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CD27, CD201, FLT3, CD48, and CD150 cell surface staining identifies long-term mouse hematopoietic stem cells in immunodeficient non-obese diabetic severe combined immune deficient derived strains

by Bianca Nowlan, Elizabeth D. Williams, Michael R. Doran, and Jean-Pierre Levesque

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CD27, CD201, FLT3, CD48, and CD150 cell surface staining identifies long-term mouse hematopoietic stem cells in immunodeficient non-obese diabetic severe combined immune deficient derived strains

**Short Title:** CD27, CD201, FLT3, CD48, and CD150 identify HSC in mice

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Tables and Figures: 6 Figures

Supplementary File: Supplementary Methods, Tables (6) and Figures (6)
Abstract

Staining for CD27 and CD201 (Endothelial protein C receptor) has been recently suggested as an alternative to stem cell antigen–1 (Sca1) to identify hematopoietic stem cells in inbred mouse strains with low or nil expression of SCA1. However, whether staining for CD27 and CD201 is compatible with low fms-like tyrosine kinase 3 (FLT3) expression and the “SLAM” code defined by CD48 and CD150 to identify mouse long-term reconstituting hematopoietic stem cells has not been established. We compared the C57BL/6 strain, which expresses a high level of SCA1 on hematopoietic stem cells to non-obese diabetic severe combined immune deficient NOD.CB17-prkdc<sup>scid</sup>/Sz (NOD-scid) mice and NOD.CB17-prkdc<sup>scid</sup> il2rg<sup>tm1Wjl</sup>/Sz (NSG) mice which both express low to negative levels of SCA1 on hematopoietic stem cells. We demonstrate that hematopoietic stem cells are enriched within the lineage-negative C-KIT<sup>+</sup> CD27<sup>+</sup> CD201<sup>+</sup> FLT3<sup>-</sup> CD48<sup>-</sup> CD150<sup>+</sup> population in serial dilution long-term competitive transplantation assays. We also make the novel observation that CD48 expression is up-regulated in Lin<sup>-</sup> KIT<sup>+</sup> progenitors from NOD-scid and NSG strains, which otherwise have very few cells expressing the CD48 ligand CD244. Finally, we report that unlike hematopoietic stem cells, SCA1 expression is similar on bone marrow endothelial and mesenchymal progenitor cells in C57BL/6, NOD-scid and NSG mice. In conclusion, we propose that the combination of Lineage, KIT, CD27, CD201, FLT3, CD48, and CD150 antigens can be used to identify long-term reconstituting hematopoietic stem cells from mouse strains expressing low levels of SCA1 on hematopoietic cells.

Article Summary

- NOD-scid immunodeficient mouse strains express low level of SCA1, which precludes identification of HSC using this antigen.
- We demonstrate in transplantation assays that most functional HSC reside in the Lineage-negative KIT<sup>+</sup> CD27<sup>+</sup> CD201<sup>+</sup> FLT3<sup>-</sup> CD48<sup>-</sup> CD150<sup>+</sup> subset alleviating the need of SCA1 staining.
Introduction

Blood myeloid and erythroid lineages are short-lived and require continuous replacement from hematopoietic stem cells (HSC) in the bone marrow (1-6). HSC are defined by their capacity to clonally reconstitute the hematopoietic system in lethally irradiated mice upon transplantation. Using cell surface markers, mouse HSC are comprised within the lineage (Lin)-negative (for B, T, myeloid and erythroid lineages), c-KIT/CD117 positive and stem cell antigen-1 (SCA1 or LY6A/E) positive (LSK) population. Multipotent long-term reconstituting HSC (LT-HSC) are LSK, fms-like tyrosine kinase 3 (FLT3)/CD135 negative, CD48 negative and signaling lymphocytic activation molecule (SLAMF1/CD150) positive (4, 5). When transplanted, these HSC can clonally and serially reconstitute hematopoiesis in lethally irradiated mice (5).

Identifying HSC in inbred mouse strains that either do not or poorly express SCA1 such as BALB/c or non-obese diabetic (NOD) mice (7, 8), or when treatments affect SCA1 expression is challenging. The SCA1 antibody detects LY6A and LY6E, which are two similar proteins of the LY6 phosphatidylinositol-anchored membrane proteins antigen family encoded by two different genes (9). LY6E is expressed by 10-15% blood leukocytes, whereas LY6A is expressed by 50-70% leukocytes (8). Inbred strains with the LY6.1 haplotype (e.g. BALB/c, C3H, DBA/1, CBA, FVB/N) do not express LY6A. This causes reduced SCA1 expression, thus compromising the classical method of identifying the HSC population based on the LSK phenotype (3, 8). Furthermore, even though the NOD strain and other immunodeficient strains in the NOD background are from the LY6.2 haplotype, they also express low levels of SCA1 (10). In addition, SCA1 expression can be affected by treatments such as irradiation, bacterial infections, and interferons which cause a transient increase in SCA1 expression in Lin- KIT+ (LK) cells in C57BL/6 mice (11, 12) further questioning the suitability of SCA1 antigen to characterize HSC in challenged mice.

CD27 and CD201 (endothelial protein C receptor – EPCR) combination has been proposed as an alternative to SCA1/c-kit staining for HSC identification in mouse strains with low expression of SCA1 or following irradiation (13). It has been demonstrated that Lin- CD27+ CD201+ cells contained all HSC activity tested in a long-term competitive repopulation assay in lethally irradiated recipient mice and this HSC phenotype remained consistent in several mouse strains including BALB/c and NOD or following irradiation (13).

Several reports suggest that mouse HSC expresses both CD27 and CD201 (14, 15). CD27 is a member of the TNF receptor family expressed on T, B, and NK cells, involved in proliferation, differentiation, and IgG production. CD27 was detected on 90% of LSK cells in
C57BL/6 mice (15). Likewise, high expression of CD201, was also observed on 90% of LSK cells (14). CD201+ cells are multipotent in both colony assays and mouse transplant reconstitution. CD201 and CD150 are co-expressed in the embryonic mouse hematopoietic development of a long-term reconstituting population of HSC throughout life (16, 17). In addition, CD201 is also expressed on multipotent human CD34+ HSC (18) showing that CD201 expression pattern is conserved between human and mouse HSC unlike the CD34 antigen (6). As few HSC markers are shared between both species, this is becoming a significant cross-species HSC marker.

Recently, the use of NOD.CB17-prkdc\textsuperscript{scid} il2rg\textsuperscript{tm1Wj1}/Sz (NSG) mice for human xenografts has increased (19-21) relative to the parental (NOD.CB17-prkdc\textsuperscript{scid}/Sz, NOD-scid) mice. NSG mice do not express functional interleukin-2 receptor and therefore lack NK cells in addition to lack of B and T cells from the parental NOD-scid strain resulting in a more profound immunosuppression more amenable to human xenograft engraftment (21).

