Cell Stem Cell
Brief Report

Il2rg Gene-Targeted Severe Combined Immunodeficiency Pigs

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SUMMARY

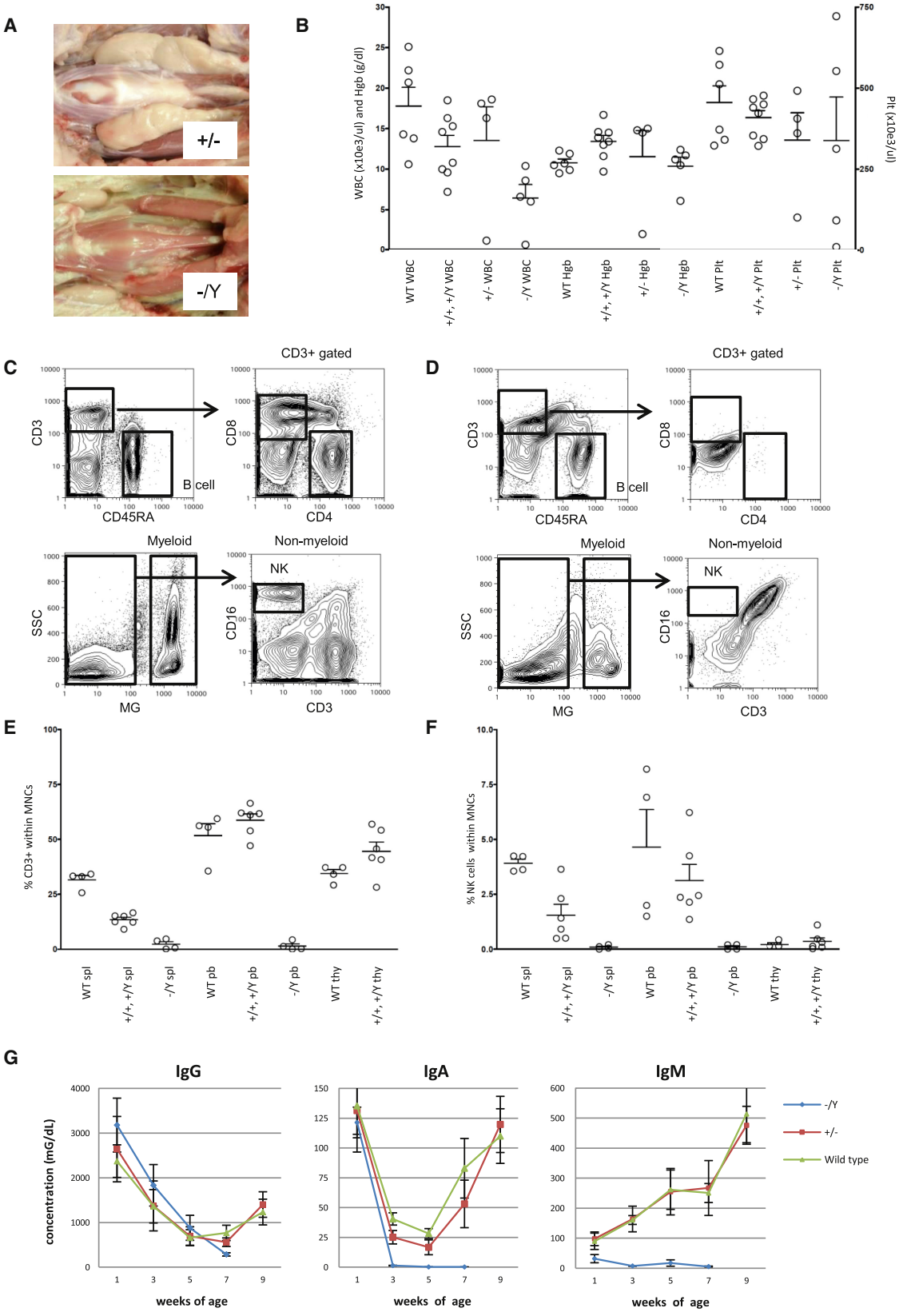
A porcine model of severe combined immunodeficiency (SCID) promises to facilitate human cancer studies, the humanization of tissue for xenotransplantation, and the evaluation of stem cells for clinical therapy, but SCID pigs have not been described. We report here the generation and preliminary evaluation of a porcine SCID model. Fibroblasts containing a targeted disruption of the X-linked interleukin-2 receptor gamma chain gene, *Il2rg*, were used as donors to generate cloned pigs by serial nuclear transfer. Germline transmission of the *Il2rg* deletion produced healthy *Il2rg*$^{+/-}$ females, while *Il2rg*$^{-/Y}$ males were athymic and exhibited markedly impaired immunoglobulin and T and NK cell production, robustly recapitulating human SCID. Following allogeneic bone marrow transplantation, donor cells stably integrated in *Il2rg*$^{-/Y}$ heterozygotes and reconstituted the *Il2rg*$^{-/Y}$ lymphoid lineage. The SCID pigs described here represent a step toward the comprehensive evaluation of preclinical cellular regenerative strategies.

