When these mice were transplanted with HSC, they showed mild GVHD, and human cells could be maintained for a longer time period due to the shift of T cells to Th2 cells.

These improved humanized mouse models can be used to study human diseases. The evaluation of the use of such models in this context will be left to experts in different areas of biomedical disciplines, such as regeneration, development, infectious diseases and vaccines, and cancer and therapeutics.