Metastatic cancer cells and human HSC can hijack the mouse BM HSC niche (22), thus any treatments affecting xenografts should also examine the drug effect on the host mouse HSC content in order to detect potential drug adverse effects. However, there are no reliable flow cytometry methods to assess the impact of human xenografts or prototype anti-cancer therapies on the host mouse HSC in these strains.

In this study, we examined CD27 and CD201 expression on bone marrow (BM) cells in NOD-scid and NSG mice. We demonstrate staining protocols using CD27 and CD201 with FLT3, CD48, and CD150 are complementary to enrich functional HSC in these strains. These antibodies could be combined to prospectively enrich HSC as validated by serial dilution transplantations in recipient mice. We also investigated the overexpression of CD48 in NOD-scid and NSG mice. Furthermore, we identified that low SCA1 expression was limited to hematopoietic cells, whereas BM mesenchymal stromal cells (MSC) and endothelial cells expressed SCA1 at levels similar to C57BL/6 mice.

**Methods**

**Mice**

Mouse experiments were approved by both University of Queensland's and Queensland University of Technology Animal Ethics Committees. C57BL/6 and NOD-scid mice were purchased from Australian Resource Centre (Cannin Vale, WA, Australia). NSG mice (Jackson Laboratories, Bar Harbor, ME), were bred at the Translational Research...
Institute Biological Research Facility (Brisbane, Australia). Mice were 7-8 weeks old at experimentation.

**Sample isolation**

BM was flushed from femurs using phosphate buffered saline (PBS) containing 2% fetal bovine serum (FBS). Spleens were harvested from mice and processed in PBS and 2% FBS using the Miltenyi gentleMACS using a C-type tube (Bergisch Gladbach, Germany). Blood was collected via cardiac-puncture in 3.2% sodium citrate. Each fraction was counted using a Coulter AcT Diff Analyzer (Beckman Coulter, BC).

To isolate BM stromal/endothelial cells, bones were harvested from NSG and C57BL/6 mice. BM was flushed and discarded and bones treated with 1 mg/mL collagenase type-1 (Worthington) as previously described (23). Blood cells were depleted using the EasySep™ Mouse Mesenchymal Stem/Progenitor Cell Enrichment Kit (Cat no. 19771 StemCell Technologies) following manufacturer's protocol.

BM-MSC were isolated from NSG femurs using a modification of previously described protocol (24) (see supplementary methods).

**Flow cytometry**

All antibodies and stains used are described in Supplementary Table S1.

HSC stains were performed on 5x10^6 BM cells. Lineage stains were on 10^6 BM or spleen cells. Cells were stained in PBS and 2% FBS containing 0.1 µg/mL purified rat anti-CD16/CD32 (Fc Block) (BD Bioscience), with the appropriate antibody cocktail. Cells were then washed and resuspended in PBS plus 2% FBS containing 2 µg/mL dead cell discriminator dye 7-amino-actinomycin D (7-AAD) (Invitrogen) and analyzed on a CyAn flow cytometer (BC).

Stromal and endothelial cells were stained with the “endosteal” stain (Supplementary Methods and Table S1). Samples were analyzed on a Fortessa flow cytometer (BD Bioscience).

Flow cytometry data were analyzed with FlowJo v10 software (FlowJo LLC, Ashland, OR).
Transplantations

Male donor BM cells were enriched for c-KIT by magnetic-activated cell sorting. Lin\textsuperscript{-} KIT\textsuperscript{+} (LK) CD27\textsuperscript{+}CD201\textsuperscript{+}FLT3\textsuperscript{+}CD48\textsuperscript{-}CD150\textsuperscript{-} and LK CD27\textsuperscript{+}CD201\textsuperscript{+}FLT3\textsuperscript{-}CD48\textsuperscript{-}CD150\textsuperscript{-} (NOT GATE) were sorted on a FACS Aria Fusion sorter (BD Bioscience). Sorted cells were washed, counted and defined cell doses were resuspended in saline with 2% heat-inactivated FBS containing 100,000 irradiated (15 Gy) BM carrier cells. Grafts were then injected retro-orbitally into female recipients 24 hours post 2.5 Gy total-body \(\gamma\) irradiation (\(^{137}\text{Cs}, \text{Gammacell 40 Exactor, Best Theratronics, Ontario, Canada}).

Engraftment was monitored with regular bleeds. At 18 weeks post-transplant, BM, spleen, and blood were harvested. Chimerism by donor male cells was determined by Y-chromosome PCR based on previous protocols (6, 25) as outlined in supplementary methods. Engraftment was considered positive when female recipients had >1% male DNA in the blood.

Results

Comparison of SCA1, CD48 and CD150 expression in LK CD27\textsuperscript{+} CD201\textsuperscript{+} cells in C57BL/6, NOD-scid and NSG mice

Bone Marrow cells from C57BL/6, NOD-scid, and NSG mice were stained with a cocktail of antibodies combining the traditional markers (Lineage, c-KIT, SCA1, FLT3, CD48, CD150) (26) together with more recently proposed markers CD27 and CD201 (13). After gating live cells, LK cells were examined for CD27 and CD201 expression (see gating strategy in Supplementary Figure S1). LK cells had a similar profile for CD27 and CD201 expression as previously reported for C57BL/6 and NOD strains (13) (Figure 1A-C). The LK CD27\textsuperscript{+} CD201\textsuperscript{+} population labelled 0.019\% \pm 0.007\% (mean \pm standard deviation) of live BM nucleated cells in C57BL/6 mice, 0.100\% \pm 0.012\% in NOD-scid and 0.041\% \pm 0.014\% in NSG mice (Figure 1D). When calculated as cells per femur, NOD-scid mice had significantly more LK CD27\textsuperscript{+} CD201\textsuperscript{+} cells than C57BL/6 and NSG mice (Supplementary Table S2 and Figure 1E). When the LK CD27\textsuperscript{+} CD201\textsuperscript{+} were back-gated for SCA1 and c-KIT expression, SCA1 staining was low in the NOD-scid and NSG mice compared to C57BL/6 which were predominately SCA1\textsuperscript{*} (Figure 1F-I). This resulted in a large proportion of the phenotypic
HSC defined by the LK CD27⁺ CD201⁺ phenotype (13) in NOD-scid and NSG mice falling in the SCA1⁻ gate compared to C57BL/6 (Figure 1J). Consequently, any calculation of phenotypic HSC numbers using the classic LSK phenotype may underestimate the actual number of HSC in NOD-scid and NSG mice when calculated as cells per femur (Figure 1K).