The common gamma chain, IL2RG, is an IL-2 receptor subunit (Takeshita et al., 1992) shared by IL-4, IL-7, IL-9, IL-15, and IL-21 receptors (Kondo et al., 1993; Noguchi et al., 1993a; Russell et al., 1993; Giri et al., 1994; Kimura et al., 1995; Asao et al., 2001). IL2RG is present in T, NK, NKT, and dendritic cells (Ishii et al., 1994) and plays an essential role in lymphoid development by activating, through its cytoplasmic domain, Janus kinase 3 (Nakamura et al., 1994; Nelson et al., 1994, 1997).

Mammalian *IL2RG* orthologs are typically located on the X chromosome; in humans, *IL2RG* mutations result in X-linked severe combined immunodeficiency (XSCID) in which T and NK cells are absent or profoundly reduced in number, while B cells are numerically normal (or increased) but functionally impaired (Noguchi et al., 1993b; Leonard, 1996; Fischer et al., 1997). Gene-targeted mice lacking *Il2rg* also exhibit immunological defects (Cao et al., 1995; Ohbo et al., 1996) including the ablation of NK cell activity. NOD/SCID/*Il2rg*^{null}, NOG, and *Rag2*^{null}/*Il2rg*^{null} mice permit the functional reconstitution of human hematopoietic and immune systems following the injection of purified human hematopoietic stem cells (Traggiai et al., 2004; Ishikawa et al., 2005; Shultz et al., 2005). Unfortunately, phenotypic differences exist between XSCID humans and *Il2rg* null mice, including a pronounced numerical B cell reduction in the latter. Two dog breeds develop SCID caused by *Il2rg* mutations (Felsburg et al., 1999; Perryman, 2004), but dogs are poorly characterized research models.

In contrast, the pig more closely resembles humans regarding anatomy, hematology, physiology, size, and longevity. By enabling long-term follow-up, pig models will permit the evaluation of human cancer and stem cell transplantation over clinically relevant time frames. We here describe disruption of the porcine *Il2rg* gene to generate SCID pigs, their phenotypic characterization, and proof-of-principle transplantation studies.

A conventional positive-negative selection *Il2rg* gene targeting vector (TV) (Figure S1A) enabled the functional inactivation of porcine *Il2rg* by removing exon 6 (Kanai et al., 1999). Following fetal fibroblast transfection, selection, and PCR screening, 1 of 3 TV-targeted cell lines was expanded for nuclear transfer (Table S1). Even after screening, PCR-positive colonies often contain a substantial proportion of nontargeted cells (not shown) that would result in a corresponding proportion of nontargeted cloned pigs following nuclear transfer. To ensure that all clones harbored a targeted *Il2rg* locus, we adopted a serial cloning strategy. Nine embryonic day 35 (E35) or E39 nuclear transfer embryos were collected and screened by PCR and Southern blotting. PCR (not shown) and Southern blotting (Figure S1B) revealed that six contained the genomic configuration predicted for a single-targeted *Il2rg* allele. Fibroblasts were cultured from one targeted embryo and used in secondary nuclear transfer to


A
+/-
-/Y
B
WBC (x10e3/ul) and Hgb (g/dl)
Plt (x10e3/ul)
WT WBC
+/+, +/Y WBC
+/- WBC
-/Y WBC
WT Hgb
+/+, +/Y Hgb
+/- Hgb
-/Y Hgb
WT Plt
+/+, +/Y Plt
+/- Plt
-/Y Plt
C
CD3+ gated
CD3
CD45RA
B cell
CD8
CD4
Myeloid
Non-myeloid
SSC
MG
NK
CD16
CD3
D
CD3+ gated
CD3
CD45RA
B cell
CD8
CD4
Myeloid
Non-myeloid
SSC
MG
NK
CD16
CD3
E
% CD3+ within MNCs
WT spl
+/+, +/Y spl
-/Y spl
WT pb
+/+, +/Y pb
-/Y pb
WT thy
+/+, +/Y thy
F
% NK cells within MNCs
WT spl
+/+, +/Y spl
-/Y spl
WT pb
+/+, +/Y pb
-/Y pb
WT thy
+/+, +/Y thy
G
IgG
IgA
IgM
concentration (mG/dL)
weeks of age
weeks of age
weeks of age
-/Y
+/-
Wild type

