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

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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.

1.4 Generation of Humanized Mice in the Future

As outlined earlier, the CIEA research group has developed various improved humanized NOG mouse models, and this work is ongoing. Here, based on our experience, we describe how improved humanized mouse models can be generated in the future (see Fig. 1.1).

As part of our vision for improving immunodeficient mouse models, the following items are needed: the creation of recipients for xenotransplants, the development of techniques to create artificial organs, and the development of methods to create a plentiful and stable supply of stem cells. Here, we focus on humanized mouse models with functioning human immune systems, given that such models are needed to research mechanisms underlying human immune disorders and to develop drugs to treat these disorders.

1.4.1 Improvements in Immunodeficient Mouse Models

Presently, NOG and NSG mice are the best-suited recipients for the generation of humanized mouse models. However, these mice still have cells responsible for innate immunity, such as granulocytes, macrophages, and dendritic cells. It is possible that elimination of innate immunity cells will generate more appropriate recipients.

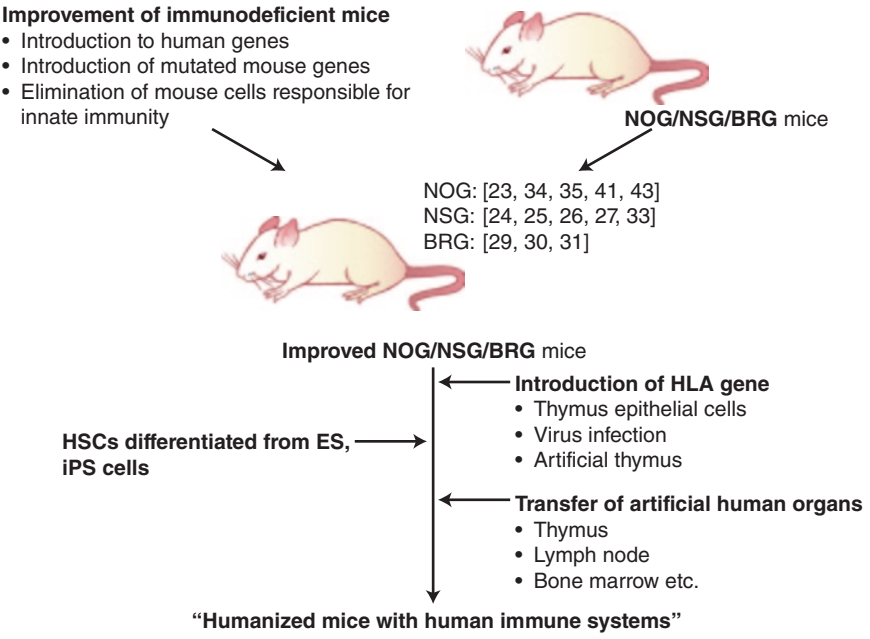

Fig. 1.1 Schematic diagram illustrating the possible future generation of humanized mice

Another possible improvement is to introduce human genes into immunodeficient mice that support the human cell growth and differentiation, as mouse factors cannot always compensate for the absence of such human factors. Indeed, we introduced human cytokine genes to generate improved humanized mouse models. Further crossing of these mouse models is expected to allow the creation of additional useful models. Nonetheless, it is time-consuming and technically difficult to cross three or more transgenic mouse strains to generate mice that express multiple transgenes. Furthermore, in mice with multiple transgenes, the transgenes can influence each other, and consequently, mice might not exhibit the expected characteristics. Knock-in technology using ES cells is an alternative strategy to generate improved humanized mouse models [29, 31, 32]. Recently, we successfully developed ES cells derived from NOG mice embryos. By performing homologous recombination using these cells, we can easily generate knock-in NOG mice, in which mouse genes are replaced with human genes. Remarkable progress in transgenic technology was recently made, that is a genome editing using CRISPR/CAS, TALENs and zinc-finger nucleases to generate targeting animals without using ES cells [36-38]. These techniques are expected to be a useful future tool for more quickly generating improved humanized NOG mice models.

1.4.2 Artificial Organ Formation

Humanized mouse models with human organs are uncommon. Nevertheless, human ovary and endometrium were successfully generated using such models [39, 40]. Furthermore, a human liver has been formed after implantation of human liver cells into urokinase-type plasminogen activator transgenic NOG mice (uPA-NOG), fumarylacetoacetate hydrolase KO BRG mice (BRG-FAH), herpes simplex virus type 1 thymidine kinase transgenic NOG mice (TK-NOG), and FK506-binding domain-fused caspase 8 transgenic BRG mice (AFC8-BRG), in which mouse liver cells were spontaneously or artificially destroyed [41–43]. These mice also appear to be useful as models for hepatitis C and B infection and for drug metabolism research [44].

