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Humanized mice: novel model for studying mechanisms of human immune-based therapies

Louis Gonzalez, Natasa Strbo, and Eckhard R. Podack

Department of Microbiology and Immunology, Miller School of Medicine, University of Miami, Miami, FL, USA

Eckhard R. Podack: EPodack@med.miami.edu

Abstract

The lack of relevant animal models is the major bottleneck for understanding human immunology and immunopathology. In the last few years, a novel model of humanized mouse has been successfully employed to investigate some of the most critical questions in human immunology. We have set up and tested in our laboratory the latest technology for generating mice with a human immune system by reconstituting newborn immunodeficient NOD/SCID- $\gamma_c^{-/-}$ mice with human fetal liver-derived hematopoietic stem cells. These humanized mice have been deemed most competent as human models in a thorough comparative study with other humanized mouse technologies. Lymphocytes in these mice are of human origin while other hematopoietic cells are chimeric, partly of mouse and partly of human origin. We demonstrate that human CD8 T lymphocytes in humanized mice are fully responsive to our novel cell-based secreted heat shock protein gp96^{HIV}-Ig vaccine. We also show that the gp96^{HIV}-Ig vaccine induces powerful mucosal immune responses in the rectum and the vagina, which are thought to be required for protection from HIV infection. We posit the hypothesis that vaccine approaches tested in humanized mouse models can generate data rapidly, economically and with great flexibility (genetic manipulations are possible), to be subsequently tested in larger nonhuman primate models and humans.

Keywords

Humanized immunity; NOD/SCID- $\gamma_c^{-/-}$ mice; Vaccine; gp96 chaperone

Introduction

The development of novel techniques and systems to study biological processes in humans, both in an in vitro and in vivo setting, is always in high demand. Small animal models are the most efficient method of studying human afflictions; however, many aspects of mammalian biological systems, including immune systems, are species specific. Moreover, rodents are refractory to certain human-specific infectious agents, for example they are unable to support productive HIV infection, even when made to express human co-receptors for the virus [1–3]. Over the past two decades, the construction of humanized animal models through the transplantation and engraftment of human tissues or progenitor cells into immunocompromized mouse strains has allowed for the development of a reconstituted

human tissue scaffold in a small animal system [4]. In recent years, the technology of constructing chimeric mice with a humanized immune system has markedly improved [5]. Early versions of humanized mice, CB-17 SCID mice which lack both T and B lymphocytes, supported productive HIV infection and allowed investigators to begin to address important questions in HIV biology in vivo [6–10]. The implantation of uneducated human immune cells and associated tissues provided the basis for the SCID-hu Thy/Liv and hu-PBL-SCID models [11, 12]. Engraftment efficiency of these tissues was further improved through the integration of the nonobese diabetic (NOD) mutation leading to the creation of NOD/SCID, NOD/SCID- $\gamma_c^{-/-}$ and NOD/Rag2 $^{-/-}$ - $\gamma_c^{-/-}$ models, which further minimized the response of the murine innate immune system [13–15]. The IL-2R- α common gamma chain is required for high affinity ligand binding and signaling through multiple cytokine receptors, including those for IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 [16]. Immunodeficient mice bearing a targeted mutation within the *IL2R* gene support higher levels of human hematolymphoid engraftment than all previous immunodeficient mouse strains and permit the engraftment of a functional human immune system [15, 17–24]. These models marked an important advancement: the use of human CD34 $^{+}$ hematopoietic stem cells (HSC). Human cord blood or fetal liver CD34 $^{+}$ HSC had been used to reconstitute Rag2 $^{-/-}$ - $\gamma_c^{-/-}$ and NOD/SCID- $\gamma_c^{-/-}$ mice, resulting in higher levels of sustained human immune cell engraftment [18, 21, 25, 26]. In summary, today there are 3 main immunodeficient mouse strains that are used for creating humanized mice: NOD.Cg-Prkdc^{scid}IL-2R^{tm1Wjl} (NSG mice) [20], NODShi.Cg-Prkdc^{scid}IL-2R^{tm1Sug} (NOG mice) [18] and strains based on C;129S4-Rag2^{tm1Flv}IL-2R^{tm1Flv} as well as BALB/c-Rag1 $^{-/-}$ or BALB/c-Rag2 $^{-/-}$ strains of mice (BRG mice) [21, 25] (Fig. 1). Recent evidence shows that these mouse models differ in their ability to support the engraftment of a functional human immune system. Overall, NSG and NRG mice support higher levels of human HSC engraftment and T- and B-cell development than do BRG mice [25]. There are four different technological approaches for the engraftment of a functional human immune system in the above-mentioned immunodeficient mouse strains, each with distinct advantages and caveats, discussed elsewhere [5, 27–30] and Fig. 1.