produce 31 cloned F_0 piglets; all were females heterozygously targeted at their *Il2rg* genes as judged by PCR (not shown) and Southern blotting (Figure S1C).

Fourteen of the 31 were stillborn and 3 of the 17 live-born died neonatally of unknown cause(s) (Table S1). Ten survivors died from pneumonia and severe arthritis (five were euthanized) between postnatal day 7 (P7) and P70. The remaining four (#5, 9, 15 and 20) survived for >1 year.

Stillborn and neonatal fatalities often had spleens with hypoplastic lymphoid aggregations (Figure S1D). Most (24/31, 77%) had undetectable or severely hypoplastic thymi (Figure S1E and Table S2). F_0 clones that died within 10 weeks also lacked detectable thymi and had few, if any, T cells either in their spleens or circulating (Figures S1F and S1G). In contrast, levels of $CD4^+$ and $CD8^+$ T cells in four long-lived F_0 lines were comparable to those of WT controls. Analysis of peripheral blood (PB) mononuclear cell (PBMC) RNA corroborated this: *Il2rg*, *CD4*, and *CD8* transcript levels were reduced in athymic $Il2rg^{+/-}$ clones that perished relative to respective levels in long-lived clones and WT controls (Figure S1H).

Thus, most $Il2rg^{+/-}$ clones exhibited SCID-like phenotypes, albeit that they had one WT allele. We attributed this high proportion to aberrant X-inactivation, a previously observed epigenetic cloning phenotype (Senda et al., 2004; Nolen et al., 2005; Jiang et al., 2008). However, epigenetic cloning phenotypes are corrected by germline transmission (Shimozawa et al., 2002). To confirm this and isolate the $Il2rg^{+/-}$ phenotype, we analyzed progeny derived by fertilization from $Il2rg^{+/-}$ cloned female #9.

Female $Il2rg^{+/-}$ #9 inseminated with WT sperm produced 19 F_1 (12 m, 7 f) offspring, and of these F_1 offspring, two $Il2rg^{+/-}$ females produced 21 F_2 (13 m, 8 f) when inseminated with WT sperm. Autopsies of representative F_1 and F_2 progeny revealed that, as expected, all $Il2rg^{-/Y}$ males had undetectable thymi, whereas $Il2rg^{+/-}$ females had thymi of normal size (Figure 1A and Table S3).

Hematological parameters in PB exhibited a significantly ($p = 0.0041$) reduced white blood cell (WBC) count in F_1 $Il2rg^{-/Y}$ males ($6.4 \pm 1.6 \times 10^3/\mu l$, $n = 5$) compared to WT controls ($17.8 \pm 2.3 \times 10^3/\mu l$, $n = 6$), while hemoglobin levels and platelet counts were unaffected (Figure 1B). F_1 $Il2rg^{+/-}$ females and WT littermates yielded comparable PB T, NK, and B cell numbers, indicative of intact acquired and innate immunity (Figures 1C, 1E, and 1F). In contrast, $Il2rg^{-/Y}$ males harbored significantly reduced PB T cells ($Il2rg^{-/Y}$ males, $1.5\% \pm 1.0\%$; $Il2rg^{+/Y}$ males, $57.3\% \pm 4.3\%$; $n = 4$ each, $p < 0.0001$) and NK cells ($Il2rg^{-/Y}$ males, $0.1\% \pm 0.1\%$; $Il2rg^{+/Y}$ males, $3.6\% \pm 1.1\%$; $n = 4$ each, $p = 0.0162$) (Figures 1D, 1E, and 1F). In proportion to the PB reductions, $Il2rg^{-/Y}$ spleens exhibited significant numerical reductions of T cells ($Il2rg^{-/Y}$ males, $2.3\% \pm 1.1\%$; $Il2rg^{+/Y}$ males, $13.0\% \pm 1.4\%$; $n = 4$ each, $p = 0.0011$) and NK cells ($Il2rg^{-/Y}$ males, $0.1\% \pm 0.0\%$; $Il2rg^{+/Y}$ males, $0.8\% \pm 0.2\%$; $n = 4$ each, $p = 0.0162$) (Figures 1E and 1F). B cells and myeloid cells accounted for the majority of $CD45^+$ leukocytes in $Il2rg^{-/Y}$ males, indicating that their immune deficiency was limited to T and NK cell lineages. The presence in $Il2rg^{-/Y}$ males of $CD33^+$ myeloid cells with both mononuclear and polynuclear properties suggests the differentiation of both granulocyte lineages and antigen-presenting cells, including monocytes and dendritic cells (Figures 1C and 1D).