Implanting human cells into NOG mice is considered the preferred method to generate humanized mice with artificial organs. To generate xeno-organs in mammals, the laboratory of Dr. Nakauchi at Tokyo University recently developed a novel technique termed "blastocyst complementation." In this technique, normal ES cells are injected into blastocysts of organ-deficient mammals. This technique was successfully used to develop a rat pancreas in a mouse [45], which grew to the same size as a normal mouse pancreas. By injecting human ES or induced pluripotent (iPS) cells, rather than rat ES cells, it may be possible to develop mice with human organs. For example, by injecting human ES or iPS cells into blastocysts of thymus-deficient BRG-nu mice, which we have already developed, there is potential to develop mice with a human thymus. However, the use of this technique is currently limited in Japan due to bioethical concerns. In another attempt, there is an interest report on artificial lymph nodes by the research group of Dr. Takeshi Watanabe at Kyoto University [46].

1.4.3 A Plentiful and Stable Supply of Stem Cells is Needed to Generate Humanized Mice

Stem cells, such as HSC, are needed to generate humanized mice, but they are relatively difficult to obtain and are heterogeneous. These difficulties are particularly pronounced when haplotype-matched HSC are used in NOG mice that express HLA. To overcome these issues, techniques are needed that allow HSC to be generated from ES or iPS cells. There are several reports on the generation of CD34+ cells from ES cells in vitro [47, 48]. Still, HSC generated from ES/iPS cells have not been successfully developed into multilineage hematopoietic cells in vivo. A plentiful and stable supply of HSC is expected to be useful for future routine generation of humanized mouse models.

1.4.4 Mice with Human Immune Systems

Mice with human immune systems are needed for vaccine development and to evaluate immunological responses to newly developed drugs. The development of mice with humanized thymus and lymph nodes is considered key to the generation of mice with human immune systems. Following transfer of haplotype-matched HSC, antigen-specific IgG antibodies are produced in HLA-DR Tg NOG/NSG mice, and CTL are induced in HLA-A2 NSG mice. Results indicate that expression of HLA assists in the generation of mice with human immune systems. Hence, an immediate focus should be placed on the generation of HLA-NOG mice that express a dominant HLA haplotype, such as D4 or D9 of HLA class I and A2 or A24 of HLA class II. By crossing mice that express HLA-DR or HLA-A, there is the potential to generate improved humanized mouse models in which human antibodies are produced, and human CTL are induced. Another approach is to inject multiple DNA isolated from persons with different HLA haplotypes into mouse embryos in order to generate mice that express multiple HLA haplotypes. Using this technique, mice can be generated that simultaneously express two or three types of HLA class I/II. Still, the expression levels of these antigens in such mice remain obscure.

There are many HLA haplotypes, which have practical applications in personalized medicine. For example, an iPS cell bank is being established and promoted specifically for the Japanese population to provide cell transplantation therapy. This resource is located at the Center of iPS Cell Research and Application (CiRA) at Kyoto University in Japan. CiRA has estimated that to cover 90% of the Japanese population, approximately 140 iPS cell lines homozygous for HLA-A, HLA-B, or HLA-DR are needed. It is not possible to generate HLA-NOG mice that cover all potential haplotypes of Japanese individuals. Still, the number of HLA haplotypes required could be less than 140. In future work, several techniques could be used to overcome this challenge. First, it may be possible to generate human thymus epithelial cell lines of various HLA haplotypes. These cells could be implanted into mouse

thymus and educate human T cells to interact with B cells and antigen-presenting cells. Unfortunately, no such cell lines are currently available. Second, it may be possible to express HLA either specifically in mouse thymus or systemically by infecting mice with a virus vector. Nonetheless, virus vectors that can potentially be used to infect the thymus have only recently been developed. In 2010, Chu et al. [49] reported that the adeno-associated virus may be a candidate, but its efficacy remains uncertain. Third, it may be possible to generate an artificial thymus from human ES or iPS cells. This would be a major advancement in the generation of humanized mice with human immune systems. However, a technical advance is required in order to generate an artificial thymus that covers all potential HLA haplotypes.

Human immune responses occur in HLA-NOG mice, but they appear to be weaker than true human immune responses. The reason for this may be that NOG mice have severe leukopenia and marked atrophy of the thymus and lymph nodes. Dr. Paul Denton et al. [50] reported that following transplantation of human fetal BLT, NOD-*scid* mice develop intestinal human T cells throughout the small and large intestine, whereas NSG and BRG mice do not. This failure of human cells to develop in the intestines of NSG and BRG mice is due to the inactivation of IL-2Rg in these mice. Inactivation of Il-2Rg is expected to impair the differentiation of lymphoid tissue inducer cells responsible for lymph node development. This can be overcome by specifically expressing IL-2Rg in the lymph nodes of IL-2Rg-deficient mice, including NOG mice.

In conclusion, there are issues associated with currently available humanized mouse models. These issues need to be resolved to generate improved humanized mouse models through which specific human diseases are reproduced. In turn, these models have the potential for use in the study of the mechanisms underlying diseases and in the development of drugs to treat diseases.

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