Engraftment of human hematopoietic stem cells is more efficient in female NOD/SCID/IL-2Rgcnull recipients

Faiyaz Notta1,2*, Sergei Doulatov1,2*, and John E. Dick1,2

1Division of Stem Cell and Developmental Biology, Campbell Family Institute for Cancer Research/Ontario Cancer Institute, Toronto, Ontario, M5G 1L7, Canada
2Department of Molecular Genetics, University of Toronto, Toronto, Ontario, M5G 1L7, Canada

* These authors contributed equally to this work.

Short title: Gender dependent engraftment of human HSCs

Contact information:
John E. Dick, Ph.D.
Toronto Medical Discovery Tower, Rm 8-301
101 College Street, Toronto
Canada M5G 1L7
Ph: 416-581-7472; FAX: 416-581-7471
Email: jdick@uhnres.utoronto.ca
Repopulation of immunodeficient mice remains the primary method to assay human hematopoietic stem cells (HSCs). Here we report that female NOD/SCID/IL-2Rgnull mice are far superior in detecting human HSCs (Lin\(^{-}\)CD34\(^{+}\)CD38\(^{-}\)CD90\(^{+}\)CD45RA\(^{-}\)) in comparison to male recipients. When multiple HSCs were transplanted, female recipients displayed a trend (1.4-fold) towards higher levels of human chimerism (female vs. male: injected femur – 44.4±9.3 vs. 32.2±6.2, n=12f, 24m, p=0.1). Strikingly, this effect was dramatically amplified at limiting cell doses where female recipients had ~11-fold higher chimerism from single HSCs (female vs. male: injected femur – 8.1±2.7 vs. 0.7±0.7, n=28f, 20m, p=0.0001). Secondary transplants from primary recipients indicate that females more efficiently support the self-renewal of human HSCs. Therefore, gender associated factors play a pivotal role in the survival, proliferation and self-renewal of human HSC in the xenograft model and recipient gender must be carefully monitored in the future design of experiments requiring human HSC assays.

Introduction

The dynamic interplay between donor HSCs and the recipient microenvironment governs the survival and proliferation of HSCs in a transplant setting. A complex network of cells within the microenvironment regulates HSC function via direct interaction or secretion of cytokines that act in an autocrine or paracrine manner\(^1\). However, the effects on HSCs by molecules that communicate in an endocrine fashion, such as steroid hormones, remain
understudied. Interestingly, gender-associated hormones including androgens and estrogen have been directly implicated in regulating hematopoietic cell function\textsuperscript{2-4}. During the course of our functional analyses of HSCs using the recently developed NOD/SCID-IL2R\textsubscript{g}null (NSG) mice\textsuperscript{5}, we observed the recipient gender plays a critical role in the engraftment and proliferation of human HSCs. Specifically, female NSG mice are far superior to their male counterparts in engrafting and detection of single human HSCs.

**Materials and Methods**

*Collection of human cord blood* – Human cord blood (CB) was collected according to guidelines established by Trillium Health Centre and University Health Network. Mononuclear cells from pooled CBs were harvested using a Ficoll gradient and subsequently depleted of mature cells (Lin\textsuperscript{–}) via negative selection using StemSep system (Stem Cell Technologies). Cells were viably frozen in 10% DMSO solution and stored at -80 or -150\degree C.

*Fluorescence activated cell sorting (FACS)* – Freshly thawed Lin\textsuperscript{–}CB was stained with CD34 APC, CD38 PC7, CD45RA FITC, and CD90 PE in PBS + 5%FCS at concentration of 10\textsuperscript{7} cells/mL for 30 mins at 4\degree C. Cells were sorted on FACS Aria II (BD) and purities of >99\% were commonly achieved in post-sort analysis (data not shown). Cells were counted in trypan blue prior to transplant and resuspended in 25\textmu L IMDM/mouse in a 28.5g 0.5cc insulin syringe.