Next, we determined if CD27 and CD201 staining was compatible or complementary with FLT3⁻, CD48⁻ and CD150⁺ staining to phenotypically identify LT-HSC. Live LK CD27⁺ CD201⁺ cells were gated for FLT3⁻, CD150, and CD48 expression analyzed for each mouse strain (Figure 2A-C). A similar CD150⁺ and CD48⁻ LT-HSC profile was observed between the three strains. The frequency of LK CD27⁺ CD201⁺ FLT3⁻ CD48⁻ CD150⁺ cells amongst live BM nucleated cells was similar between C57BL/6 and NOD-scid mice but reduced in NSG mice (Supplementary Table S2, Figure 2D). When calculated as cells per femur, C57BL/6 and NOD-scid mice had similar levels of phenotypic LT-HSC per femur, whereas NSG had a significantly lower number of phenotypic LT-HSC cells per femur (Supplementary Table S2, Figure 2E).

As it has been proposed that in the absence of SCA1 staining, the LK FLT3⁻ CD48⁻ CD150⁺ phenotype is sufficient to quantify mouse HSC (27), we further examined the expression of CD27 and CD201 in this population. In C57BL/6 mice, only 17.6% LK FLT3⁻ CD48⁻ CD150⁺ were positive for both CD27 and CD201 (Supplementary figure S2). As it has been previously reported that all HSC reconstitution activity is within the Lin⁻ CD27⁺ CD201⁺ population (13), this suggest that it is necessary to add CD27 and CD201 stain in order to further enrich HSC within the Lin⁻ CD117⁺FLT3⁻ CD48⁻CD150⁺ population. Likewise, in NOD-scid and NSG mice, only 28.6-32.9% LK FLT3⁻ CD48⁻CD150⁺ were positive for both CD27 and CD201.

High expression of CD48 in NOD-scid and NSG mice

In this analysis of CD48 and CD150 HSC detection (Figure 2A-C), we noticed that CD48 was more highly expressed in NOD-scid and NSG mice compared to C57BL/6 as revealed by CD48 expression overlays of LK CD27⁺ CD201⁺ FLT3⁻ cells from the different mouse strains (Figure 2F). In addition, the CD48 mean fluorescence intensity for the whole LK CD27⁺ CD201⁺ FLT3⁻ population was significantly reduced in C57BL/6 mice compared to the other mouse strains (Figure 2G).

The ligand for CD48 is CD244 (28) and is expressed by NK cells, some T cells, and monocytes (29). As the NOD-scid and NSG mice are devoid of functionally mature B and T cells, and NSG lack NK cells (Supplementary Figures S3-4) we speculated that CD48
upregulation in NSG and NOD-scid mice may be due to low expression of the ligand CD244. To assess this, we performed a lineage and CD244 stain on BM and spleen cells (Figures 3-4) to measure CD244 expression on each cell subset defined in Supplementary Figures S3-4. In C57BL/6 mice, subsets of CD244+ cells were observed on all BM lineages (Figure 3C,F) but predominately on NK cells (Supplementary Table 3, Figure 3F). Within the C57BL/6 spleen (Figure 4), CD244 was highly expressed on a subset of NK cells as well as monocytes, macrophages, and the neutrophils/myeloid progenitors. In NOD-scid and NSG mice, the frequency of CD244+ was less than 1% of all lineages examined (Figures 3-4).

We detected some lymphocyte type cells that were B220+ in BM and spleen in both NOD-scid and NSG mouse BM (Supplementary Figures S3-4). In addition, NSG mice contained rare NK1.1+ cells whereas both NSG and NOD-scid had few CD3ε+ cells.

**LT-HSC are enriched in the Lin⁻ KIT⁺ CD27⁺ CD201⁺ FLT3⁻ CD48⁻ CD150⁺ subset in NSG mice**

Finally, we tested whether the combination of CD27 and CD201 with FLT3, CD48, and CD150 markers could identify functional LT-HSC in NSG mice by serial dilution transplantation assay into non-lethally irradiated syngeneic recipients (Figure 5). As it has been previously shown that the whole CRU activity is contained within the Lin⁻ CD27⁺ CD201⁺ fraction of the BM in NOD mice (13), we further characterized the functional properties of these cells stained additionally with FLT3, CD48, and CD150 antibodies. We sorted two subsets of the CD27⁺ CD201⁺ population from the BM of male NSG mice, namely 1) LK CD27⁺ CD201⁺ FLT3⁻ CD48⁻ CD150⁺ cells (CD48⁻CD150⁺ gate) and 2) LK CD27⁺ CD201⁺ FLT3⁻ cells that were not in the CD48⁻ CD150⁺ gate (NOT GATE) (Figure 5H, isotype controls in Supplementary Figure S5). We transplanted serial dilutions of these two populations (Figure 5-H) into sub-lethally irradiated (2.5 Gy) female NSG recipient mice together with 100,000 lethally irradiated whole BM as carrier cells. At 8, 12, and 16 weeks a small amount of blood was lysed for longitudinal analysis of donor engraftment by genomic quantitative PCR using primers specific for the Y chromosome Sry gene compared to biallelic mouse Il6 gene. In preliminary experiments, we validated this method of quantifying relative male cell number by mixing a known amount of male versus female cells to demonstrate that the assay readout reflected the linear dilution series (Supplementary Figure S6). A level of >1% donor male cells at the 18 week harvest point was considered to be a successful reconstitution of the host (Supplementary table S4).
In transplanted recipients, we measured chimerism between 8 and 18 weeks. Robust long-term male donor chimerism in recipients that received 50 or 150 CD48⁻ CD150⁺ gate cells whereas there was very “low” frequency of long-term chimerism in recipients of NOT GATE cells (Figure 5I-J). Poisson’s distribution analysis (Figure 5K) showed a 32-fold enrichment (p = 1.86 x10⁻⁵) in competitive repopulation unit (CRU) frequency in the CD48⁻ CD150⁺ gate (1 in 179 cells) compared to the NOT GATE cells (1 in 5,786 cells) confirming that the FLT3⁻ CD48⁻ CD150⁺ phenotype complements the CD27⁺ CD201⁺ phenotype to further enrich in functional LT-HSC. It is also important to note that the 1 / 5,786 CRU frequency found in the NOT gate was due to a single recipient of the highest donor cell dose which had a very low level of engraftment (less than 3%) compared to recipient of CD48⁻ CD150⁺ cells (Supplementary Table S4). Therefore the CRU frequency in the NOT gate could be overestimated. Nevertheless, by multiplying the CRU frequency obtained from each gate by the number of cells in each gate, we found that 70% of the CRU contained within LK CD27⁺ CD201⁺ FLT3⁻ cells was within the CD48⁻ CD150⁺ subset (Supplementary Table S5).

