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Generation of Immunodeficient Mice Bearing Human Immune Systems by the Engraftment of Hematopoietic Stem Cells

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ABSTRACT

Immunodeficient mice are being used as recipients of human hematopoietic stem cells (HSC) for *in vivo* analyses of human immune system development and function. The development of several stocks of immunodeficient *Prkdc* ^{scid} *(scid)*, or recombination activating 1 or 2 gene *(Rag1 or Rag2)* knockout mice bearing a targeted mutation in the gene encoding the IL2 receptor gamma chain (*IL2r γ*) have greatly facilitated the engraftment of human HSC and enhanced the development of functional human immune systems. These “humanized” mice are being used to study human hematopoiesis, human-specific immune therapies, human-specific pathogens, and human immune system homeostasis and function. The establishment of these model systems is technically challenging, and levels of human immune system development reported in the literature are variable between laboratories. The use of standard protocols for optimal engraftment of HSC and for monitoring the development of the human immune systems would enable more direct comparisons between humanized mice generated in different laboratories. Here we describe a standard protocol for the engraftment of human HSC into 21-day old *NOD-scid IL2r γ* (NSG) mice using an intravenous injection approach. The multi-parameter flow cytometry used to monitor human immune system development and the kinetics of development are described.

ACKNOWLEDGEMENT

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GUIDELINES

INTRODUCTION

1.1 Immunodeficient Mice Bearing Mutations Within the IL2ry Gene as Recipients of Human HSC

The generation of immunodeficient mice that support the engraftment of human immune systems has enabled the *in vivo* study of human immune system development and function(1). Early efforts to engraft human immune systems into mice utilized the C.B-17 strain bearing the *Prkdc* ^{scid} (*scid*, severe combined immune deficiency) mutation (2), which permitted low levels of human immune cell engraftment after injection with peripheral blood mononuclear cells (PBMC) or hematopoietic stem cells (HSC) but overall immune system function was limited (1). The development of NOD -*scid* mice (3) improved engraftment of human immune systems but overall function and levels of take were still suboptimal for the study of human immunobiology (4, 5). The introduction of immunodeficient *Prkdc* ^{scid} (*scid*), or recombination activating 1 or 2 gene (*Rag1 or Rag2*) knockout mice bearing a mutated IL-2 receptor gamma chain (*IL2r γ* ^{null}) facilitated greatly the in vivo engraftment and function of human immune cells (6-9). The IL2rγ-chain is required for high affinity signaling of IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 cytokines, and *IL2r γ* ^{null} mice have severe defects in NK-cell activity in addition to T and B cell development (10). These new strains of immunodeficient *IL2r γ* ^{null} mice are now being used for studies of human hematopoiesis, innate and adaptive immunity, autoimmunity, infectious diseases, cancer biology, and regenerative medicine (11).

1.2 HSC Engraftment of Immunodeficient NOD-scid IL2rγ^{null} Mice

NOD.Cg-*Prkdc* ^{scid} *Il2rg* ^{tm1Wjl} */Sz (NODscid IL2r γ* ^{null} or NSG) mice support engraftment of human HSC from a variety of sources, including G-CSF mobilized peripheral blood, bone marrow aspirates, umbilical cord blood, and fetal liver (12-15). In vivo human hematopoietic repopulation through transplantation of human CD34+ HSC in NSG recipients allows high levels of human HSC engraftment, differentiation of human T cells in the murine thymus and human B cells, differentiation of human myeloid subsets and human immune function *in vivo* (12-14, 16-20). A critical aspect for generating HSC-engrafted immunodeficient mice is the use of standardized protocols that enable consistent and robust human immune system development. For example, age of the mouse recipient has important implications for development of human immune cell subsets. One study has shown that newborn NSG mice support more efficient human T cell development after HSC injection than adult NSG mice (8-12 weeks) (21).