We have set up and tested in our laboratory the latest technology for generating mice with a human immune system by reconstituting newborn immunodeficient NOD/SCID- $\gamma_c^{-/-}$ mice (NSG) with human fetal liver-derived hematopoietic stem cells. To overcome the problems associated with low HSC engraftment, we developed an effective conditioning regimen in recipient mice that includes a combination of total body irradiation (1 Gy) with an antibody regiment capable of selective depletion of endogenous mouse HSC (anti-mouse c-kit antibody). In this model, we have achieved robust repopulation of mouse lymphoid tissues with human immune cells and have generated robust cellular immune responses systemically and in mucosal compartments. Furthermore, recently we modified the model by implanting human fetal thymic tissue subcutaneously accompanied by intrahepatic injection of human autologous fetal liver HSC. We believe this improved humanized mouse model will allow us to study questions surrounding innovative vaccines and their protective effects not readily approachable through human studies.

Generation of humanized mice

Breeding and purification of human CD34 $^{+}$ HSC

The construction of humanized mice, as shown in Figs. 1 and 2, is a very complex process and requires a breeding colony of NOD/SCID- $\gamma_c^{-/-}$ mice, a source of fetal tissue, purification of fetal CD34 $^{+}$ HSC, an irradiator and the surgical expertise necessary for proper implantation of human fetal thymus. We have established our own protocol for the

generation of humanized mice (Fig. 1). In brief, mice are bred in two parallel cycles that are offset by 7 days. During the breeding cycle, NOD/SCID- $\gamma_c^{-/-}$ mice are placed in timed matings on day 0 and separated on day 4; pregnant mice are segregated to single cages on day 18 and monitored through day 23 for litters.

Human fetal liver and thymus from elective terminations, 12–23 weeks of gestational age (Advanced Bioscience Resources, Alameda, CA), are acquired on a fee for service basis, and the tissue is delivered approximately every 14 days. Upon receipt, liver tissue is digested with collagenase to make single cell suspensions. After density gradient centrifugation, CD34⁺ cells are enriched using immunomagnetic beads according to the manufacturer's instructions (CD34⁺ selection kit; Stem Cell Technologies, Vancouver, Canada). Purity of isolated CD34⁺ HSC is evaluated by flow cytometry at >90 %. Purified cells are aliquoted and frozen for later HLA typing and transplantation into neonatal NOD/SCID- $\gamma_c^{-/-}$ mice. HSC designated for transplant are thawed on day 21 of the breeding cycle and placed into culture containing 10 % human serum, IL-3, IL-6 and SCF (10 ng/ml) for 3 days. On the day of transplant, cells are harvested from culture and washed in HBSS to remove traces of serum and exogenous cytokine.

Isolated donor CD34⁺ HSC are assessed for purity as well as for their potential to proliferate and differentiate into progenitor cells using CFU and LTC-IC assays in addition to being HLA typed. Briefly, cells are plated in methylcellulose media supplemented with SCF, GM-CSF, IL-3 and EPO, at various cell concentrations, in triplicate. After 14 days, colonies are identified and analyzed. Because no established references could be found for progenitor counts from fetal liver cells, we compared our colony counts to known counts for CD34⁺ bone marrow. None of our tissues gave rise to colonies in numbers significantly different than those for bone marrow.

Transplantation of human CD34⁺ HSC and characterization of human immune reconstitution