**SCA1 expression in unaltered in BM endothelial and mesenchymal cells in NSG mice**

As the NSG mice and NOD-SCID mice blood cells have a low SCA1 expression in HSPC (Figure 1I), we compared SCA1 expression in BM endothelial cells and MSC from C57BL/6 mice and NSG mice (Figure 6). Endostea cells were collected from collagenase-treated femurs, magnetically enriched in non-hematopoietic cells, and stained against CD45, lineage, CD31, CD51, SCA1, and PDGFRα antibodies (Gating Strategy 6A-D). CD45⁻ Ter119⁻ CD31⁺ BM endothelial cells (Figure 6E, 6F) expressed equivalent levels of SCA1 in C57BL/6 and NSG mice (Figure 6E-F, I). Likewise, BM MSC defined as CD45⁻ Ter119⁻ CD31⁻ CD51⁺ cells (Figure 6G, 6H) expressed similar levels of SCA1 in the PDGFRα⁺ subset which defines the PaS cells (30) (Figure 6J).

Finally, we found that plastic adherent BM mesenchymal stromal cells derived from NSG mice also expressed high levels of SCA1 (Figure 6K).

**Discussion**

Considering that all the LT-HSC reconstituting activity resides within the Lin⁻ CD27⁺ CD201⁺ population (13), we sought to determine the expression profile of these cells for FLT3, CD48 and CD150 antigens which are classically used to identified LT-HSC and various subsets of multipotent progenitors (4, 5, 26). We found that in all three strains irrespective of SCA1 expression levels, only a small subset of LK CD27⁺ CD201⁺ cells was
also FLT3–CD48–CD150+, a phenotype that defines LT-HSC when used in combination with SCA1 positivity (26). Conversely, only a minority of LK FLT3–CD48–CD150+ cells were double positive for CD27 and CD201. Using a stringent serial dilution long-term transplantation assay, we demonstrate that CRU are 32-fold enriched in the small FLT3–CD48–CD150+ subset of the LK CD27+CD201+ population from NSG mice despite negative to low levels of SCA1 expression. This demonstrates that CD27 and CD201 positivity is complementary to the FLT3–CD48–CD150+ phenotype to identify functional LT-HSC and can be used to replace SCA1. This is particularly advantageous in strains that express low levels of SCA1 in hematopoietic cells such as NOD-scid and NSG strains, or because of treatments that increase or decrease SCA1 expression, such as irradiation and LPS administration (13, 27). We also noted a lower CRU frequency compared to the reported 50% CRU frequency in LK CD48CD150+ cells sorted from C57BL/6 (6). Competitive assays in lethally irradiated recipient mice with congeneric whole BM cells as a source of competing HSC were used in these studies (6) whereas in our present study, we sub-lethally irradiated our recipient mice (2.5 Gy) without exogenous competing HSC. This irradiation dose depresses circulating granulocytes and monocytes for only 8 days without transplant (not shown) and therefore spares an unknown number of host HSC. Consequently, this sublethal irradiation of the hosts creates a competitive assay between the residual female host HSC and the transplanted male HSC. This could in part explain the relatively low frequency of reconstituting cells that we measured in LK CD27+CD201+FLT3–CD48–CD150+ cells from NSG mice. An additional factor to consider for this relatively low frequency of reconstituting cells in the LK CD27+CD201+FLT3–CD48–CD150+ fraction from NSG mice is the known engraftment defect of HSC caused by the scid mutation which would consequently reduce the reconstitution potential of the sorted cell populations (31, 32).

Our flow cytometry data revealed that the expression of CD48 was unusually higher in LK CD27+CD201+FLT3– cells from the NOD-scid and NSG mice compared to C57BL/6. We found that the expression of CD244, the physiological ligand of CD48, was dramatically reduced in myeloid cells and lymphocytes from NOD-scid and NSG BM and spleen. Although NOD-scid and NSG mice have very low frequencies of T and NK cells that would express CD244, expression of CD244 in all myeloid lineages was also markedly reduced in the BM and spleen of NOD-scid and NSG mice. Therefore, it is tempting to speculate that CD48 upregulation in NOD-scid derived strains is caused by the low expression of its ligand CD244. However, this potential mechanism will need to be confirmed in C57BL/6 mice deficient in the CD244 gene.

Interestingly, NOD-scid mice still contain an NK cell population (33) but we did not detect higher numbers of NK1.1+ cells compared to NSG mice. As we did not perform
functional assays, we cannot conclude from our experiments whether NSG mice had less functional NK compared to the NOD-scid mice. Literature does indicate that CD3+ and primitive B cells are present in these mice but do not develop into mature functional lymphocytes (33). The scid mutation is known to eliminate the B and T cells at the education stage of development during VDJ recombination (34). This means that the BM will develop immature B and T precursors, which migrate into the circulation but cannot fully mature into functional lymphocytes. With age, NOD-scid mice are known to have some ‘leakiness’ and develop functional B and T cells while NSG do not (33). Previous studies on these mice have focused on the spleen and/or blood (20, 33) which are locations of mature B and T cells and did not examine the BM in which these cells develop initially. As we used 8-week old mice, this small percentage of CD3ε+ and B220+ cells may represent immature lymphoid cells. The use of a more mature B cell markers such as CD19 and surface IgM (sIgM) could have confirmed the absence of mature CD19+ sIgM+ B cells in these mice (33, 35).

