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Ⅳ. 研究成果の刊行物・別刷

NOD/Shi-*scid IL2rγ*^{null} (NOG) Mice More Appropriate for Humanized Mouse Models

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Abstract "Humanized mice," in which various kinds of human cells and tissues can be engrafted and retain the same functions as in humans, are extremely useful because human diseases can be studied directly. Using the newly combined immunodeficient NOD-*scid IL2rγ*^{null} mice and *Rag2*^{null} *IL2rγ*^{null} humanized mice, it has became possible to expand applications because various hematopoietic cells can be differentiated by human hematopoietic stem cell transplantation, and the

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T. Nomura et al. (eds.), *Humanized Mice.*
Current Topics in Microbiology and Immunology 324.

human immune system can be reconstituted to some degree. This work has attracted attention worldwide, but the development and use of immunodeficient mice in Japan are not very well known or understood. This review describes the history and characteristics of the NOD/Shi-*scid IL2rγ*^{null} (NOG) and BALB/cA-*Rag2*^{null} *IL2rγ*^{null} mice that were established in Japan, including our unpublished data from researchers who are currently using these mice. In addition, we also describe the potential development of new immunodeficient mice that can be used as humanized mice in the future.

Abbreviations Asialo GM1: asialoganglioside gangliotetraosylceramide; BM: bone marrow; CB: cord blood; GM-CSF: granulocyte-macrophage colony-stimulating factor; HSC: hematopoietic stem cell; IFN-γ: interferon gamma; IL: interleukin; IL-2Rγ: interleukin 2 receptor gamma chain; MHC: major histocompatibility complex; MSC: mesenchymal stem cell; NK: natural killer; PBMC: peripheral blood mononuclear cell; RNAi: RNA interference; SCF: stem cell factor; TCR: T cell receptor

1 Introduction

Clarification of the genetic base that covers the entire human and mouse genetic makeup [55, 107] has helped to accelerate research on human diseases and potential therapeutic applications based on known genetic information. In addition, the search for genes related to diseases, their expression patterns, the interaction between genes, and functional analysis of the gene products can now be pursued more quickly over a much wider spectrum. The results obtained from these analyses will be reflected in gene therapy and therapeutic developments and in future preventive medicine. Since a human being or a human pathogen becomes the target, the testing of such medicines requires the involvement of a human subject or an in vivo experimental model that is highly similar to a human. Therefore, the clearer the gene and product functions become in humans, the more the interspecies gap will become an issue, and one of the means of overcoming the interspecies gap is to use laboratory animals in which human cells and tissues are engrafted and function as in humans.

Over a long period of time, attempts have been made to develop laboratory animals with engrafted human cells, tissues, and organs. With each of these attempts, there has been a history of improvement and development of better immunodeficient animals. Historically, it started with the discovery by Isaacson et al. [39] of nude (formally *Foxn1*^{nu}) mice that have no thymus and Beige (*Lyst*^{bg}) mice [49], XID (*Btk*^{xid}) mice [5], and their combinations [46, 74]. Because of the lack of a thymus in these nude mice, T cells do not develop and thus it is possible to implant human tumors. Therefore these mice have been widely used in research on anticancer drugs against human tumors [28]. However, the engraftment of normal human cells and tissues in nude mice was not as successful as hoped for. The breakthrough in overcoming the limitations of these mice came from Bosma et al. in 1983 [8]

with their discovery of CB-17-*scid* (formally *Prkdc* ^{scid}) mice. These mice exhibit severe immunodeficiencies since they have neither functional T nor B cells. Since a similar mutation causes pediatricsevere combined immunodeficiency (SCID) syndrome, the mice were named after the *scid* mutation. Subsequently, the immunodeficiency was found to be generated because of mutation of the DNA-dependent protein kinase (*Prkdc*) genes that are involved in the double-chain DNA restoration and in the V(D)J rearrangements of TCR and immunoglobulin [52, 67]. In this mouse, normal human hematopoietic cells could be developed, which is difficult with nude mice [64, 72]. Thus, in order to improve the engraftment level of human hematopoietic cells in these mice, SCID congenic mice based on the genetic background of other inbred mice combined with spontaneous mutation or transgenic mice were developed, allowing for better engraftment of human cells and tissues in this mouse line [6, 14, 36, 68]. Through these attempts, it was found that engraftment levels for human cells were very high in the NOD-*scid* mice, which were established by introducing the *scid* gene into the nonobese diabetes model of NOD inbred mice [53, 57, 82, 104]; It was then reported that the high engraftment level observed in these mice was due to the multiple immunological disorders of the background NOD mice strain, that is, it was due to the reduction of hemolytic complement activity, macrophage function, and NK activity [93]. These mice have been used for about 10 years. Beginning in the first half of the 1990s these mice became the "gold standard" for xenotransplantation involving human cells, especially for research into the differentiation of the human hematopoietic cells from stem cells, which resulted in extensive progress being made [31, 63]. Further attempts have also been undertaken to develop mice that show higher engraftment levels than the NOD-*scid* mice developed through combinations with other immunodeficient mice. Further investigations determined that the engraftment level of human cells in the recently developed NOD-*scid IL2rγ*^{null} mice and *Rag2*^{null} *IL2rγ*^{null} mice when combined with *IL2rγ*^{null} mice was extremely high, and that greater differentiation of various hematopoietic cells occurred after human stem cell transplantation compared with the conventional NOD-*scid* mice [41, 92, 103]. Various human cells differentiated from hematopoietic stem cells (HSCs) in these mice will result in successful reconstitution of the human immune system. Therefore, these mice are considered to be closer to true "humanized mice" at the present time.