HSC-injection into newborn mice is challenging, as the injection sites (intrahepatic, intracardiac and facial vein)

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require technical expertise and in some instances survival is problematic (6). Here we describe a standard protocol for the engraftment of human HSC into 21-day old NSG mice using an intravenous injection approach. A description of the multi-parameter flow cytometry used to monitor human immune system development is shown. The kinetics of human immune system development in 21-day old NSG mice were compared to those in HSC-engrafted newborn NSG mice.

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MATERIALS TEXT

1. Human Cord Blood Preparation (See NOTE 1)

1.1 Citrate phosphate dextrose anticoagulant solution, USP (CPD) blood pack unit (Fenwal, Lake Zurich, IL). UCB specimens were provided by the University of Massachusetts Memorial Umbilical Cord Blood Donation Program under Institutional Review Board (IRB) approval.

1.2 Hespan (6% Hetastarch / 0.9% NaCl Solution) (Braun Medical Inc, Bethlehem, PA). Hespan is used for volume reduction of UCB and preserves recovery of HSC (22).

1.3 Histopaque-1077 (Sigma-Aldrich, St Louis, MO, USA). Histopaque is a density medium and is used for separating mononuclear cells from blood (23).

1.4 Bovine Serum Albumin (BSA) (Fisher Scientific, Pittsburgh, PA)

1.5 DNase I recombinant, RNAse free (10,000 U/ml) (Roche, Indianapolis, IN)

1.6 Phosphate buffered saline, PBS

1.7 RPMI 1640 (Gibco, Life technologies, Grand Island, NY USA)

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1.8 50mL centrifuge tubes (BD Falcon, Franklin Lakes, NJ, USA)

1.9 Water bath

2. CD3 T cell Depletion (See NOTE 2)

2.1 Human CD3 microbead kit (Miltenyi Biotech, Auburn, CA, USA)

2.2 MidiMACS Separator (Miltenyi Biotech, Auburn, CA, USA)

2.3 MACS multistand (Miltenyi Biotech, Auburn, CA, USA)

2.4 LD columns (Miltenyi Biotech, Auburn, CA, USA)

2.5 MACS Buffer: PBS supplemented with 0.5% fetal bovine serum (FBS, Atlanta Biologicals, Lawrenceville, GA, USA), 2 mM EDTA, and sterilized with a vacuum flask (0.2µm filter).

3. Flow cytometry analysis

3.1 FACS buffer: PBS supplemented with 1% FBS, and 0.1% sodium azide.

3.2 Flow cytometry analysis of T cell depleted UCB

- Human CD3, Clone UCHT1 **BD Biosciences Catalog #555333**
- Human CD34, Clone 581 **BD Biosciences Catalog #555821**
- Human CD45, Clone HI30 **BD Biosciences Catalog #555485**

3.3 Fluorescent antibodies for screening of HSC-engrafted NSG mice.

- Anti-Mouse CD45 (mLy5), Clone 30-F11 **BD Biosciences Catalog #557235**
- Anti-Human CD45, Clone HI30 **BD Biosciences Catalog #555485**
- Anti-Human CD3, Clone UCHT1 **BD Biosciences Catalog #555333**
- Anti-Human CD20, Clone 2H7 **BD Biosciences Catalog #556632**
- Anti-Human CD33, Clone WM53 **BD Biosciences Catalog #562854**
- Anti-Human CD14, Clone HCD14 **BioLegend Catalog #325618**

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4. HSC injection

The materials listed below are necessary for intravenous injection of HSC into mice that are 21 to 28 days old. The materials used for injecting newborn mice with HSC have been listed in detail previously (6).

4.1 Immunodeficient mice: NOD-*Prkdc*^{scid}*IL2rg*^{tm1Wjl}(NSG) mice between 3 to 4 weeks of age. (**See NOTE 3**)

4.2 ^{137}Cs gamma irradiator

4.3 Autoclaved, filtered, ventilated device for holding mice during irradiation.

4.4 **1 mL** syringe with 27-G needle

4.5 Heating pad or warming lamp

4.6 Tailvein restrainer for mice (Braintree Scientific, BrainTree, MA)

NOTE 1
UCB is a reliable source of functional human HSC. Alternative sources include G-CSF mobilized peripheral blood, bone marrow aspirates and fetal liver (24). Each source will have differences in preparation, in cell yields and engraftment capacity and the characteristics of the developed human immune system.