*Xenotransplantation and detection of human chimerism* – All animal experiments were performed with the approval of the Animal Care committee at the University
Health Network. Ten to 12 wk old NSG mice were sublethally irradiated (200 – 225cGy) 24h prior to transplantation. Cells were transplanted intrafemorally as previously described. Briefly, 27g needle was used to drill a hole in the right femur, which was followed by injection of cells using a 28.5g insulin syringe. Mice were sacrificed 16 – 20wk post-transplant. To evaluate human engraftment, cells were collected from injected femur (IF – right femur), non-injected bones (BM – including left femur, right and left tibiae), spleen (SP), thymus (TH) and stained with CD45PC7, CD45PC5 (Beckman Coulter), CD19 and CD33, CD4 (Beckman Coulter) and CD8 (all BD Biosciences unless indicated). Threshold for human engraftment was considered to be 0.1% double positive for CD45. All flow cytometry analysis was performed on the LSRII (BD Biosciences).

Statistical Analysis: Data is represented as mean ± s.e.m. The significance of the differences between groups was determined by using Mann-Whitney test.

Results and Discussion

In the hematopoietic system, HSC possess extensive self-renewal and differentiation capacities and remain the only cell type capable of long-term multilineage engraftment. Human HSCs capable of this feat reside in the Lin- CD34+CD38-CD90+CD45RA-7-10 compartment. This population was flow sorted from Lin- CB and intrafemorally transplanted at equivalent cell doses into sublethally irradiated adult male and female NSG mice (Fig. S1A). Human chimerism was assessed by flow cytometry 16-24wk post-transplant. The cell dose varied (245 – 5000 cells), and was considered non-limiting, since all mice
transplanted at these doses displayed human multi-lineage engraftment (Fig. S1B and Fig. S1C). When multiple HSCs were transplanted, female NSG mice displayed a trend towards higher level of human engraftment in comparison to males (female vs. male: IF – 44.4±9.3 vs. 32.2±6.2, p=0.14; BM – 32.8±7.8 vs. 21.1±4.3, p=0.16; SP – 29.1±7.1 vs. 16.7±4.0, p=0.12; TH – 40.5±15.2 vs. 25.3±9.5, p=0.12; n=13 females, 24 males, 3 independent experiments) (Fig 1A,B). Females displayed 1.4-, 1.6-, 1.7-, and 1.6-fold increases in engraftment in the IF, BM, SP, and TH, respectively, in comparison to males (Fig. 1C). No differences in lineage distribution of human cells engrafted in male or female recipients (Fig. S1C). Therefore, Lin-CD34+CD38-CD90+CD45RA- CB cells repopulate at modestly higher levels in females compared to age matched male NSG mice.

To assess the effects of gender on the repopulation capacity of single human HSCs, we performed limiting-dilution cell dose transplants with Lin-CD34+CD38-CD90+CD45RA- fraction (doses: 10 – 25). Transplantation of doses equivalent to a single HSC unveiled striking differences in total engraftment between male and female recipients (female vs. male: IF – 8.1±2.7 vs. 0.7±0.7, p=0.0001; BM – 4.8±1.7 vs. 0.1±0.04, p<0.0001; SP – 3.2±0.8 vs. 0.1±0.1, p<0.0001; TH –2.1±1.2 vs. 0, p=0.04; n=28F, 20M, 4 independent experiments) (Fig. 1D,E). Female mice had 11.3-, 76.5- and 26.7-fold higher mean engraftment levels in IF, BM and SP, respectively (Fig. 1F). Furthermore, 23/28 females and 3/20 males were engrafted at limiting dose, indicating that females were approximately 5–fold more sensitive in detecting human HSCs. Thus,
proliferation and detection of HSCs at the clonal level, but not their lineage potential (Fig. S1C), are markedly enhanced in female NSG recipients. Interestingly, only clonal doses reveal these dramatic differences indicating that transplantation of saturating levels of HSCs can mitigate gender effects on engraftment. These results are consistent with Ballen et al.\textsuperscript{11}, where high numbers of CB cells transplanted in NOD/SCID (NS) mice did not reveal significant effects of recipient gender on human engraftment. Therefore, female NSG recipients more efficiently support the repopulation of single human HSCs.