2 Development of Complex Immunodeficient Mice in Japan

*2.1 NOD/Shi-*scid IL2rγ^{null} *Mice*

A schematic diagram of the development of NOD/Shi-*scid IL2rγ*^{null} (NOG) mice in Japan is shown in Fig. 1. The development of various immunodeficient mice worldwide was reviewed by Shultz et al. [91] with the formal naming of them according

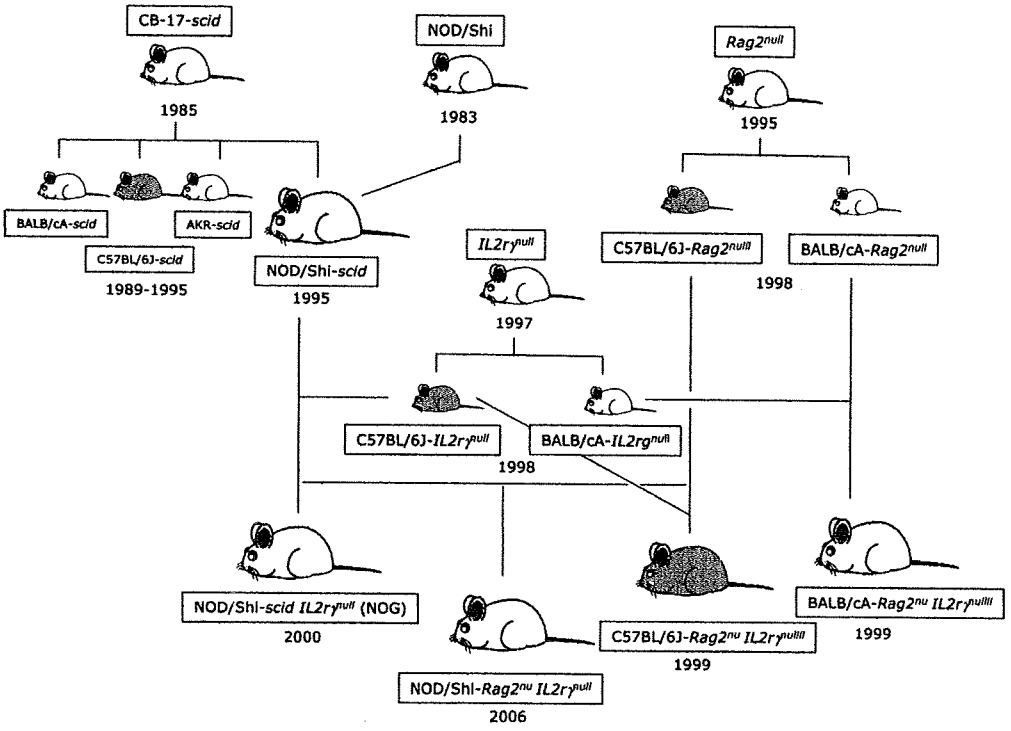

Fig. 1 Schematic diagram of the development of various immunodeficient mice in Japan. Strain nomenclature: CB-17-*scid*, C.Bka*Igh*^{b}-*Prkdc* ^{scid}/IcrJic; NOD-*scid*, NOD.CB17-*Prkdc* ^{scid}/ShiJic; BALB/cA-*Rag2*^{null}, C.129S1-*Rag2*^{tm1Fwa}/AJic; C57/B6J-*Rag2*^{null}, B6.129S1-*Rag2*^{tm1Fwa}/JJic; BALB/cA-*IL2rγ*^{null}, C.129S1-*Il2rg*^{tm1Sug}/ShiJic; C57/B6J-*IL2rγ*^{null}, B6.129S1-*Il2rg*^{tm1Sug}/Jic; NOG (NOD/Shi-*scid IL2rγ*^{null}), NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Sug}/ShiJic; BALB/cA-*Rag2*^{null} *IL2rγ* ^{nul}, C.Cg-*Rag2*^{tm1Fwa} *Il2rg*^{tm1Sug}/AJic; C57B6J-*Rag2*^{null} *IL2rγ*^{nul}, B6.Cg-*Rag2*^{tm1Fwa} *Il2rg*^{tm1Sug}/JJic; NOD/Shi-*Rag2*^{null} *IL2rγ*^{nul}, NOD.Cg-*Rag2*^{tm1Fwa} *Il2rg*^{tm1Sug}/ShiJic

to International Committee on Standardized Genetic Nomenclature for Mice. NOG mice were established based on NOD/Shi-*scid* mice, one of the SCID congenic strains developed by the Central Institute for Experimental Animals (CIEA) [53]. Based on the NOD/LtSz-*scid* mice, Shultz et al. at the Jackson Laboratory developed NOD/LtSz-*scid IL2rγ*^{null} mice. Our original purpose for developing this mouse was not to improve the engraftment level, but to clarify the role of the subpopulation of T and B cells in the development of nonobese diabetes, which occurred in autoimmune NOD mice. Afterwards, it became clear that this strain of mice exhibited a better engraftment level for xenotransplants, including human hematopoietic cells compared to the other SCID congenic mice, and thus they have become widely used for xenotransplantation. However, differentiation of single $CD4^+$ and $CD8^+$ T cells have rarely been observed even in these mice. In these NOD-*scid* mice, the elimination of NK activity or NK cells by anti-NK antibody, anti-asialo GM1 antibody [32, 48], and anti-IL-2Rβ antibody Tmβ2 [100] treatment has been found to augment the engraftment of human cells [115]. This finding has led to the generation of new immunodeficient mice in which NK cells or NK activities are abolished.