NOTE 2
The standard protocol for our laboratory is to use CD3 depleted UCB cells for injecting CD34+ HSC into mouse recipients. The depletion of CD3+ T cells is essential to prevent development of acute xenogeneic graft-versus-host disease (GVHD) in the mice. Alternatively, purified CD34+ cells can also be injected into the recipient mice (25). One advantage for the CD3 depletion approach is that accessory cells (CD34-negative) present within the UCB specimen have been shown to enhance engraftment of human HSC in immunodeficient mice (26).

NOTE 3
Immunodeficient mice bearing mutations within the IL2-receptor common gamma chain are the ideal recipients for the engraftment of human HSC. NSG mice were developed at The Jackson Laboratory by back-crossing a complete null mutation at the*IL2rg*locus onto the NOD.Cg-*Prkdc*^{scid}(NOD/SCID) strain (12). Alternative mouse strains bearing mutations within the*IL2rg*locus have been described previously (7, 24).

BEFORE STARTING

The protocols described below involve the manipulation of primary human cells. All work should be done in a standard laminar flow hood and with appropriate personal protective equipment. Waste materials should be disposed of using protocols approved by an Institutional Biosafety Committee. All mouse injections and handling

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should be done using protocols approved by an Institutional Animal Care and Use Committee.

Methods

1 Preparation of umbilical cord blood (UCB)

1.1 Allow histopaque and RPMI supplemented with 0.5% BSA to warm to room temperature.

1.2 Transfer UBC to **50 mL** conical tubes (30 mls per tube).

1.3 Add hespan to each tube of UBC to a final concentration of 20% per volume and mix gently.

1.4 Incubate for 30 minutes at room temperature. The incubation period is to allow red blood cells (RBC) to sediment.

1.5 Remove bottom layer of RBC with **10 mL** pipette, leaving approximately **2 mL** of the RBC volume.

1.6 Add RPMI supplemented with 0.5% BSA to each tube to bring to a total volume of **30 mL** .

1.7 Slowly underlay **14 mL** of histopaque to each tube containing umbilical cord blood. Ensure a clear interface is maintained between the histopaque and the cell-containing medium.

1.8 Spin for 30 min at 300 × g at room temperature with centrifuge brake off.

1.9 Remove the top layer of plasma, leaving **2 mL** volume above buffy coat layer.

1.10 Remove buffy coat layer, transfer to a new **50 mL** conical tube.

1.11 Add **30 mL** of RPMI supplemented with 0.5% BSA and centrifuge for 5 min 400 × g and **4 °C** .

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1.12 Discard supernatant

1.13 Pool pellets from each **50 mL** conical tube in a total volume of 10 mls of RPMI supplemented with 0.5% BSA. (**See NOTE 4**)

> **NOTE 4**
> DNAse (15 ul 10,000 U/ml DNAse/30 ml) can be added if pellet is viscous after resuspension.

1.14 Perform viability count.

1.15 Reserve 100,000 cells for flow cytometric analysis.

1.16 Centrifuge cells for 5 min at 400 × g and **4 °C** and proceed to step 2.

2 Depletion of CD3+ T cells

Our laboratory uses reagents from Miltenyi Biotech to deplete human CD3+ T cells from UCB samples. The manufacture's protocol was followed for depletion of human T cells (**See NOTE 2**)

2.1 Resuspend CD3-depleted UCB cells in 1 ml RPMI supplemented with 0.5% BSA and perform viability counts.

2.2 To validate depletion of CD3+ T cells, stain recovered cells with antibodies specific for human CD3 and human CD45. T cell levels (cells staining double positive for CD3 and human CD45) should be below 0.5% of total cells. Cells saved from step 1.15 should be used as a positive staining control.