Beside hematopoietic cells, SCA1 is expressed by various cell types such as mesenchymal and endothelial cells and is considered a progenitor/stem cell marker in many adult mouse tissues (36). In particular, SCA1 is known to be expressed by immature mesenchymal stromal cells in the BM and skeletal muscle, as well as by BM endothelial cells (30, 37, 38). The literature is conflicting as to whether SCA1 expression on stromal cells is dependent on the mouse strain. For instance, some groups have identified that cultured plastic adherent MSC derived from BALB/c (39, 40) and CBA (40) mice are SCA1+ (both haplotype LY6.1 mice). In contrast, other groups reported that the SCA1 staining on MSC was restricted to plastic adherent cultured MSC from the C57BL/6 and FVB/N strains whereas DBA1-derived MSC expressed low levels and BALB/c-derived MSC were negative (41). As SCA1 has been recently described as an activation marker facilitating cell cycling (8, 42) and mesenchymal progenitor cell self-renewal in vivo (43), culturing these cells in vitro could activate SCA1 expression and may explain this discordance. In our experiments, we find that low SCA1 expression is restricted to HSPC in NSG mice. Both freshly isolated BM endothelial cells (Figure 5G) and primitive mesenchymal progenitor cells (Figure 5H) from NSG mice expressed high levels of SCA1 similar to C57BL/6 mice as previously reported (44, 45). The high expression level of SCA1 on mesenchymal cells from NOD-scid and NSG mice is consistent with the absence of the osteoporotic phenotype that is observed in SCA1 knockout mice (43). Our results therefore suggest that lower SCA1 expression may be limited to hematopoietic cells in NSG mice and may be a result of the original source of the scid mutation that was derived from the BALB/c background, a LY6.1 haplotype mouse or from the NOD background (20, 46).
In conclusion, co-staining for CD27 and CD201 can be used in place of SCA1 to identify HSC in NOD-scid and NSG mice where SCA1 expression is weak. However, when Lin\(^-\) CD27\(^+\) and CD201\(^+\) phenotype is combined with FLT3\(^-\) CD48\(^-\) CD150\(^+\) phenotype, HSC with long-term engraftment potential are further enriched in NOD-scid and NSG mice. Compared to recent studies that focused only on Lin\(^-\) CD27\(^+\) CD201\(^+\) cells (32, 47), we show that within this population the small subset that is FLT3 and CD48 negative and CD150 positive is enriched in LT-HSC activity in NSG mice in a rigorous serial dilution long-term competitive transplantation assay. This alleviates the need for SCA1 staining, which is expressed at very low levels in these mice. In addition, we identify a non-reported up-regulation of CD48 in NOD-scid and NSG mice possibly due to the low expression of its ligand CD244. Finally, the low SCA1 expression in NSG mice seems limited to the hematopoietic compartment as SCA1 expression remains high in primary BM endothelial and mesenchymal cells. Overall, our new strategy may provide a more accurate method to quantify murine HSC within xenograft models using NOD-scid derived strains. For instance, in previous work (48, 49) humanized scaffolds seeded with human MSC were transplanted into NOD-scid mice and once humanized ectopic bone organoid established, injected with human BM or cord blood CD34\(^+\) cells. The relative quantification of the seeding of humanised ectopic bone scaffolds by human versus mouse HSC was difficult due to low SCA1 expression by NOD-scid and NSG HSC. Likewise, in a common xenotransplanted model of NSG mice engrafted with human cold blood CD34\(^+\) HSC, we were able to demonstrate that hypoxia-inducible factor prolyl hydroxylase inhibitor can rescue human HSPC mobilization defect in NSG mice but we were unable to show a similar effect on mouse HSC due to their low SCA1 expression (50). Therefore, this new staining strategy identifying Lin\(^-\) KIT\(^+\) CD27\(^+\) CD201\(^+\) FLT3\(^-\) CD48\(^-\) CD150\(^+\) cells as mouse HSCs in NOD-scid-derived strains will enable a more accurate measurement of the relative colonisation or distribution of mouse bones or ectopic bone organoids by endogenous mouse HSC versus xenotransplanted human HSC.

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**Author Contributions:**

BN conceptualized the work, planned and completed experiments, analyzed data, created figures, wrote and reviewed the manuscript. JPL, EDW, and MRD conceptualized the work, wrote, and edited the manuscript.

**Conflict of Interest:**

All authors report no conflict of interest with this paper.
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Figure Legends

Figure 1. CD27 and CD201 expression in Lin⁻ KIT⁺ BM HSPC from different NOD-scid-derived mouse strains. Expression of CD27 and CD201 in viable Lin⁻ KIT⁺ BM HSPC BM cells from C57BL/6 (A), NOD-scid (B) and NSG mice (C). (D) Frequency of Lin⁻ KIT⁺ CD27⁺ CD201⁺ cells within BM leukocytes and (E) absolute number of Lin⁻ KIT⁺ CD27⁺ CD201⁺ cells per femur in the three strains. Comparison of SCA1 and KIT expression in Lin⁻ KIT⁺ CD27⁺ CD201⁺ cells (Red overlay) compared to Lin⁻ KIT⁺ CD27⁺ CD201⁻ cells (blue overlay) in C57BL/6 (F), NOD-scid (G) and NSG (H) mice. (I) Overlay of SCA1 expression in Lin⁻ KIT⁺ CD27⁺ CD201⁺ cells from C57BL/6 (black), NOD-scid (blue) and NSG mice (red). (J) Percentage of Lin⁻ KIT⁺ CD27⁺ CD201⁺ cells that are SCA1⁺. (K) Number of Lin⁻ CD27⁺ CD201⁺ cells that are SCA1⁺ per femur. Data are average ± SD of n=5 mice per group. P-values were calculated by ANOVA with Tukey corrections, * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001, **** p ≤ 0.0001.

Figure 2. Complementarity of CD27 and CD201 with FLT3, CD48 and CD150 staining in different mouse strains. Gating strategy is in Supplementary Figure S1. Lin⁻ KIT⁺ CD27⁺ CD201⁺ FLT3⁻ BM cells were gated and analyzed for CD150 and CD48 expression in C57BL/6 (A), NOD-scid (B) and NSG (C) mice. Frequency (D) and total number (E) of Lin⁻ KIT⁺ CD27⁺ CD201⁺ FLT3⁻ CD48⁻ CD150⁺ cells per femur in each strain. (F) Overlay of CD48 expression in Lin⁻ KIT⁺ CD27⁺ CD201⁺ FLT3⁻ BM cells from C57BL/6 (grey shaded), NOD-scid (blue) and NSG (red) mice. (G) Geometric mean fluorescence intensity of CD48 on Lin⁻ KIT⁺ CD27⁺ CD201⁺ FLT3⁻ cells between mouse strains. Data are average ± SD of n=5 mice per group. P values were calculated by ANOVA with Tukey corrections with multiple comparisons, * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001, **** p ≤ 0.0001.

Figure 3. CD48 ligand CD244 is poorly expressed in the BM of NOD-scid and NSG mice. (A) Viable single cells were gated into a lymphoid (CD11b⁻ low side scatter) and myeloid (CD11b⁺) cells. (B) Myeloid cells were further separated using CD169 and F4/80 antigens. Monocytes were CD11b⁺ F4/80⁺ CD169⁻; macrophages CD11b⁺ F4/80⁺ CD169⁺; and neutrophils and remaining myeloid progenitors were CD11b⁺ F4/80⁻ CD169⁻. (C) CD244 expression was measured in each subset in each mouse strain and plotted as numbers CD244⁺ cells per femur. (D) Lymphoid cells were separated using B220 and NK1.1 antigens to identify B (CD11b⁻ B220⁺ NK1.1⁻) and NK (CD11b⁻ NK1.1⁺) cells. (E) The B220⁻ NK1.1⁻ gate was then plotted for CD3ε expression to identify T cells (CD11b⁻ B220⁻ NK1.1⁻ CD3ε⁻). (F) CD244 expression on lymphoid subsets in each mouse strain. Numbers CD244⁺ cells in each subset per femur. Data are mean ± SD of n=5 mice per group. P-values were
calculated by ANOVA with Tukey corrections with multiple comparisons: ** p ≤ 0.01, *** p ≤ 0.001, **** p ≤ 0.0001.