We next evaluated humoral immune status in *Il2rg*-targeted pigs (Figure 1G). Serum IgG and IgA levels were high at 1 week (P7) and decreased gradually from 3 to 5 weeks in $Il2rg^{-/Y}$ (males), WT littermates, and $Il2rg^{+/-}$ female controls. After 7 weeks, IgG and IgA levels re-elevated in WT controls, while levels of both remained low in $Il2rg^{-/Y}$ males. Serum IgM was low at 1 week and increased gradually in controls but remained low in $Il2rg^{-/Y}$ males. Because both IgG and IgA at 1 week of age are entirely transferred via the colostrum in pigs, these results indicate that there had been no de novo Ig production in $Il2rg^{-/Y}$ males after weaning at 4 weeks. Impaired antibody production by $Il2rg^{-/Y}$ B cells is likely due to the absence of critical $CD4^+$ T helper cells. Consistent with their impaired immunity, all F_1 $Il2rg^{-/Y}$ males became systemically ill in the conventional housing conditions used, while F_1 $Il2rg^{+/-}$ females appeared healthy.

Collectively, this shows that when produced by conventional breeding, $Il2rg^{-/Y}$ F_1 males, but not $Il2rg^{+/-}$ females, present SCID phenotypes. SCID-like phenotypes observed in many $Il2rg^{+/-}$ female clones are attributable to aberrant, nonrandom X-inactivation during somatic cell cloning. Following germline transmission, *Il2rg*-targeted phenotypes resembled those of X-linked SCID in other species, with greatly reduced T and NK cell development and function (Cao et al., 1995; Puck et al., 1987). *Il2rg*-targeted pigs harbor B cells and thereby recapitulate human XSCID more closely than do *Il2rg*-targeted mice.

We next performed proof-of-principle allogeneic transplantation experiments using *Il2rg*-targeted pigs as recipients. Preliminary conditioning with orally administered fludarabine and busulfan produced significantly decreased WBC and platelet counts (not shown), which might promote the engraftment of transplanted cells. However, 2 of 6 $Il2rg^{-/Y}$ males died within 2 weeks postadministration, suggesting that the regimen was lethal for some piglets. Bone marrow (BM) cells from WT siblings were intravenously transplanted to four P11-12 $Il2rg^{-/Y}$ males with (#113, #115) or without (#605, #610) conditioning. Ubiquitously GFP-expressing (#184) BM cells (Watanabe et al., 2005)

Figure 1. Phenotypes of F_1 and F_2 Progeny Derived from $Il2rg^{+/-}$ Clone #9 by Germline Transmission

(A) Thymic phenotype in an $Il2rg^{+/-}$ female at 10 weeks and an $Il2rg^{-/Y}$ male at 9 weeks.

(B) Peripheral blood (PB) white blood cell (WBC), hemoglobin (Hgb), and platelet count (Plt) at 2 months of age in WT controls, $Il2rg^{+/+}$ female littermates, $Il2rg^{+/-}$ female littermates, and $IL2rg^{-/Y}$ males.

(C) Identification of acquired and innate immune subsets in an $Il2rg^{+/+}$ female by surface phenotype of $CD45RA^+CD3^-$ B cells, $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells, and $CD3^-CD16^+$ NK cells among the nonmyeloid fraction and myeloid cells.

(D) Analysis as for (C), but of an $IL2rg^{-/Y}$ male.

(E) Proportion of $CD3^+$ T cells in the spleen (spl), PB, and thymus (thy) in control $Il2rg^{+/+}$ females and male $Il2rg^{+/Y}$ littermates and nonlittermates (WT).

(F) Analysis as for (E), except showing the proportion of $CD3^-CD16^+$ NK cells.