2.3 To determine the CD34+ HSC levels, stain the recovered cells with antibodies specific for human CD34 and human CD45. HSC are identified as CD45 dim and CD34 positive.

2.4 Resuspend cells in RPMI supplemented with 0.5% BSA. Keep cells on ice until injection.

3 HSC injection

The protocol for injecting newborn mice with HSC has been described in detail previously (6). Here we will focus on

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intravenous injection of HSC into 21 to 28 day old mice.

3.1 Irradiate recipient mice by whole body gamma irradiation. For young (21 to 28 days old) NSG mice a conditioning dose of 100 cGy is normally well tolerated and enables efficient engraftment. For engraftment of newborn NSG mice, 100 cGy is also used. HSC injection should be performed between four and 24 hours after irradiation.

3.2 Warm recipient mice using warming lamp.

3.3 Inject CD3-depleted UCB cells to obtain a total of 1×10^5CD34+ cells per injection into the lateral tail vein. For newborn mice show in here, 5×10^4CD34+ cells were injected by intracardiac route into 24 to 72 hour old NSG pups.

3.4 Ensure that bleeding from injection site has stopped and return recipient mouse to cage.

4 Evaluation of Human Cell Chimerism Levels by Flow Cytometry

HSC-engrafted NSG mice are normally screened for human cell chimerism levels in peripheral blood between 12 and 15 weeks post-injection. This evaluation is easily done by flow cytometric analysis of peripheral blood cells to validate human immune system development. For analysis, all cells positive for mouse CD45 are excluded from the gating strategy. Human immune cells are identified as staining positive for human CD45 and subpopulations are defined from those cells. The immune cell subsets that develop will be dependent on age of the recipient mouse and the specific time point evaluated. The results shown below compare human immune system development between HSC-engrafted newborn NSG mice and HSC-engrafted young (21-day old) NSG mice.

4.1 Levels of human CD45+ cells. Newborn and 21-day old NSG mice were irradiated and injected IV by intracardiac route (newborn) or via the tail vein (3 weeks old) with T cell depleted UCB containing 1×10^5CD34$^+$cells as described above. Between 6 to 18 weeks after HSC injection, the percentages of human CD45+ cells in the blood were determined by flow cytometry (Figure 1). Each shape represents an individual animal and mice injected with the same HSC are identified by symbols of similar shape and color. Higher proportions of human CD45+ cells were detected in the blood of 21-day old, HSC-engrafted NSG mice at 6, 9,12 and 18 weeks as compared to newborn mice (Figure 1). In addition higher levels of human CD45+ cells were detected in the spleen and bone marrow of the 21-day old NSG mice compared with levels of circulating cells (Figure 2). These results show that 21-day old NSG mice and newborn NSG mice support HSC engraftment and that 21-day old mice show faster kinetics of human immune system development.

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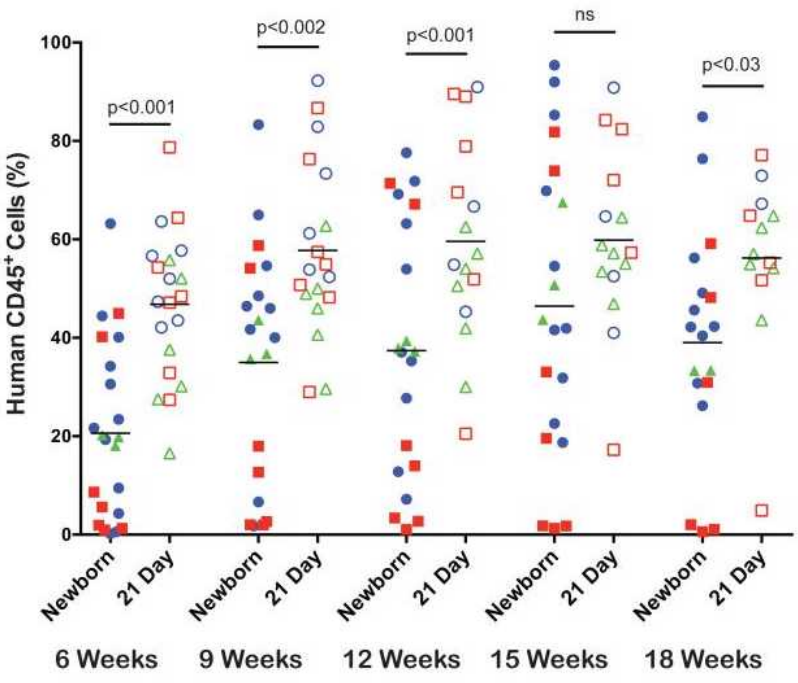