At the time that genetic manipulation of the NOD-*scid* mice was being attempted, it was well known that *b2m*^{null} mice [116], *Perforin*^{null} mice [44], and

Granzyme B^{null} mice [89] have no or impared NK activity and *IL2rγ*^{null} mice [11, 22, 42, 78] and *IL2rβ* transgenic (Tg) mice [98] lack NK cells. Introducing these targeted genes into the NOD-*scid* mice led to the development of an improved NOD-*scid* mouse. The Shultz group developed NOD/LtSz-*scid b2m*^{null} mice [13], the related NOD/LtSz-*Rag1*^{null} *Pfn*^{null} mice [89], and more recently NOD/LtSz-*scid IL2rγ*^{null} mice [92]. We also attempted to improve NOD/Shi-*scid* mice by backcrossing the latter two mice, since this would result in NOD-*scid* mice that lacked NK cells. Among these two mouse lines, the development of NOD/Shi-*scid* mice with the IL2rβ transgene was stopped midway because of their low production efficiency. In 2000, NOD/Shi-*scid IL2rγ*^{null} (NOG) mice with the *IL2rγ*^{null} gene were successfully established at last and were able to be maintained with good production efficiency. Subsequently, as was reported in 2002 [41], experimental studies with human hematopoietic stem cells transferred to these mice demonstrated that they were extremely efficient for humanized mice. NOG mice were established by a 10th generation backcrossing of *IL2rγ*^{null} mice to NOD/Shi-*scid* mice. There are two differences between Shultz's NOD-*scid IL2rγ*^{null} mice and our mouse line. These differences are related to the background substrain of the NOD mice and the *IL2rγ*^{null} mice used for backcrossing during development of the NOD-*scid* mice. However, the difference in multiple immunological disorders between NOD/Shi-*scid* and NOD/LtSz-*scid* mice has yet to be found [41]. The *IL2rγ*^{null} mice that we used were generated by Ohbo et al. [78]. In these mice, exon 7 was targeted and there was a truncated form of IL-2Rγ without intracellular signaling, which could be stained with an anti-IL-2Rγ antibody. The *IL2rγ*^{null} mice of Cao et al. [11] were used by Shultz et al., and in these mice, exon 1 was targeted. Therefore, the formal names for the NOD-*scid IL2rγ*^{null} mice are NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tm1Sug}/Jic and NOD.Cg-*Prkdc* ^{scid}*Il2rg*^{tm1Wjl}/SzJ, respectively. We named our mice after our "NOG mice." To date, the difference in engraftment level is not completely clear, because engraftment efficiency has never been compared between these mice, although it is considered to be basically the same.

2.2 *Other* IL2rγ^{null} *Combined Immunodeficient Mice*

In addition to the development of NOG mice, another type of immunodeficient mice replacing the *Rag2*^{null} genes for the *scid* mutation has been developed, because these inactive genes cause the same phenotypic T and B cell deficiency in the mice. In 1998, Goldman et al. were the first to report this combination mouse [29], in which the proliferation of B-lymphoblastoid cells and engraftment rate for human peripheral blood lymphocytes (PBL) were higher than those seen for NOD/LtSz-*scid* mice. The genetic backgrounds were a mixture of 129Ola, BALB/c, and C57BL/6 mice. Afterwards, Kirberg et al. reported on H2^{d}- *Rag2*^{null} *IL2rγ*^{null} mice [51], but the genetic background of the strain was outbred. We have also developed *Rag2*^{null} *IL2rγ*^{null} mice that have genetic backgrounds that consist of respective BALB/cA, C57BL/6J, or NOD/Shi inbred strains (Fig. 1). Dr. Shultz and his coworkers also described NOD/LtSz-*Rag1*^{null} *IL2rγ*^{null} in their review [91].

The NOG mice that we developed were mainly used in Japan for various research studies on xenotransplantation, including human hematopoiesis. BALB/cA and C57BL/6J-*Rag2*^{null} *IL2rγ*^{null} mice were sent to the United States and later on to Europe, with the successful development of humanized mice that used irradiated newborn BALB/c-*Rag2*^{null} *IL2rγ*^{null} mice, as reported by Traggiai et al. in 2003 [103].

3 NOG Mice

3.1 Immunodeficiencies in NOG Mice

NOG mice have multiple immunodeficiencies that are principally derived from three strains of mice. These include:

1. Reduced innate immunity derived from a NOD inbred strain, which involves a macrophage dysfunction, and a defect of complement hemolytic activity and reduced NK activity [93]. The NOD/Shi inbred strain was first discovered by Makino et al. as autoimmune non-obese-type diabetes mice [50, 58].
2. Lack of functional T and B cells that is derived from a mutation of protein kinase (*Prkdc*: protein kinase, DNA activated, catalytic polypeptide), which is the causative gene of the *scid* mutation [8, 52].
3. Lack of NK cells, dendritic cell dysfunctions, and other unknown deficiencies due to inactivation of the IL-2Rγ gene [38, 78, 79, 97].

When transfer of human umbilical cord blood CD34$^+$ cells to NOG mice was performed, growth and differentiation of various hematopoietic cells were observed. This high level of engraftment and differentiation in NOG mice cannot be traced only to elimination of the NK cells from the NOD-*scid* mice. In fact, remarkable differences (3- to 4-fold) in the engraftment rate and differentiation of human hematopoietic cells have been observed between NOG and NOD/LtSz-*scid b2m*^{null} mice that lack NK activity [41]. In addition, the engraftment rates in NOG mice appear to be relatively uniform, although in some of the NOD-*scid* mice it was noted that engraftment often failed to occur. The unknown factors that might be responsible for supporting the higher engraftment have yet to be elucidated. One of these factors may be the lack of interferon gamma (IFN-γ) production in dendritic cells of NOG mice as described below.