Newborn 1-day-old NOD/SCID- $\gamma_c^{-/-}$ mice are irradiated with a single sublethal dose of 1 Gy whole body irradiation and then housed with dams for 24 h (Fig. 2a). Transplantation in mice older than 4 days is difficult because the liver is more difficult to visualize through the skin as the mouse ages. Importantly, it has been demonstrated that newborn mice (less than 3 days) support higher transplant efficiency [17, 21, 25, 31, 32]. Allowing the mice to recover from irradiation for 1 day, they are injected with $1-2 \times 10^6$ pre-cultured HSC, intrahepatically in a volume of 30 μ l. The pups are immediately returned to their dams and monitored for the next few hours. At day 7, mice are injected i.p. with 100 μ g of anti-c-kit antibody (ACK2) (a generous gift from Dr. I. Weissman). It has been shown that administration of ACK2 leads to the transient removal of more than 98 % of endogenous HSCs in immunodeficient mice [33]. We reasoned that human HSC engraftment might be limited by the occupancy of appropriate niches by endogenous, mouse HSC and that the specific removal of host HSCs could significantly improve engraftment. The pups are allowed to nurse until weaning at 28 days. At week 5, mice are again injected i.p. with 100 μ g of ACK2. Weaned mice are further allowed 2–3 weeks of maturation before being screened for initial levels of human/mouse chimerism. Mice 8–12 weeks old are bled, and heparinized peripheral blood is treated with red cell lysis buffer and analyzed for relative percentages of murine and human CD45⁺ cells by flow cytometry. Once human CD45⁺ cells are detected, analysis is extended to include human CD3, CD4, CD8, CD56, CD11c, CD19, HLA-DR and CD123 on all blood collections. A human CD45⁺ level of >2 % was considered positive. Reconstituted mice became candidates for functional analyses of the immune system when at least several members of the cohort showed measurable levels of engrafted cells in circulation (> 30 %). Mice that received antibody as a pup and adult had

significantly higher engraftment than the mice that received the ACK2 treatment when adults (week 5) (Fig. 2b). All mice survived the ACK2 treatment with no signs of distress. Consistent with prior reports [4, 10, 11, 19, 34], NOD/SCID- $\gamma_c^{-/-}$ mice generated in our hands demonstrated a high proportion of human CD45⁺ cells among the leukocyte population in the peripheral blood (85 % human to 15 % murine, range 98/15–55/35) that continued to increase overtime; remaining stable for at least 1 year (Fig. 3a, b).

NOD/SCID- $\gamma_c^{-/-}$ mice, which lack NK cell activity, have been reported to support improved human immune engraftment compared to that of NOD/SCID mice [17, 20]. Interestingly, human HSC engraftment was more efficient in female NSG recipient mice than in male (Fig. 3b). Similar observations have been reported by other investigators [34, 35]. Furthermore, we found that NOD/SCID- $\gamma_c^{-/-}$ hu-mice demonstrate a high level engraftment of human CD45⁺ cells not only in their peripheral blood but also in the peripheral tissues: spleen, mesenteric lymph nodes, gut and the reproductive tract mucosal tissue (lamina propria and intraepithelial compartment) (Fig. 3c, d).

Recently, we have established the novel protocol of combined intrahepatic injection of human fetal liver HSC at day 2 and subcutaneous implantation of autologous human thymic tissue at day 4 (Figs. 1, 2). We have observed that subcutaneous co-transplant of human fetal thymus with autologous HSC increases human immune reconstitution, particularly T regulatory cells. The implantation of human fetal thymic and liver tissues accompanied by i.v. injections of human fetal HSC offers a number of reported advantages, including the robust levels of human chimerism, development of functional T cells and education of T cell progenitors on autologous human thymic epithelium [36, 37].

T, B, NK cell and DC reconstitution

We next identified human T cells, B cells, NK cells, dendritic cells (DC) and T regulatory cells (Treg) in NOD/SCID- $\gamma_c^{-/-}$ humanized mice, using polychromatic flow cytometry with the gating scheme shown in Fig. 4, depicting a representative sample of spleen leukocytes from a 12-week-old humanized mouse. As shown in Fig. 4, the majority of human CD45⁺ lymphocytes in the spleen were T cells (~50 %), followed by B cells (30 %), NK cells (1.2 %) and DC (1 %). Most of the CD3⁺ cells were CD4⁺ T cells. Intracellular staining for Foxp3 revealed the presence of T_{reg} cells. As demonstrated in Fig. 4, both CD11c⁺ myeloid DCs (mDC) and CD123⁺ plasmacytoid DCs (pDC) could be identified among human CD45⁺, lineage-negative (CD16⁻, CD3⁻, CD19⁻), HLA-DR⁺ cells. 1 % of human CD45⁺ cells in the blood were mDC, whereas pDC represented 0.2 % of human CD45⁺ cells. Similarly, greater numbers of mDCs than pDCs have been noted in human peripheral blood [38, 39].