(G) Changes with time postpartum in serum IgG (left), IgA (middle), and IgM levels. All error bars indicate SEM. See also Figure S1 and Tables S1–S3.

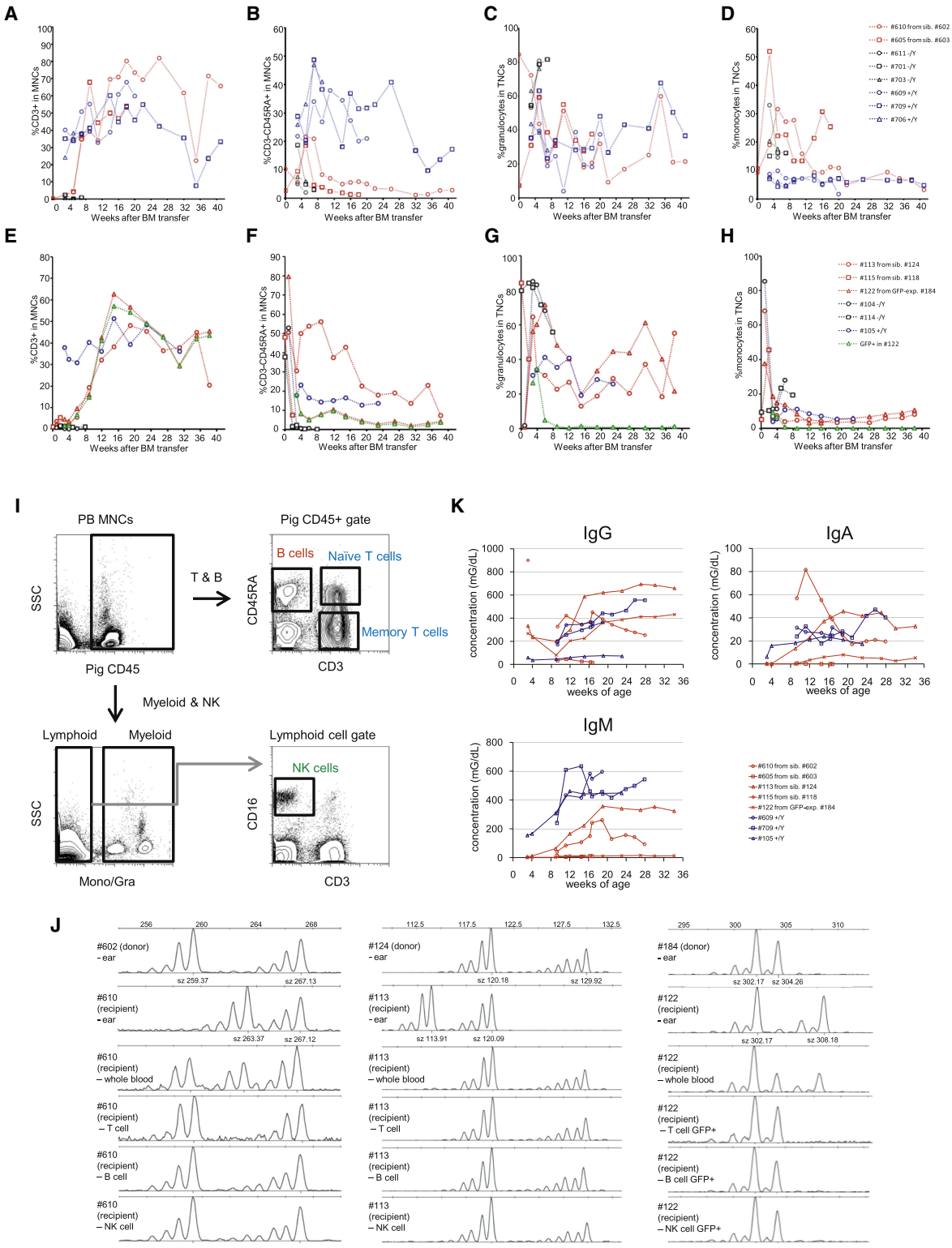


Figure 2. Allogeneic Bone Marrow Transfer into *Il2rg*$^{-/Y}$ Males

(A–H) Flow cytometric quantification of each subtype of leukocytes in *Il2rg*$^{-/Y}$ males (red lines) at different times after BM transfer. Controls are WT littermates (blue lines) and *Il2rg*$^{-/Y}$ males (black lines) without BM transfer. (A)–(D) correspond to BMT without conditioning, the case for #605 and #610. (E)–(H) correspond to BMT with conditioning, the case for #105, #113, and #122. (A) and (E) show the proportion of CD3^{+} T cells in PB at different times after BM transfer. (B) and (F) show proportion of CD45RA^{+}CD3^{-} B cells in PB at different times after BM transfer. (C) and (G) show proportion of granulocytes in PB at different times after BM transfer. (D) and (H) show proportion of monocytes in PB at different times after BM transfer.