**Figure 4.** CD48 ligand CD244 is poorly expressed in the spleen of NOD-scid and NSG mice. (A) Viable single cells were gated with CD11b into a lymphoid (CD11b⁻ low side scatter) and myeloid (CD11b⁺) cells. (B) Myeloid cells were further separated using CD169 and F4/80 antigens. Monocytes were CD11b⁺ F4/80⁺ CD169⁻; macrophages CD11b⁺ F4/80⁺ CD169⁺; and neutrophils and remaining myeloid progenitors were in the CD11b⁺ F4/80⁻ CD169⁻ gate. (C) CD244 expression was measured in each subset in each mouse strain and plotted as numbers CD244⁺ cells per femur. (D) Lymphoid cells were separated using B220 and NK1.1 antigens to identify B (CD11b⁻ B220⁺ NK1.1⁻) and NK (CD11b⁻ NK1.1⁺) cells. (E) The B220⁻ NK1.1⁻ gate was then plotted for CD3ε expression to identify T cells (CD11b⁻ B220⁻ NK1.1⁻ CD3ε⁻). (F) CD244 expression on lymphoid subsets in each mouse strain. Numbers CD244⁺ cells in each subset per femur. Data are average ± SD of n=5 mice per group. P-values were calculated by ANOVA with Tukey corrections with multiple comparisons, * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001, **** p ≤ 0.0001.

**Figure 5.** CRU are enriched in the Lin⁻ KIT⁺ CD27⁺ CD201⁺ FLT3⁻ CD48⁻ CD150⁺ population in NSG mice. (A) Transplantation outline using sorted male donor cells transplanted into sub-lethally irradiated (2.5 Gy) female NSG mice to test CRU activity. (B-H) Sorting profile from donor male c-KIT enriched BM into either the Lin⁻ KIT⁺ CD27⁺ CD201⁺ FLT3⁻ CD48⁻ CD150⁺ gate or the Lin⁻ KIT⁺ CD27⁺ CD201⁺ FLT3⁻ NOT GATE. (I) Frequencies of donor engraftment in blood at 18 week harvest. (J) Time-course of male donor cell engraftment in each transplanted mouse. Warm colours are for dilutions of sorted Lin⁻ KIT⁺ CD27⁺ CD201⁺ FLT3⁻ CD48⁻ CD150⁺ cells, cold colours are dilutions of Lin⁻ KIT⁺ CD27⁺ CD201⁺ FLT3⁻ NOT GATE sorted cells. J) Frequency of donor engraftment at 18 weeks post-transplantation and Poisson’s distribution of donor HSC. tx: transplant.

**Figure 6.** Comparative SCA1 expression in BM endothelial and mesenchymal cells from C57BL/6 and NSG mice. (A) Live single cells were gated for non-hematopoietic CD45⁻ Ter119⁻ cells (B). From these CD45⁻ Ter119⁻ cells, endothelial cells were gated as CD31⁺ (C). The remaining CD31⁻ non-endothelial cells were gated for CD51⁺ mesenchymal cells (D). SCA1 expression in BM endothelial cells was similar in C57BL/6 (E) and NSG mice (F) as seen in overlay (I). CD45⁻ Ter119⁻ CD31⁻ CD51⁺ mesenchymal cells were further gated for PDGFRα and SCA1 expression. SCA1 expression in mesenchymal cells was comparable in C57BL/6 mice and NSG mice in dot-plots and overlay (G, H, J). (K) SCA1 expression in MSC cultured from NSG mice. These plots are pooled from 4 separate mice for each strain.
Figure 2.

A. C57bl/6

B. NOD-scid

C. NSG

D. Frequency of Ly-6K^+CD27^+CD201^+FLT3^-CD150^+CD48^- in BM

E. Total Ly-6K^+CD27^+CD201^+FLT3^-CD48^-CD150^+ per femur

F. CD48 Geometric Mean Fluorescence Intensity

G. CD48 Geometric Mean Fluorescence Intensity
Complementarity of CD27, CD201, FLT3, CD48, and CD150 cell surface staining to identify long-term reconstituting mouse hematopoietic stem cells in immunodeficient non-obese diabetic severe combined immune deficient derived strains

Short Title: CD27, CD201, FLT3, CD48, and CD150 identify HSC in mice

Bianca Nowlan, Elizabeth D. Williams, Michael R. Doran, and Jean-Pierre Levesque

Supplementary Methods

Flow cytometry
HSC stain was performed on 5x10^6 BM cells from a femur flushed with 1 mL of PBS + 2% FBS. Cells were stained in PBS+ 2% FBS containing 0.1 µg/mL purified rat anti-mouse CD16/CD32 antibody (Fc Block) (BD Bioscience), lineage (CD5, CD3ε, Ter119, CD11b, Ly6G/Ly6C, CD41)-FITC, anti-SCA1-PE, CD150-Brilliant Violet (BV)-605, c-KIT-APC-Cy7, CD48-Pacific Blue, FLT3-PE-CF594, CD27-PECy7 and CD201-biotin. Control stain with isotype-matched antibody controls instead of CD27, CD201, and CD48 antibodies were used to position gates correctly. Cells were incubated for 40 minutes and washed before a secondary stain of streptavidin-APC in PBS+ 0.5% bovine serum albumin (BSA) for 30 minutes. Samples were then washed and resuspended in PBS with 2% FBS containing 2 µg/mL dead cell discriminator dye 7-amino-actinomycin D (7-AAD) (Invitrogen) and analyzed on a CyAn flow cytometer (Beckman Coulter). Full gating strategy for HSC is provided in Supplementary Figure S1. All antibodies used in the experiments are listed in Supplementary Table S1.
Lineage stain was performed on 10^6 BM or spleen cells from each mouse. Cells were stained in PBS+ 2% FBS containing Fc Block, CD11b-PECy7, B220-APC-Cy7, CD3ε-Pacific Blue, NK1.1-PE, F4/80-AlexaFluor647, CD169-FITC, and CD244-biotin. Control stain with isotype-matched antibody controls instead of CD244 were used to position gates correctly. Cells were incubated for 40 minutes and washed before a secondary stain of streptavidin-BV-605 in PBS plus 5% BSA for 30 minutes. Samples were then washed and resuspended in PBS plus 2% FBS containing 7-AAD and analyzed on a CyAn flow cytometer (Beckman Coulter). Gating strategy for lineage stain is in Supplementary Figures S3-4.