3.2 Gene Expression

To determine the factors responsible for higher engraftment of human cells in NOG mice, the expression of genes related to the immunological responses in spleen

cells were compared among the different NOG mice, NOD/Shi-*scid* mice treated with anti-asialo GM1 antibodies to eliminate NK cells, and NOD/Shi mice. As seen in Fig. 2, there was a remarkable reduction in the gene expression of IFN-γ, and in IP10, Mig, and RANTES that was secondarily induced by IFN-γ [108]. Proinflammatory cytokine IFN-γ is well known as an important factor that is responsible for various cell signal transductions [1, 7]. A recent report on IFN-γ inducing killer dendritic cells that found that these cells play a role in the rejection of xenotransplants [12, 99] suggests that lack of IFN- γ may be a critical factor for supporting higher engraftment of xenotransplants in NOG mice. This is consistent with our unpublished data showing that NOD-*scid*-derived CD11c^{+} spleen cell transfer into NOG mice reduced the engraftment of human PBL in NOG mice. Clarification of this issue may lead to discovery of new xenotransplant rejection mechanisms and, moreover, lead to further improvement of immunodeficient mice.

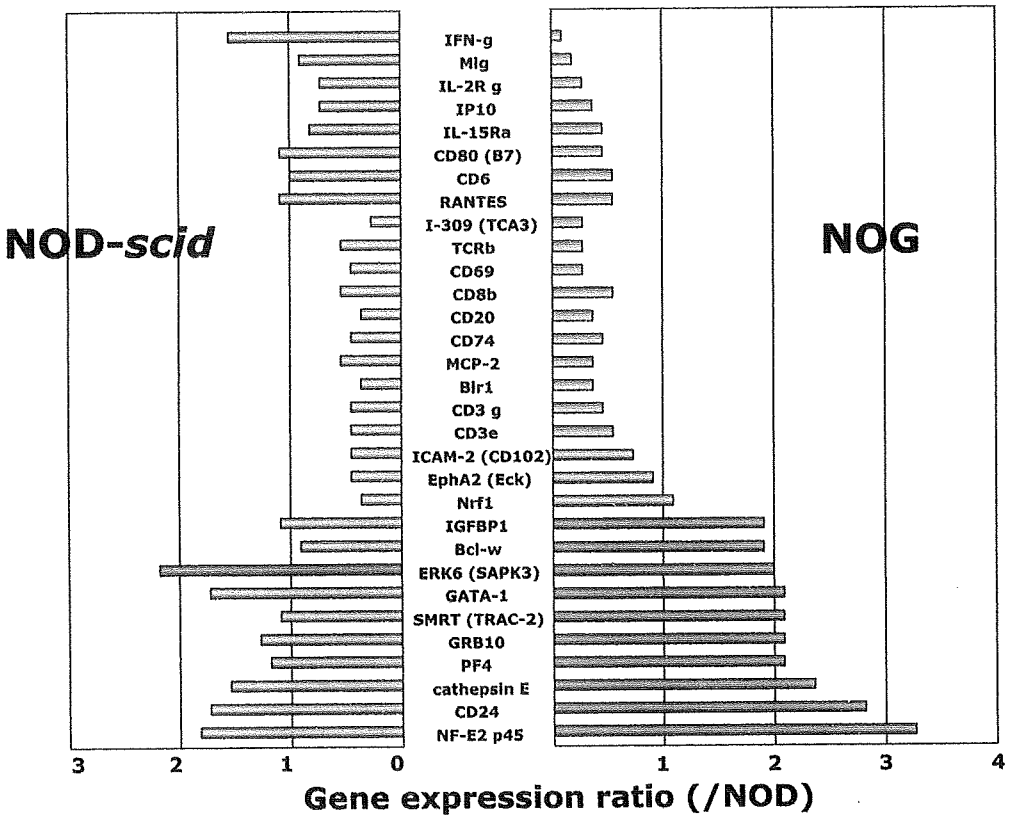

Fig. 2 Expression of the genes associated with immunological responses in NOD, NOD/Shi-*scid* and NOG mice. At 48 h after intraperitoneal infection of 1×10^{7} *Listeria monocytogenes*, spleens were removed and the RNA was extracted. Gene expression was examined by microarray (Toyobo Inc.). The *yellow areas* express the intensity of gene expression in the NOD mice. The *blue bars* express higher intensity, and the *gray bars* express lower intensity when compared with those of the NOD/Shi mice

3.3 Other Characteristics

Although NOG mice are extremely immunodeficient, when kept under strict SPF conditions, NOG mice can live for more than one and a half years, which is the same life span as that of conventional inbred mice. NOD/LtSz-*scid* *IL2rγ*^{null} mice also can survive over one and a half years. However, half of the NOD/LtSz-*scid* mice die within one year [90]. The body weight change of NOG mice was almost the same as that seen for the NOD/Shi-*scid* mice, with weights of 23.0 ± 2.0 g for males and 19.7 ± 1.3 g for females at 8 weeks of age, and 28.1 ± 1.1 g for males and 23.0 ± 1.2 g for females at 16 weeks of ages. Lymphoid organs in both the NOG and BALB/cA-*Rag2*^{null} *IL2rγ*^{null} mice were remarkably immature and exhibited atrophy. The average spleen weight (23 mg for females and 24 mg for males) for NOG mice was less than that noted for the NOD/Shi-*scid* mice (27 mg for females and 28 mg for males). The thymus of the NOG mice was also smaller than that of NOD/Shi-*scid* mice and can be detected at a rudimental level by histological observation. The lymph nodes could not be seen macroscopically.

Rag1, *Rag2*, and *Prkdc* (a causative gene of the *scid* mutation) are indispensable genes for the rearrangement of TCR and immunoglobulin, with the inactivation of these genes in mice resulting in a similar phenotype that lacks both T and B cells [25, 70, 88]. *Prkdc* genes also act as DNA repair machinery. Mice with the *scid* mutation have additional phenotypes, such as irradiation sensitivity, high occurrence of thymic lymphoma, and leakiness, which has been described as a phenomenon where immunoglobulin and aberrant T cells occur in aging CB-17-*scid* mice. Thus, there is a possibility that NOG mice will show these phenomena simply because they have the *scid* mutation gene.