In the initial phase of engraftment, transplanted HSCs home to the bone marrow and lodge in a supportive microenvironment. To determine if this process was influenced by recipient gender, Lin\textsuperscript{−} CB cells were injected intravenously (IV) and were quantified 16h after transplantation. Homing efficiency of both total Lin\textsuperscript{−} CB and HSCs were similar between male and female mice indicating that gender-specific factor(s) influence long-term, but not initial, phases of HSC engraftment (Fig. S2A,B).

The self-renewal potential of HSCs can be evaluated using long-term repopulation assays or secondary transplants. Long-term repopulation indirectly measures this capability, since self-renewal divisions must be executed by HSCs to maintain chimerism. Historically however, secondary transplants remain the most commonly used technique to assay for self-renewal. We performed parallel secondary transplants from both male and female primary recipients. Human cells from primary females generated higher levels of human engraftment in parallel secondary female, compared to male, recipients (Fig. 2A). To rule out
whether male mice harbored quiescent HSCs that did not proliferate efficiently, we also performed secondary transplants into females from 12 primary males that had undetectable (<0.1%, n=8) or low (>0.1%, n=4) engraftment levels. No engraftment was observed in secondary recipients (Fig. 2B), indicating that male mice do not harbor quiescent HSCs. This data supports the conclusion that female NSG recipients better support the self-renewal of human HSCs.

In conclusion, we report a striking and previously unappreciated role of gender in the engraftment potential of human HSCs in the xenotransplant model. In our perspective, two rationales exist to reconcile this dichotomy: female NSG mice might be more immunodeficient than males, or gender-associated factors, such as steroid hormones, can positively or negatively regulate human HSCs. Residual B-, T- and NK cells could not be detected over background levels by flow cytometry suggesting that the IL2Rgε deletion is fully penetrant in male mice (data not shown). Moreover, NOD/SCID (NS) males display the same disparity in human engraftment (McDermott et al., manuscript in preparation), and therefore the role of recipient gender in regulating HSCs is beyond the scope of IL-2R signaling network. Furthermore, cellular toxicity to the murine hematopoietic system observed after sub-lethal irradiation at increasing doses was comparable between age-matched male and female mice ruling out the possibility that the increased body weight of male mice serves to mitigate the effects of sub-lethal irradiation (Fig. S2C-E).

The role of sex hormones in immune function is well documented and the expression of cognate receptors on HSCs implies the potential to respond to
ligand binding. However, the expression of receptors such as estrogen (ER) and progesterone (PR) receptor varies during ontogeny\textsuperscript{4}. Consistent with murine studies, adult human BM and not CB CD34\textsuperscript{+} express detectable levels of ER and PR, while androgen receptor (AR) is expressed in both\textsuperscript{4}. While further experiments are required to identify gender-specific mechanisms of HSC engraftment, our study has revealed their key role and supports the concept that recipient gender as a critical variable in the context of stem cell-transplantation studies.

Acknowledgements:

We would like to thank A. Poepppl and L. Jin for their technical assistance and the rest of the Dick Lab for the critical review of this manuscript. We thank K. Moore and the obstetrics unit of Trillium Hospital (Mississauga, Ontario) for providing cord blood samples and co-op students for processing cord blood samples. We also thank P. A. Penttilä, L. Jamieson and S. Zhao, at our UHN/Sickkids flow cytometry facility. This work was supported by funds from Canadian Institutes for Health Research (CIHR) studentships (FN,SD), The Stem Cell Network of Canadian National Centres of Excellence; the Canadian Cancer Society and the Terry Fox Foundation; Genome Canada through the Ontario Genomics Institute; Ontario Institute for Cancer Research with funds from the province of Ontario; the Leukemia and Lymphoma Society; the Canadian Institutes for Health Research; and a Canada Research Chair. This research was funded in part by the Ontario
Ministry of Health and Long Term Care (OMOHLTC). The views expressed do not necessarily reflect those of the OMOHLTC.