All collected endosteal cells depleted of hematopoietic cells by magnetic enrichment as per manufactures protocol and were stained with PBS + 2% FBS containing Fc Block, CD45-BV785, Ter119-FITC, CD31-BV421, PDGFRα-APC, SCA1-PE-Cy7 and CD51-PE for 40 minutes. Control stain with isotype-matched antibody controls instead of PDGFRα, CD51, and SCA1 to were used to position gates correctly. Samples were washed and resuspended in PBS + 2% FBS containing 7-AAD and analyzed on a Fortessa flow cytometer (Beckman Dickson).

For the transplant sort experiments, 'full minus one' (FMO) color controls were used to determine correct gating strategy (Supplementary Figure S5).

**Measurement of male cell engraftment**

DNA was extracted using a modification of the whole blood protocol with a Genomic DNA spin column kit (Bioline BIO-52066). Blood samples were made up to 200 μL with PBS with 4 mM CaCl₂ for extraction with the kit. An aliquot of BM flush and splenocyte preparation equaling a fifth of one femur or a fifth of a whole spleen was used for genomic DNA preparation. Samples were digested at 56 °C for 3 hours and then centrifuged of 10,000 rpm to exclude all cell debris and DNA-containing supernatants collected. 10 ng DNA was used in a quantitative PCR reaction using the SYBR green master mix (Thermofisher 4309155) with 4 nM of primers for the sry gene versus the il6 gene. The level of male engraftment was determined by correlating the signal of the sry primers (2) (forward: 5'-TTATGGTGTTGGTCCC GTGT-3' and reverse: 5'-GGCCTTTTTTTCGGCTTCTGT) compared to total genomic expression of il6 gene (forward: 5' GACCACAGGACCATCCAATTTCATTTTGAAA and reverse: 3' GACCACAGGAGGAATGTCCACAAA): 50 cycles at 95°C for 30 seconds followed by a minute at 60°C. A calibration and validation of male genomic DNA were made by mixing male and female BM cells in known ratios before extraction to confirm the linearity of Y chromosome
DNA amplicons relative to the proportion of male cells (Supplementary Figure S6). The proportion of male cells in test samples was calculated by linear regression extrapolation from the calibration regression. Mice which were counted as positively reconstituted when more than 1% were of male origin (Supplementary Table 4).

Competitive reconstitution units (CRU) frequencies were calculated by Poisson’s statistic using the L-Calc software (Stem Cell Technologies). The number of CRU contained in each gate was then calculated by multiplying for each individual mouse the number of cells in each sort gate by the frequency of CRU in each gate (Supplementary Table S5).

**Isolation culture and staining of Mesenchymal Stromal Cells**

Mouse MSC were isolated from male NSG mice based on plastic adherence as previously reported (1). Briefly femurs and tibias were collected and gently crushed in a mortar with pestle to remove the BM. Bone fragments were washed repeatedly with PBS+ 2% FBS. Bone chips were then incubated in 3 mg/ml collagenase type 1 (Worthington) made in Iscove's Modified Dulbecco's Media (IMDM) for 20 minutes at 37 °C with shaking. Cells were then filtered with 70 µm cell strainer and placed into culture with α-Minimum Essential Medium (MEM) supplemented with 10% FBS, 1% penicillin-streptomycin and 100 µM phospho-ascorbic acid in atmosphere with 5% CO₂ at 37 °C. Medium was changed twice a week and passages were counted at each expansion step. Tissue culture reagents were purchased from Thermofisher unless mentioned otherwise.

After first passage, mouse MSC were depleted of mouse leukocytes by sorting the CD45⁻ population with a Beckman Coulter Astrios cell sorter. At passage 2, mouse MSC were stained with anti-SCA1 antibody or isotype-matched control to measure its expression on the Cytoflex (Beckman Coulter) flow cytometer.

**Statistics**

Statistics were performed with Graph Pad Prism 7 (La Jolla, CA) using one-way ANOVA analysis with Tukey Correction. The frequencies of competitive repopulation units (CRU) in the transplantation assays were calculated by Poisson’s distribution statistics using the L-Calc software (Stem Cell Technologies, Vancouver, Canada).
Supplementary References

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Supplementary Table S1. Flow Antibodies used in experiment separated into each test

| HSC stain | antibody | company | clone | catalogue number | Antibody concentration |
|-----------|----------|---------|-------|------------------|-----------------------|
|           | CD5 FITC | Biolegend | 53-7.3 | 100606           | 2.5 µg/mL             |
|           | CD3ε FITC | Biolegend | 17A2  | 100204           | 2.5 µg/mL             |
|           | B220 FITC | Biolegend | RA3-6B2 | 103206          | 2.5 µg/mL             |
|           | Ter119 FITC | Biolegend | TER-119 | 116206       | 2.5 µg/mL             |
|           | CD11b FITC | Biolegend | M1/70 | 101206           | 2.5 µg/mL             |
|           | Ly6G/Ly6C (Gr-1) FITC | Biolegend | RB6-8C5 | 108406       | 2.5 µg/mL             |
|           | CD41 FITC | Biolegend | MWRReg30 | 133904     | 2.5 µg/mL             |
|           | Sca1 PE | Biolegend | D7  | 108108           | 2 µg/mL               |
|           | CD150 Brilliant Violet 605 | Biolegend | TC15-12F12.2 | 115927       | 1 µg/mL               |
|           | CD117 (c-KIT) APC-Cy7 | Biolegend | 2B8 | 105826           | 1 µg/mL               |
|           | CD48 Pacific Blue | Biolegend | HM48-1 | 103418      | 2.5 µg/mL             |
|           | CD201 (EPCR) Biotin | Biolegend | RCR-16 | 141508      | 2.5 µg/mL             |
|           | CD27 PE-Cy7 | Biolegend | LG.3A10 | 124216      | 1 µg/mL               |
|           | CD135 (Flt3) PE-CF594 | BD Bioscience | A2F10.1 | 562537      | 2 µg/mL               |
|           | Streptavidin APC | Biolegend | 405207 | 1 µg/mL       |                      |
| Lineage stain | antibody | company | clone | catalogue number | Antibody concentration |
|           | CD11b PE-Cy7 | Biolegend | M1/70 | 101216           | 1 µg/mL               |
|           | B220 APC-Cy7 | Biolegend | RA3-6B2 | 103224       | 1 µg/mL               |
|           | CD3ε Pacific Blue | Biolegend | 17A2  | 100214           | 2.5 µg/mL             |
|           | NK1.1 PE | Biolegend | PK136 | 108708           | 2 µg/mL               |
|           | F4/80 Alexa Fluor 647 | Serotec | A3-1 | MCA497A647       | 2.5 µg/mL             |
|           | CD169 FITC | Biolegend | 3D6-112 | 142406      | 2.5 µg/mL             |
|           | CD244 Biotin | Biolegend | m2B4 (B6)458.1 | 133505     | 2.5 µg/mL             |
|           | Streptavidin Brilliant Violet 605 | Biolegend | 405232 | 0.5 µg/mL      |                      |
| Endosteal stain | antibody | company | clone | catalogue number | Antibody concentration |
|           | CD45 BV785 | Biolegend | 30-F11 | 109839           | 2 µg/mL               |
|           | Terr-119 FITC | Biolegend | TER-119 | 116206       | 2.5 µg/mL             |
|           | CD31 BV421 | Biolegend | 390  | 102423           | 2 µg/mL               |
|           | CD140 (PDGFRα) APC | Biolegend | APA5  | 135908           | 2 µg/mL               |
|           | Sca1 PE-Cy7 | Biolegend | D7  | 108114           | 2 µg/mL               |
|           | CD51 PE | Biolegend | RMV-7 | 104106           | 2 µg/mL               |
| Tissue Culture MSC stain | antibody | company | clone | catalogue number | Antibody concentration |
|           | Sca1 PE-Cy7 | Biolegend | D7  | 108129           | 2 µg/mL               |
|           | Rat IgG1, κ | Biolegend | 400416 | 2 µg/mL       |                      |
|           | CD45 APC | Biolegend | 30-F11 | 103112           | 2 µg/mL               |
Supplementary Table S2. Cell frequencies and total cells per femur between mouse strains