Irradiation sensitivity is higher in NOG mice than in BALB/cA-*Rag2*^{null} *IL2rγ*^{null} mice. In our studies, we found that when mice at 10 weeks of age were exposed to whole body irradiation with 3 Gy, about one-third of the irradiated mice were killed by 3 weeks. However, this was not seen when the irradiation level was 2.5 Gy. In contrast, NOD/LtSz-*scid* *IL2rγ*^{null} mice survived in irradiation up to levels of 3.5 Gy [92]. This difference may be associated with the radiation source and the radiation dose per minute. On the other hand, for BALB/cA-*Rag2*^{null} *IL2rγ*^{null} mice with the *Rag2*^{null} for *scid* mutation, the quantity of irradiation used can be higher than that used in NOG mice because of their X-ray resistance [27, 103]. Whole body irradiation in newborn NOD-*scid* *IL2rγ*^{null} and BALB/cA-*Rag2*^{null} *IL2rγ*^{null} mice was performed with 1 Gy and 3.5 Gy, respectively.

It is well known that thymic lymphoma occurs frequently in CB-17-*scid* mice [15]. From our unpublished data, we found that thymoma frequently occurred 30-50 weeks after birth for CB-17-*scid* mice, and thymomas were observed in 69 (about 27%) of 255 mice during the 2 years of observation. A much higher incidence of thymoma has been reported in NOD/LtSz-*scid* mice [83]. In NOD/Shi-*scid* mice, 18.4% had a thymus tumor at 10 months of age, which is remarkably high when compared with the 9% incidence seen in the CB-17-*scid* mice at 10 months of age (unpublished data). On the other hand,

thymomas are rare in NOG mice. To date, thymomas have been observed in only 4 of more than 5,000 mice, including mice with transfer of various human cells, including human umbilical cord blood CD34$^+$ cells. The disadvantage of NOD-*scid IL2rγ*^{null} mice has generally been considered to be the high incidence of thymoma as compared to BALB/cA-*Rag2*^{null} *IL2rγ*^{null} mice [4, 103]. However, this could be a misunderstanding, because the reasons why thymomas are so frequently observed in NOD-*scid* mice and why they do not occur in NOG mice are really not all that clear. The reason might be a lack of factors such as IL-2, IL-4, and IL-7, which are responsible for the growth of T cells by inactivation of the IL-2R γ gene [97].

Another feature of mice with the *scid* gene involves the incidence of T and B cells that are associated with aging, which is referred to as "leakiness" [9]. For CB-17-*scid* mice, more than 90% of the mice begin to produce more than 1 μg/ml of immunoglobulin in the serum by one and a half years after birth. However, when compared with the leakiness observed in CB-17-*scid* mice, little is found in NOD/Shi-*scid* mice, with no leakiness at all recognized in NOG mice. The reason for this remains to be elucidated.

4 Humanized Mice Based on NOG Mice

4.1 Hematopoietic Cells

Many studies have been performed using NOD-*scid* mice to examine differentiation of hematopoietic stem cells (HSCs) [20, 21]. The most attractive characteristic of NOD-*scid IL2rγ*^{null} mice in this research field is that various human hematopoietic cells develop with high engraftment and have been shown to be able to survive for a long time with the transfer of human umbilical cord blood CD34$^+$ stem cells. Thus, the human immune system can be reconstructed in these mice. In Table 1, the results obtained to date using NOD -*scid IL2rγ*^{null} and *Rag2*^{null} *Il2rγ*^{null} mice are summarized.

The degree of differentiation and growth of human hematopoietic cells in these mice via transfer of HSCs varies depending upon the donor cells, cell number, mouse age at transfer, and days after transfer (Table 2). Constant detection of differentiated human cells in various organs was possible when at least 5×10^4 CB CD34$^+$ cells were transferred. Human cells could be engrafted in 2 of 6 mice in which only 100 CD34$^+$ cells were transferred [41]. As a source of inoculated stem cells, CD34$^+$ cells derived from cord blood seem to be more efficient for engraftment, although all CD34$^+$ cells from cord blood, bone marrow, and peripheral blood can successfully grow and differentiate in NOG mice [61]. Three serial passages of engrafted CD34$^+$ cells in NOG mice to a different NOG mouse in bone marrow of these mice was found to be possible [Dr. Kiyoshi Ando of Tokai University, personal communication].

Table 1 Results of human stem cell transfer experiments using NOD–*scid IL2rγ*^{null} and *Rag2*^{null} *IL2rγ*^{null} mice