Authorship:

Contributions: F.N., S.D. and J.E.D designed research and wrote the manuscript. F.N. and S.D. performed experiments.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: John E. Dick, Toronto Medical Discovery Tower, Rm 8-301, 101 College Street, Toronto, Canada M5G 1L7, Email: jdick@uhnres.utoronto.ca

References:


Figure legends:

**Figure 1:** Female NOD/SCID/IL-2Rgc<sup>null</sup> mice more efficiently support human HSC detection and proliferation than syngeneic male mice. Male and female recipients transplanted with Lin<sup>−</sup>CD34<sup>+</sup>CD38<sup>−</sup>CD90<sup>+</sup>CD45RA<sup>−</sup> cells (refer to Fig. S1) were sacrificed 16-20wk post-transplant and human chimerism was assessed by flow cytometry in the injected femur (IF), non-injected bones (BM – left femur, 2 tibiae), spleen (SP) and thymus (TH). **(A)** Representative analysis from primary male and female recipient transplanted with Lin<sup>−</sup>CD34<sup>+</sup>CD38<sup>−</sup>CD90<sup>+</sup>CD45RA<sup>−</sup> cells. **(B)** Mean human engraftment levels in the IF, BM, SP and TH of male (n=24, open circle) and female (n=13, closed circle) NSG recipients transplanted with Lin<sup>−</sup>CD34<sup>+</sup>CD38<sup>−</sup>CD90<sup>+</sup>CD45RA<sup>−</sup> cells (range: 245-5000). This analysis was consistent in 3 independent experiments. **(C)** Bars represent fold difference in engraftment levels between male and female recipients from **B.** **(D)** Representative analysis from primary male and female recipients transplanted with 25 Lin<sup>−</sup>CD34<sup>+</sup>CD38<sup>−</sup>CD90<sup>+</sup>CD45RA<sup>−</sup> cell dose. **(E)** Mean human engraftment levels in the IF, BM, SP and TH of male (n=20, open circle) and female (n=28,
closed circle) NSG recipients transplanted with Lin−CD34−CD38−CD90−CD45RA− cells (range: 10-25), from 4 independent experiments. (F) Bars represent fold difference in engraftment levels between male and female recipients from E. *P < 0.05, **P = 0.01 – 0.001, ***P < 0.001.

Figure 2: Female NOD/SCID/IL-2Rgcnul l mice more efficiently support the self-renewal of human HSCs. (A) Whole bone marrow (IF and BM) was harvested and transplanted in parallel into secondary male and female recipients at equivalent cell dose. Mice were sacrificed 12wk post-transplant and human cell engraftment was assessed by flow cytometry. Shown is representative analysis from 2 of 4 independent cases. (B) Whole bone marrow from primary male mice displaying low (>0.1%, n=4) or undetectable (<0.1%, n=8) levels of human engraftment were transplanted into secondary female recipients to evaluate if males harbored quiescent HSC. No engraftment was detected in secondary females. Shown is representative analysis from 3 cases.
Figure 1

A. M vs. F

B. % human engraftment

C. Fold difference engraftment (female/male)

D. M vs. F

E. % human engraftment

F. Fold difference engraftment (female/male)
Engraftment of human hematopoietic stem cells is more efficient in female NOD/SCID/IL-2Rgnull recipients

Faiyaz Notta, Sergei Doulatov and John E. Dick

Advance online articles have been peer reviewed and accepted for publication but have not yet appeared in the paper journal (edited, typeset versions may be posted when available prior to final publication). Advance online articles are citable and establish publication priority; they are indexed by PubMed from initial publication. Citations to Advance online articles must include digital object identifier (DOIs) and date of initial publication.