Figure 1. Human cell chimerism levels in peripheral blood of NSG mice engrafted with human HSC

Newborn and 21-day old NSG mice were irradiated and injected IV via intracardiac route (newborn) or the tail vein (21-day old) with T cell depleted UCB containing $CD34^+$cells. At 6– 18 weeks after HSC injection, the percentages of human CD45 positive cells were determined by flow cytometry. Each shape represents an individual animal, and points of the same color and shape are from independent experiments (N=3).

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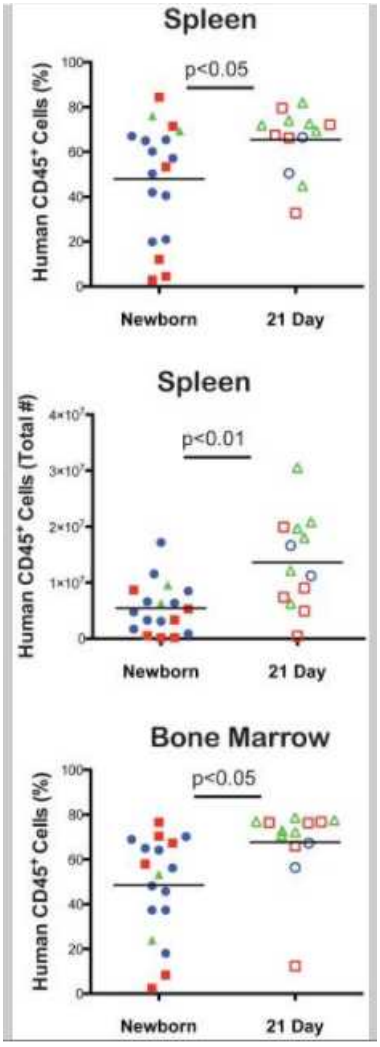

Figure 2. Human cell chimerism levels in spleen and bone marrow of NSG mice engrafted with human HSC

Newborn and 21-day old NSG mice were irradiated and injected IV via intracardiac route (newborn) or the tail vein (21-day old) with T cell depleted UCB containing $CD34^+$cells. 18 weeks after HSC injection, the percentages and number of human CD45+ cells in the spleen and percentages in the bone marrow were determined by flow cytometry. Each shape represents an individual animal, and points of the same color and shape are from independent experiments (N=3).

4.2 Human T cell development. The kinetics of human CD3+ T cell development were compared between newborn and 21-day old NSG mice that had been injected with human HSC as described above (Figure 3.) The levels of human T cells detected in the peripheral blood were low at the 6 and 9 week time points for both groups of HSC-engrafted NSG mice and began to increase by week 12. At weeks 15 and 18 the levels of T cells were significantly higher in newborn NSG mice as compared to 21-day old NSG mice. These data suggest that both newborn and 21-day old NSG mice support human T cells development, but the kinetics are accelerated in mice injected as newborns (**See NOTE 5**)

NOTE 5
Immunodeficient mice expressing HLA class I and II have been used support HLA-rerstricted human T cell development and are commercially available (27).