	Mouse strain	Adult or newborn	Inoculation route	Cell source	Results	References
1	NOG	Adult/240 cGy	Tail vein	CB CD34$^+$ 1×10^5, 4×10^4	First report on NOG mice, 15.9%* in PB** (2 months)	Ito et al. (2002)
2	NOG	Adult/250 cGy/ 7–9 weeks old	Tail vein	CB CD34$^+$ 8–20×10^4	54.6% in PB, 72.9% in BM (over 19 weeks)/6.8% in PB, 41.9% in BM of NOD–*scid*	Yahata et al. (2002)
4	NOG	Adult/240 cGy/ 8–10 weeks old	Tail vein	CB CD34$^+$ 1×10^4, 5×10^4	72.6% in SP (4 months)/functional T, B, and NK cells/human cell reconstitution in organs	Hiramatsu et al. (2003)
5	NOG	Adult/250 cGy/ 9 weeks old	Tail vein	hCB CD34$^+$ 1.8–5.6×10^5/hBM CD34$^+$ 1.9–14×10^5/hMPB CD34$^+$/ 5–18×10^5	CB: 47.5% in PB, 66.7% in BM, 63.7% in SP/BM: 8.1% in PB, 22.5% in BM, 14.0% in SP/MPB: 10.7% in PB, 45.4% in BM, 13.8% in SP (14 weeks)	Matsumura et al. (2003)
6	NOG	Adult/300 cGy/6–10 weeks old	Tail vein	CB CD34$^+$ 1×10^4–1.2×10^5	18.8% in PB, 43.9% in SP (2 months)/26.1% in PB, 52.3% in SP/67.7% in PB, 81.9% in SP/lymphoid–like structure in spleen, R–5, X–4 HIV–1 infection, production of anti–HIV–1 antibodies	Watanabe et al. (2007)
7	NOD/LtSz–*scid IL2rγ*^{null}	Newborn/100 cGy	Facial vein	CB CD34$^+$ 1×10^5, CB CD34+, CD38–2 x 10^4	68.9% in PB, 72.9% in BM, 54.5% in SP (3 months)/OVA–specific IgM and IgA$^+$/ erythrocytes$^+$, DCs$^+$, other progenitors$^+$	Ishikawa et al. (2005)
8	NOD/LtSz–*scid IL2rγ*^{null}	Adult/ 325 cGy	Tail vein	CB CD34$^+$ 7×10^5	34.9% in BM, 59.9% in SP, 36.6% in THY, 6.3% in PB (10 weeks)/78.3% in THY, 20.7% in PB (+FC–IL–7)	Shultz et al. (2005)
9	H2D–*Rag2*^{null} *IL2rγ*^{null}	Adult/350 cGy	Tail vein	CB CD34$^+$ 5×10^5	33% in BM, 4% in PB (7 weeks)	Rozenmuller et al. (2004)

10	BALB/cA–*Rag2*^{null} *IL2rγ*^{null}	Newborn/2×200 cGy (3– to 4–h interval) 375 cGy/min	Intrahepatically	CB CD34$^+$ 3.8-12×10^4	5% to ~70% in BM, 0 to –83% in BM/positive EB infection, anti-TT IgG formation, white pulplike structure in spleen	Traggiai et al. (2004)
11	BALB/cA–*Rag2*^{null} *IL2rγ*^{null}	Newborn/350 cGy	Intrahepatically	CB CD34$^+$ 1×10^6	5%–89% in SP (12 weeks)/HIV–1 infection	Berges et al. (2006)

* % of human CD45$^+$ cells

** PB: peripheral blood, BM: bone marrow, SP: spleen, THY: thymus

Table 2 Cell differentiation after human HSC transfer in NOG mice

Cell	Development*
Lymphocytes	++
T cells	+++
B cells	++
NK cells	+
Macrophages	+
Dendritic cells	+
Mast cells	+
Monocytes	+
Neutrophils	– to +
Platelets	+
Erythrocytes	– to +

*12–16 weeks after human HSC transfer

After human stem cell transfer to NOG mice, the human (h) CD19$^+$ B cell differentiates first. Most hCD45$^+$ cells in the peripheral blood are B cells by about 6-10 weeks after cell transfer. T cells will differentiate later, around 8-16 weeks. hCD4 single positive and hCD8 single positive T cells, which are rarely observed in NOD-*scid* mice, can be detected in the peripheral blood and spleen. At the present time, T cells are considered to develop in the mouse thymus. In the thymus, hCD4hCD8 double-positive cells reached 60% to 75%. In contrast, hCD4$^+$ T cells ranged from 40% to 70% and hCD8$^+$ cells ranged from 30% to 50% in hCD45$^+$ cells of the spleen and but a few hCD4hCD8 double-positive cells were found [37, 61, 112]. These observations are consistent with known T cell development in human organs. These T cells show diversity of the TCR, which is activated by stimulation with anti-human CD3 antibodies, indicating that T cells that develop in NOG mice are functionally active. B cells express IgM and IgD on the surface and can produce antigen-specific IgM antibodies, but not a lot of antigen-specific IgG is produced even with multiple antigen challenges. Matsumura et al. [61] reported that CD5$^+$ B1 type B cells are well differentiated, but CD5$^-$ B2 type B cells were not, suggesting that CD5$^-$ B2 type B cells may be required to efficiently produce antigen-specific IgG. A lymphoid-like structure develops in the spleen and contains human macrophages and dendritic cells [106]. However, human follicular dendritic cells (FDC) have yet to be observed. Natural killer cells are also detected from 12 weeks in the peripheral blood and spleen after stem cell transfer [36]. Mast cells are recognized within the skin, lung, stomach, and intestine from 20 weeks after transfer, and connective-tissue type (tryptase$^+$/chymase$^+$) and mucosal type (tryptase$^+$/chymase$^-$) mast cells are localized and consistent with human organs [45]. While the differentiation of lymphoid lineage cells is well recognized, this is not the case for cells of the myeloid lineage. Levels of platelets that are detected in the peripheral blood at 8 weeks after CB CD34$^+$ cell transfer range from 0.2% to 3% [75]. Erythrocytes and neutrophils are

not usually recognized in the peripheral blood. Human neutrophils in HSC-engrafted NOG mice can be detected by a zymosan-induced air pouch inflammation technique [23]. Nucleated red blood cells and megakaryocytes could be detected to some degree in mouse bone marrow transfer, indicating that the factors responsible for denucleation of these cells might not all be fully present in NOG mice. However, fully matured erythrocytes could be detected in the peripheral blood when HSCs were transferred to newborn NOD/LtSz-*scid, IL2rγ*^{null} mice [40]. It is possible that when using newborn mice, the degree of engraftment and differentiation could be accelerated, although direct experiments to compare newborns and adults have yet to be performed.