were transferred to a *Il2rg*$^{-/Y}$ male (#122) with conditioning. Recipient #115 died 15 days after BM transfer (BMT), possibly due to conditioning toxicity, and recipient #605 died of presumptive pneumonia (not shown) 140 days posttransplantation; nevertheless, it survived longer than *Il2rg*$^{-/Y}$ controls without BMT, which died within 54 days. The remaining three recipients have survived >516 days (#610) and >321 days (#113 and #122) posttransplantation, with PB T cell populations exhibiting similar dynamics (Figures 2A and 2E); T cell counts increased ~6 weeks posttransplantation and remained high. Following early variability, PB B cell counts equilibrated at detectably low levels. The exception was #113, in which B cell counts were clearly higher than those of WT controls until 16 weeks posttransplantation, gradually decreasing thereafter to a level comparable with other recipients (Figures 2B and 2F). Compared to WT controls, recipient #610 PB contained similar T cell counts and a diminished but substantial number of B and NK cells 42 weeks posttransplantation (Figures 2A, 2B, and 2I). Similar profiles were observed in #113 and 122 (Figures 2E and 2F; not shown for NK). Surviving recipients and WT littermates had comparable granulocyte and monocyte numbers (Figures 2C, 2D, 2G, and 2H).

The provenance of immune cells in surviving recipients was examined by microsatellite marker analysis of genomic DNA from the ear, whole blood, and sorted T, B, and NK cells. GFP fluorescence of immune cells was also detected in the case of #122 (Figures 2E–2H and 2J). Lymphoid lineages were totally of donor origin in all recipients. Myeloid lineages were mainly of donor origin in #113 and of mixed donor/host origin in #122 and #610. Thus, our conditioning regimen enabled donor-derived myeloid lineage reconstitution in #113, but not #122. All surviving recipient PB contained IgG, IgA, and IgM (Figure 2K), albeit at varying levels, strongly suggesting that humoral immunity had been reconstituted and that donor-derived recipient B cells produced antibodies. Thus, allogeneic BM transplantation to *Il2rg*$^{-/Y}$ SCID pigs reproducibly resulted in enduring functional donor cell engraftment and reconstituted acquired immunity.

Assuming that the allogeneic model reported here reflects the behavior of cells transplanted from different species, SCID pigs promise to become a valuable tool in xenogeneic transplantation studies of human stem cells, such as hematopoietic, embryonic, and induced pluripotent stem cells (Takahashi et al., 2007). In particular, they promise to serve as platforms for the evaluation of therapeutic outcomes over several years, possibly after further genetic manipulation such as disruption of recombination activating genes 1 or 2 (*Rag1*, *Rag2*), which play critical roles in both cellular and humoral immunity (Shinkai et al., 1992) and may facilitate efficient human stem cell engraftment. Preliminary xenotransplantation of human BM cells to porcine *Il2rg*$^{-/Y}$ recipients in the absence of preconditioning permitted limited engraftment, underscoring the importance of further genetic manipulation, and optimized preconditioning (not shown). The porcine SCID model described here therefore represents an essential step toward the translational evaluation of human stem cells for long-term clinical applications.

SUPPLEMENTAL INFORMATION

Supplemental Information includes one figure, three tables, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.stem.2012.04.021.

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(I) Identification of immune subsets in recipient *Il2rg*$^{-/Y}$ male, #610, 12 weeks posttransfer as CD45RA$^+$CD3$^-$ B cells, CD3$^+$CD45RA$^+$ naive T cells, CD3$^+$CD45RA$^-$ memory T cells, and CD3$^-$CD16$^+$ NK cells among nonmyeloid and myeloid cells.
(J) Representative microsatellite PCR analyses to distinguish between donor and recipient DNA illustrated by the discriminatory marker (SW1263 for #610, SWR1367 for # 113, and SW24 for #122).
(K) Changes of IgG (left), IgA (middle), and IgM levels in serum from recipients (red lines) and wild-type controls (blue lines).

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