Specific T cell immune response

Human CTL in humanized mice are cross-primed, expand and migrate to mucosal sites in response to allogeneic 293-gp96^{HIV-gag}-Ig vaccination. Over the last two decades, Dr. Podack and his group have developed an innovative vaccine approach based on a genetically modified form of the endoplasmic reticulum (ER) chaperone gp96, the fusion protein gp96-Ig. The gp96-Ig vaccine is composed of live, but replication incompetent cells, containing the antigens of interest and secreting gp96-Ig, which has been introduced into the cell by transfection and G418-selection [40–48]. Secreted gp96-Ig chaperones peptides (gp96^{pep}-Ig) come into ER via the TAP transporter [49]. Gp96 also chaperones proteins synthesized in the ER of the vaccine cells [50], including peptides and proteins derived from infectious agents or tumor antigens. In murine studies, nonhuman primates and in cancer patients, we have shown that gp96-Ig-based vaccines generate CD8 CTL responses to gp96-Ig-chaperoned, antigenic peptides derived from SIV or HIV and to other test antigens. Immune

responses ensue systemically and are especially powerful in the intestinal, rectal and vaginal mucosa [40, 47, 51].

To determine HIV-gp96-Ig cross-priming with human immune cells, we used humanized mice generated from NSG mice transplanted with HLA A2⁺ human fetal liver CD34⁺ cells, with ~80 % chimerism for the human CD45 molecule (Fig. 5a). As vaccine cells, we used allogeneic 293-gp96^{HIV-gag}-Ig cells secreting 1 µg gp96^{HIVgag}-Ig in 24 h. The cells were injected i.p. on day 0 and 14 into 12-week-old mice. The HIV structural protein Gag contains the peptide SLYNVATL, which is presented by HLA A2. Gag-specific CD8 CTL in HLA A2⁺ humanized mice can be detected with HLA A2-gag-tetramers or pentamers. As negative control for specificity, HLA A2-CMV pentamers were used. The mice were analyzed 5 days after the second vaccination to determine the frequency of gag-specific CD8 CTL at various sites. Human gag-specific CD8 cells expanded from undetectable to 14 % of all CD8 cells after two immunizations (Fig. 5b) in the peritoneal cavity; the assay is specific because HLA A2-pentamer carrying CMV peptides did not detect any CD8 T cells. Importantly, high frequencies of gag-specific CD8 CTL were also found after vaccination in the intestinal/vaginal lamina propria (LPL) and among intestinal/vaginal intraepithelial lymphocytes (IEL), in mesenteric lymph nodes (MLN), spleen (SP) and peripheral blood (PBL) (Fig. 5c). These data show for the first time that human CD8 CTL in humanized mice are generated by cross-presentation induced by gp96^{HIVgag}-Ig, which depends on HLA A2⁺ APC (DC)—CD8 interaction and priming and NK help. The data also show a normal trafficking pattern of human CD8 CTL to the mucosa. Therefore, human cells in humanized mice are able to receive the proper signals and programming instructions for migration within the murine environment and exhibit proper homing behavior to mucosal sites including epithelia (IEL).

In summary

1. Humanized mice have the advantage of providing a small animal model with these associated benefits: they are economical, offer greater flexibility, are susceptible to genetic manipulations and experimental protocols not ethical in humans. Using defined HLA types for reconstitution of immunodeficient mice, epitope-specific responses can be measured using HLA tetramers/pentamers.
2. To further overcome the limitations of humanized mice vis-à-vis the murine microenvironment, treatment for humanized mice with antimouse c-kit antibody (ACK2) enhanced the human HSC engraftment.
3. Allogeneic HIV-gag vaccine cells via gp96HIV-gag-Ig secretion in the murine environment induce gag-antigen cross-presentation by human HLA A2⁺ APC to prime the expansion of human HLA A2 restricted gag-specific CD8 CTL (Fig. 5). This evidence validates the humanized mouse model as an excellent system for testing novel vaccines.
4. The hu-mice studies described here will allow a comparison of the macaque model with the humanized mouse model in order to evaluate which model better reflects human HIV pathogenesis and vaccine responses.

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Fig. 1.