To date, there is no evidence on the relative merits of NOG and BALB/cA-*Rag2*^{null} *IL2rγ*^{nul} mice for humanized mice. The reason for this is the differences in handling of these mice. In NOG mice, HSCs are usually transferred into irradiated adult mice via the tail vein. In contrast, in irradiated newborns they are transferred intrahepatically in BALB/cA-*Rag2*^{null} *IL2rγ*^{nul} mice. Recent results using irradiated newborn NOD/LtSz-*scid IL2Rγ*^{null} mice by Ishikawa et al. [40] suggest that higher engraftment and differentiation of HSCs can be obtained by using newborns rather than adults. Preliminary intravenous transfer experiments using CB CD34$^+$ cells in irradiated adult mice in order to compare the engraftment rate and differentiation among NOG, BALB/cA-*Rag2*^{null} *IL2rγ*^{nul}, C57BL/6J-*Rag2*^{null} *IL2rγ*^{nul}, and NOD/Shi-*scid* mice have provided interesting results. When the engraftment rates were compared among these mice, they were found to be the highest in NOG mice, moderate in the NOD/Shi-*scid* and BALB/cA-*Rag2*^{null} *IL2rγ*^{nul} mice, and extremely low in the C57BL/6J-*Rag2*^{null} *IL2rγ*^{nul} mice. Interestingly, hCD3$^+$, hCD4$^+$, and hCD8$^+$ T cells were highly developed in the NOG and BALB/cA-*Rag2*^{null} *IL2rγ*^{nul} mice, but not in NOD/Shi-*scid* and C57BL/6J-*Rag2*^{null} *IL2rγ*^{nul} mice. Further experiments are needed to clearly determine which mice are more appropriate and how transfers should be undertaken [Dr. Kiyoshi Ando of Tokai University, personal communication].

It is known that the genetic background of inbred strains of mice can influence the engraftment of tumor cells [56]. The difference in engraftment between *Rag2*^{null} mice and SCID mice has also been reported [94]. These findings suggest that the combination of inactive genes and the genetic backgrounds of mouse strains are very important when selecting more appropriate mice for attempts to improve immunodeficient mice for a humanized mouse model. Selection of the transfer route for stem cells into immunodeficient mice may also be important from the viewpoint of homing of the transferred cells [81].

4.2 Cancer

In the field of cancer research, since the discovery of nude mice there has been a long history of engraftment of a variety of tumor cells, including primary cells and tumor cell lines [28]. A liver metastasis model [96], a multiple myeloma model [19,

69], an acute myeloid leukemia model [77], and a Hodgkin lymphoma model [18] using NOG mice have all been reported. In the liver metastasis model, human pancreatic cancer cells, which were delivered by intrasplenic injection, highly metastasized in the liver in NOG mice but not in NOD/Shi-*scid* mice. In addition, the cells with metastatic capability were easily concentrated, allowing for identification of the genes associated with the metastasis. In the multiple myeloma model, intravenous transfer in NOG mice resulted in the development of multiple myeloma that resembled human myeloma. In these mice, the myeloma only grew in bone marrow, leading to paralysis in the hind legs. These observations suggest that tumor cells can grow when supported by mouse adhesion molecules, and in such cases, cell engraftment should be extremely high.

The identification of cancer stem cells is now one of the most studied areas in the field [80, 105]. Since tumor cells can easily grow in NOG mice after inoculation of just a small number of tumor cells, these mice can be very useful in this research.

4.3 Others

In the field of human infectious diseases, extensive research on the HIV-1 infection model is being undertaken. The hu-PBL-SCID mouse model, which can be infected with HIV-1 via transfer of human PBL, is well known [71, 102]. This model uses NOG mice and is also useful in evaluating anti-HIV-1 drugs, because of the high engraftment rate of transferred PBL [76]. However, this model develops a severe graft vs. host reaction that results in death by 2-3 months after cell transfer. Recently, a new mouse model with various human hematopoietic cells including $CD4^+$ T cells has been developed through the transfer of CB $CD34^+$ using NOG [106] and *Rag2*^{null} *Il2r*γ^{nu}mice [2, 4, 30]. In this model, HIV-1 infection persists for a long time without any graft vs. host reaction and with human immunity, and therefore research on vaccine development is now possible. The usefulness of NOG mice as Epstein-Barr virus (EBV) and human T-cell leukemia virus type 1 (HLTV-1) infection models has also been reported [16, 17]. So far there have been no reports concerning hepatitis C virus (HCV) infection studies using this model. Human albumin- and α-anti-trypsin-positive cells have been detected after transfer of $CD34^+$ cells in NOG mice, although these cells were also positive for both human and mouse MHC, which resulted from human $CD3^+$ cells that fused with mouse liver cells [26]. Studies on replacement of mouse liver cells with human liver cells have been performed in another mouse model [66, 101]. By combining with NOD-*scid, IL2r*γ^{null}, or *Rag2*^{null} *IL2r*γ^{null} mice, more useful models with human liver and the human immune system will become available. Other studies using NOG mice included reconstitution of human endometrium after implantation of endometrial tissue or cells resulting in induction of a menstrual cycle dependent on human sexual hormone [60, 62], and drug evaluation for human thrombopoiesis [75]. Retroviral introduction of a green fluorescent protein

gene into HSCs can be used to perform research on the distribution or homing of transferred human cells [111, 113].

5 Further Improvement of Immunodeficient Mice

There are two approaches to further improve immunodeficient mice for "humanized mice." The first requires investigation of the inoculant side and involves cell sources including cotransplantation with mesenchymal stem cells, bone marrow, fetal livers, and inoculation routes [65, 73], while the second involves improvement on the recipient mouse side. Here, we refer to the latter. The improvement of NOD-*scid IL2rγ*^{null} or BALB/cA-*Rag2*^{null} *IL2rγ*^{null} mice is a practical approach. For this to occur, there are three approaches that can be used with current transgenic technology. First, there must be introduction of human genes into the mice that code for growth or differentiation factors. Second, depletion of cells, that is, macrophages, dendritic cells, mast cells, neutrophils, etc., which are responsible for innate immunity and which still remain in NOG mice, or their differentiation factors must be performed. Third, introduction of HLA genes into the mice must be performed in order to facilitate cell-to-cell interactions. These steps have been already performed with NOD-*scid* mice.