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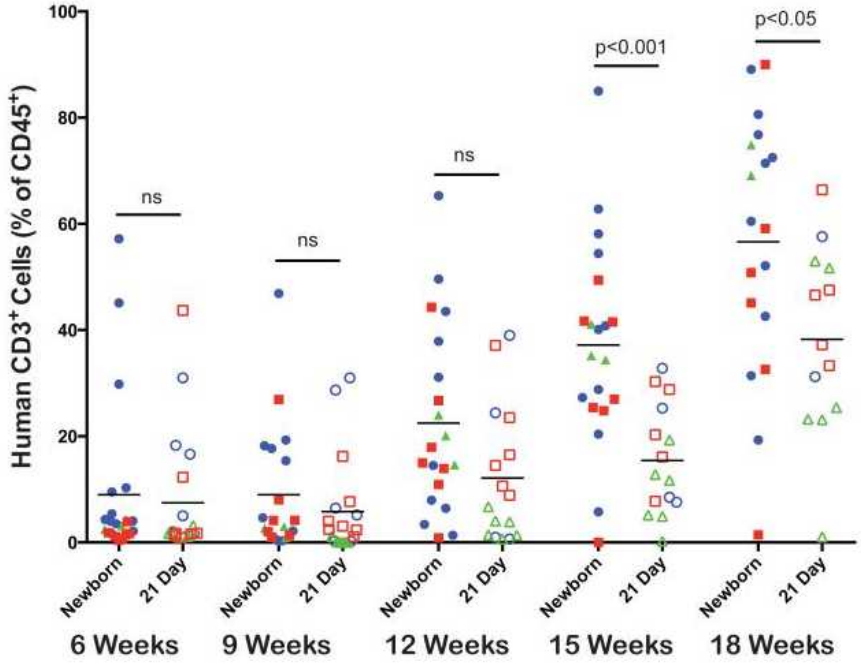

Figure 3. Human CD3+ T cell development in peripheral blood of NSG mice engrafted with human HSC

Newborn and 21-day old NSG mice were irradiated and injected IV via intracardiac route (newborn) or the tail vein (21-day old) with T cell depleted UCB containing $CD34^+$cells. At 6– 18 weeks after HSC injection, the percentages of human CD3 positive T cells were determined by flow cytometry. Each shape represents an individual animal, and points of the same color and shape are from independent experiments (N=3).

4.3 B cell development. The kinetics of human CD20+ B cell development were compared between newborn and 21-day old NSG mice that had been injected with human HSC as described above (Figure 4). Both newborn and 21-day old injected NSG mice supported B cell development and significantly higher levels were detected in 21-day old mice at 6, 15 and 18 weeks. Overall a significant proportion of the CD45+ cells in NSG mice were human B cells at all time points.

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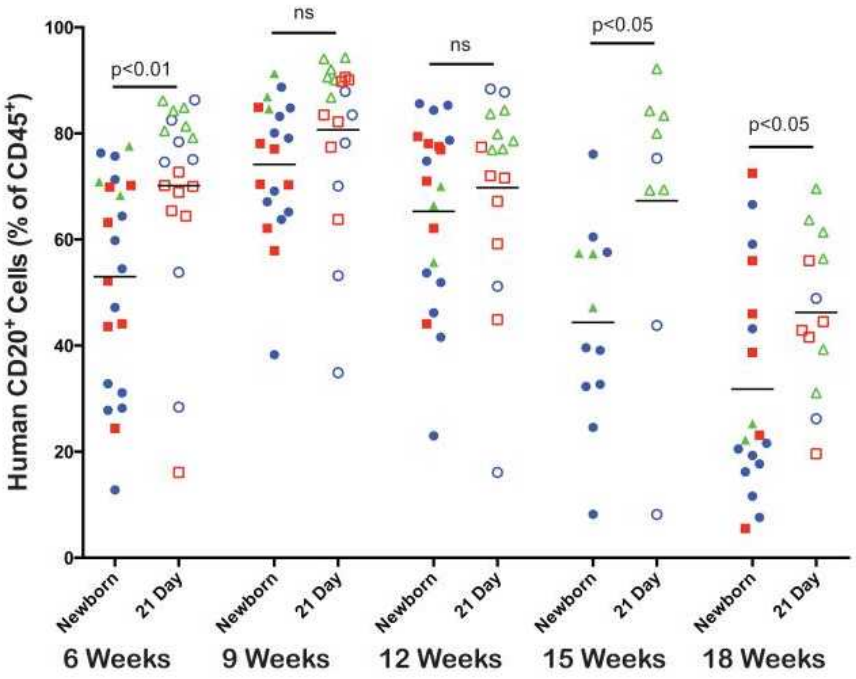