Different approaches for creating humanized mice. Different strains of immunodeficient mice :SCID (*Severe Combined Immune Deficiency*, *Prkdc^{scid}*), NSG (*NOD/scid IL-2 receptor gamma chain knock out*, *NOD.Cg-Prkds^{scid} Il2rg^{tm1Wjl}*), NOG (*NOD/scid IL-2 receptor gamma chain knock out*, *NOD/Shi.Cg-Prkds^{scid} Il2rg^{tm1Sug}*), NRG (*NOD/Rag IL-2 receptor gamma chain knock out*, *NOD.Cg-Rag1^{tm1Mom} Il2rg^{tm1Sug}*) and BRG (*BALB/c Rag IL-2 receptor gamma chain knock out*, *C.Cg-Rag1^{tm1Mom} Il2rg^{tm1Wjl}* and *C.Cg-Rag2^{tm1Fwa} Il2rg^{tm1Sug}*) were engrafted with human hematopoietic cells (HSC) and/or immune tissues to establish four different immune system engraftment models. 1. *Hu-PBL-SCID*: engraftment of human peripheral blood leukocytes (PBL) into SCID mouse. 2. *Hu-SRC-SCID*: human stem repopulating cell model, engraftment of human HSC derived from bone marrow, umbilical cord blood, fetal liver or mobilized peripheral blood into adult or neonatal NSG, NOG, NRG and BRG mice. 3. *SCID-Hu*: implantation of human fetal liver and thymus fragments under the renal capsule of SCID mice. 4. *BLT*: bone marrow, liver, thymus humanized mouse model is established by implantation of human fetal liver and thymus fragments under the renal capsule of sublethally irradiated immunodeficient mice (NSG) accompanied by intravenous injection of autologous fetal liver HSC

Fig. 2.

Generation of humanized mice and breeding schedule. **a** NOD/SCID- $\gamma_c^{-/-}$ mice are bred in two parallel cycles. Fetal liver tissue is processed into single cell suspensions that are frozen for later pre-culture with cytokines and transplant into neonates. One-day-old NOD/SCID- $\gamma_c^{-/-}$ mice are irradiated (sublethal, 1 Gy, whole body irradiation) and returned to dams for 24 h at which time they are transplanted with 10^6 – 2×10^6 pre-cultured HSC intrahepatically (i.h.). At day 2, subcutaneous implantation of autologous human thymic tissue (1–2 mm³ fragments) is performed. At week 1 and 5, mice are injected with 100 μ g anti-mouse c-kit antibody (ACK2) i.p. Ten weeks later, mice are screened for human/mouse chimerism. **b** Comparison of human engraftment levels in mice that received antimouse c-kit antibody (ACK2) as adult (5 weeks old mice) or as a pups and adult (1 and 5 weeks old) with control mice (no antibody treatment)

Fig. 3.

Human immune reconstitution in NOD/SCID- $\gamma_c^{-/-}$ mice after fetal liver CD34⁺ hematopoietic cell engraftment. **a** Chimerism (% human engraftment) is determined with anti-human and anti-mouse CD45 at weeks 8–12, 12–13, **a** and **b**. Representative flow cytometry dot plots of chimerism in several compartments: PBL, spleen, mesenteric lymph nodes (MLN), thymus, gut intraepithelial compartment (IEL) and lamina propria (LPL). **c** Immunohistochemical analysis of human CD45 expression in the spleen (SPL), mesenteric lymph nodes (MLN) and vaginal tissue

Fig. 4.

T-cell, B-cell, NK cell and DC reconstitution of NOD/SCID- $\gamma_c^{-/-}$ 12 weeks old

NOD/SCID- $\gamma_c^{-/-}$ hu-mouse was sacrificed, and human cells were identified in blood by flow cytometry following staining with anti-mouse CD45 and antihuman CD45, CD3, CD4, CD8, CD19, CD56, Lin1, HLA-DR, CD123, CD25 and Foxp3 antibody. mDC are identified as CD11c⁺ and pDC as CD123⁺ cells among human CD45⁺, lin-1 (lineage-1) negative (CD16⁻, CD3⁻, CD19⁻), HLA-DR⁺ cells. Numbers in the panels represent the percentage of cells contained in the indicated gates

Fig. 5.

Expansion of human CD8 CTL in hu-mice to 293-gp96^{HIVgag}-Ig vaccination.

NOD/SCID- $\gamma_c^-/-$ were transplanted with HLA-A2⁺ human fetal liver CD34⁺ cells. The vaccine cells, 293-gp96^{HIVgag} (secreting 1 μ g gp96 HIVgag in 24 h), were injected i.p on days 0 and 14 in 12-week-old mice and analyzed 5 days later gating on hu-CD45⁺ cells. **a** Chimerism in blood. **b** HLA A2-gag-pentamer⁺ cells in the peritoneal cavity (PEC) and within intraepithelial lymphocytes (IEL). HLA A2 CMV pentamers as specificity control. **c** Expansion and trafficking of HLA A2-gag-pentamer⁺ cells to various sites. *PEC* peritoneal exudates cells, *SP* spleen, *PBL* peripheral blood lymphocytes, *MLN* mesenteric lymph node, *LPL* lamina propria lymphocytes, *IEL* intraepithelial lymphocytes