Generation of transgenic mice that are based on NOD-*scid IL2rγ*^{null} and BALB/cA-*Rag2*^{null} *IL2rγ*^{null} mice is easy by microinjection directly into the pronuclear embryos of these mice. The introduction of targeted genes into these mice can now be rapidly completed within 1 year through the use of the speed congenic technique with multiplex PCR [59, 110] different from the conventional technique by over 7-10 backcrossings without genetic analysis to replace the background genes.

5.1 Introduction of Human Growth or Differentiation Factors

Numerous works have been published on the introduction of human genes and their products for the purpose of enhancing engraftment rate and differentiation of transferred human cells in immunodeficient mice, including CB-17-*scid*, NOD-*scid* mice [81]. By applying this technique to immunodeficient mice, further improvements may be possible. However, it is unclear which factors are appropriate for this purpose. The cross-reactivity of factors between mice and humans also may influence human cell engraftment and differentiation. Recent advances have allowed us to identify the gene responsible for self-renewal of stem cells [87, 114], and their introduction into mice may make it possible to develop new immunodeficient mice in which human stem cells can be maintained for a long time. To further differentiate and grow myeloid lineage cells, introduction of genes of GM-CSF, IL-3, erythropoietin, thrombopoietin, etc., may be efficient. The factors responsible for denucleation of erythroid progenitors or megakaryocytes may be necessary. The

genes of lymphotoxin-related molecules and chemokines that are necessary for reconstruction of human secondary lymphoid organs in these mice may also be targets for introduction [24, 95]. Genes related to inflammatory responses or human diseases, namely, IL-4, IL-5, etc. for allergic reactions, are interesting in that they may be useful in developing the disease in humanized mice [109].

5.2 *Depletion of Cells from Immunodeficient Mice*

To improve immunodeficient mice, depletion of the cells remaining in the immunodeficient mice may be an alternative, with the expectation that there might be a higher engraftment rate even when only a small number of stem cells are transferred. NOG and BALB/cA-*Rag2*^{null} *IL2rγ*^{null} mice lack T, B, and NK cells and also have a variety of immune disorders. However, these mice still have macrophages, dendritic cells, mast cells, and neutrophils, which play a role in innate immunity. To deplete such cells, the transgenic technique using genes of the herpes simplex virus thymidine kinase, diphtheria toxin A, and more recently, the diphtheria toxin receptor will be useful [3, 34, 43, 47, 85]. The partial depletion of a particular cell with the RNAi technique through the suppression of specific gene expression to a certain cell may also be effective [84, 86].

5.3 *Introduction of HLA Genes*

The introduction or replacement of HLA genes in NOG and BALB/cA-*Rag2*^{null} *IL2rγ*^{null} mice is considered to be a potent method for humanized mice. Many studies using these techniques for HLA transgenic mice have also been performed [80]. Intrathymic expression of human HLA-DR1 in NOD-*scid* mice has clearly accelerated T cell engraftment and their responses in these mice [10]. Lower production of antigen-specific IgG is considered to be one of the current issues in humanized mice based on NOG mice. One of the reasons for this may be the lack of a cell-to-cell interaction between human T cells and B cells, or macrophages, since the T cells that develop in the mice are educated in the mouse thymus. Therefore, the introduction of HLA into the mice should provide a more complete form of human immune system in the mice. However, it is also well known that HLA shows extreme diversity and that human diseases are associated with a particular HLA type [35]. Therefore, the choice of the HLA type to introduce into the mice may be not only an important but also a difficult issue to deal with. A newly developed transgenic technology using artificial chromosomes may be of help in introducing multiple HLA type genes [54]. On the other hand, there is an unsolved issue with regard to current humanized mice based on NOG mice. The observation of HLA-dependent T cell cytotoxicity in HSC-transferred NOD/LtSz-*scid*, *IL2rγ*^{null} mice [40] cannot

rule out the existence of extrathymic development of human T cells in the humanized mice. This issue is both interesting and important to clarify.

It is possible to improve immunodeficient mice through the introduction of genes with current transgenic technology, as has been described above. However, although such multiple modifications in the mice may be theoretically possible, they will lower production efficacy of the mice. It will only be after many trials are undertaken to generate various types of immunodeficient mice that a new immunodeficient mouse more appropriate for use as a humanized mouse may eventually be established.

6 Conclusion

Humanized mice having human cells, tissues, and organs, which will facilitate the study of the mechanism of pathogenesis in human diseases and help develop medicines that can be used directly to treat such diseases, are extremely important and a highly desirable goal. For humanized mice, various immunodeficient mice have been established through the introduction of targeted genes in existing immunodeficient mice. Recently developed NOD-*scid IL2rγ*^{null} and *Rag2*^{null} *IL2rγ*^{null} mice, which have a *scid* mutation gene or a *Rag2*^{null} gene and an *IL2rγ*^{null} gene, have garnered wide attention because of high engraftment and differentiation rates of the human hematopoietic cells from stem cells that occur in these mice. Therefore, these mice are now considered to be the best for use as a humanized mouse. However, because of the limitations, these mice have not yet reached the optimal level, and the process should be accelerated so that the ultimate immunodeficient mouse can be developed.

Acknowledgements We thank Dr. Masataka Nakamura, Tokyo Medical and Dental University, and Dr. Yoshito Ueyama, Tokai University School of Medicine, for cooperation in generation of NOG and *Rag2*^{null} *IL2rγ*^{null} mice. This work was supported by the Ministry of Education, Culture, Sports, Science and Technology, Ministry of Health, Labor and Welfare, Japan Science and Technology Agency (JST), and Kanagawa Academy of Science and Technology (KAST).

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