Figure 4. Human CD20+ B cell development in peripheral blood of NSG mice engrafted with human HSC

Newborn and 21-day old NSG mice were irradiated and injected IV via intracardiac route (newborn) or the tail vein (21-day old) with T cell depleted UCB containing $CD34^+$cells. At 6– 18 weeks after HSC injection, the percentages of human CD20 positive T cells were determined by flow cytometry. Each shape represents an individual animal, and points of the same color and shape are from independent experiments (N=3).

4.4 Monocyte/Macrophage development. The kinetics of human CD14+/CD33+ monocyte/macrophage development were compared between newborn and 21-day old NSG mice that had been injected with human HSC as described above (Figure 5). Both newborn and 21-day old NSG mice supported low levels of human monocyte/macrophage development with slightly higher levels detected in 21-day old mice at the 15 week time point. At all time points tested CD14+/CD33+ cells were detectable in the peripheral blood.

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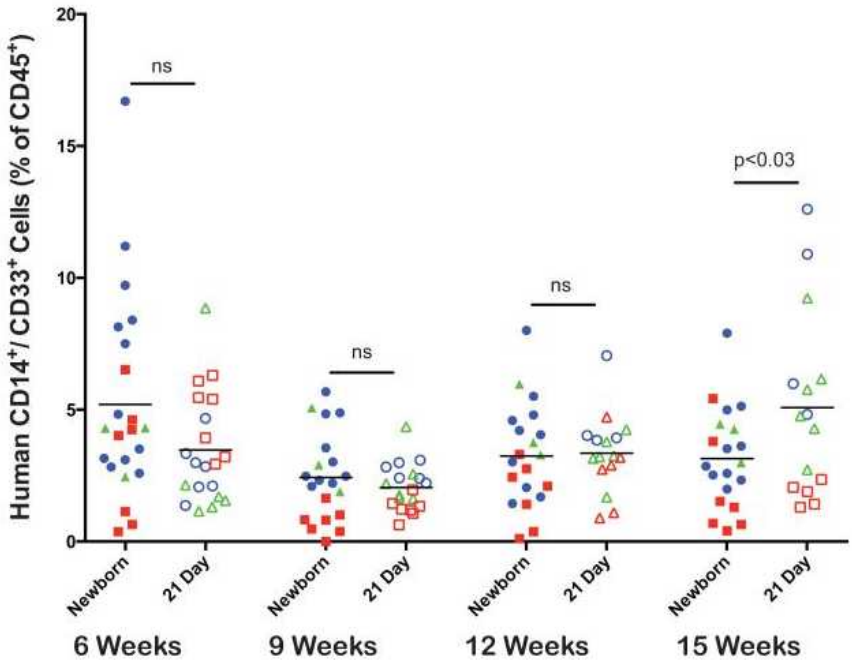

Figure 5. Human monocyte/macrophage development in peripheral blood of NSG mice engrafted with human HSC

Newborn and 21-day old NSG mice were irradiated and injected IV via intracardiac route (newborn) or the tail vein (21-day old) with T cell depleted UCB containing $CD34^+$cells. At 6– 15 weeks after HSC injection, the percentages of human CD14/CD33+ positive myeloid cells were determined by flow cytometry. Each shape represents an individual animal, and points of the same color and shape are from independent experiments (N=3).

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