|                  | C57bl/6          | NOD-scid        | NSG              |
|------------------|------------------|-----------------|------------------|
|                  | Frequency | Cells/Femur | Frequency | Cells/Femur | Frequency | Cells/Femur |
| L'K'CD27'CD201'  | 0.019%± 0.007%  | 5,872 ± 2,365  | 0.100%± 0.012%  | 23,077 ± 5,495 | 0.041%± 0.014% | 8,744 ±3,520 |
|                  | 0.0012%± 0.0004%| 362 ±125       | 0.0015%± 0.0003%| 332 ± 83     | 0.0007%± 0.0005%| 145 ± 99     |

Average ± standard deviation – 5 mice per group.
Supplementary Table S3. CD244 prevalence in bone marrow and spleen of mice strains

|                  | C57BL/6 | NOD-scid | NSG     |
|------------------|---------|----------|---------|
|                  | CD244+ Percent of parent | CD244+ Cells/ femur | CD244+ Percent of parent | CD244+ Cells/ femur | CD244+ Percent of parent | CD244+ Cells/ femur |
| **BONE MARROW**  |         |          |         |         |         |         |
| Monocytes (CD11b+ F4/80+ CD169+) | 10.85% ± 2.05% | 263,677 ± 58,364 | 0.35% ± 0.05% | 3,588 ± 865 | 0.54% ± 0.22% | 2,614 ± 738 |
| Macrophages (CD11b+ F4/80+ CD169+) | 2.79% ± 0.31% | 65,609 ± 9,949 | 0.6% ± 0.08% | 10,656 ± 2,349 | 0.73% ± 0.14% | 18,056 ± 1,578 |
| Neutrophils and myeloid progenitors (CD11b+ F4/80- CD169-) | 2.01% ± 0.35% | 88,499 ± 11,197 | 0.12% ± 0.04% | 4,934 ± 2,132 | 0.12% ± 0.06% | 2,210 ± 1,264 |
| **SPLEEN**       |         |          |         |         |         |         |
| Monocytes (CD11b+ F4/80+ CD169+) | 39.36% ± 4.36% | 110,051 ± 27,242 | 3.13% ± 1.4% | 1,797 ± 1,303 | 2.08% ± 0.83% | 955 ± 467 |
| Macrophages (CD11b+ F4/80+ CD169+) | 31.06% ± 2.09% | 6,075 ± 1,480 | 10.51% ± 8.21% | 1,431 ± 1,113 | 6.97% ± 4.66% | 633 ± 336 |
| Neutrophils and myeloid progenitors (CD11b+ F4/80- CD169-) | 23.86% ± 2.38% | 696,150 ± 9,656 | 0.27% ± 0.08% | 140 ± 73 | 0.29% ± 0.04% | 161 ± 35 |
| B cells (CD11b+ B220+) | 4.04% ± 1.77% | 5,318 ± 2,027 | 2.21% ± 0.59% | 586 ± 171 | 7.61% ± 5.74% | 1,094 ± 404 |
| NK cells (CD11b+ Nk1.1+) | 78.44% ± 2.04% | 128,456 ± 20,757 | 62.31% ± 12.27% | 4,858 ± 593 | 84.59% ± 6.04% | 7,687 ± 1,139 |
| T cell (CD11b+ B220+ CD3+) | 1.55% ± 0.41% | 74,026 ± 30,276 | 0.73% ± 0.26% | 3,570 ± 1,510 | 1.36% ± 0.47% | 2,621 ± 969 |
| **BONE MARROW**  |         |          |         |         |         |         |
| Monocytes (CD11b+ F4/80+ CD169+) | 39.36% ± 4.36% | 110,051 ± 27,242 | 3.13% ± 1.4% | 1,797 ± 1,303 | 2.08% ± 0.83% | 955 ± 467 |
| Macrophages (CD11b+ F4/80+ CD169+) | 31.06% ± 2.09% | 6,075 ± 1,480 | 10.51% ± 8.21% | 1,431 ± 1,113 | 6.97% ± 4.66% | 633 ± 336 |
| Neutrophils and myeloid progenitors (CD11b+ F4/80- CD169-) | 23.86% ± 2.38% | 696,150 ± 9,656 | 0.27% ± 0.08% | 140 ± 73 | 0.29% ± 0.04% | 161 ± 35 |
| B cells (CD11b+ B220+) | 4.04% ± 1.77% | 5,318 ± 2,027 | 2.21% ± 0.59% | 586 ± 171 | 7.61% ± 5.74% | 1,094 ± 404 |
| NK cells (CD11b+ Nk1.1+) | 78.44% ± 2.04% | 128,456 ± 20,757 | 62.31% ± 12.27% | 4,858 ± 593 | 84.59% ± 6.04% | 7,687 ± 1,139 |
| T cell (CD11b+ B220+ CD3+) | 1.55% ± 0.41% | 74,026 ± 30,276 | 0.73% ± 0.26% | 3,570 ± 1,510 | 1.36% ± 0.47% | 2,621 ± 969 |

Average ± standard deviation – 5 mice per group
Supplementary Table S4: Transplant results from mice including 12 and 16 week bleeds and 18 week harvest of blood, spleen and bone marrow.

| Group               | Cells tx | mouse # | Bleed (Weeks) | Harvest 18 weeks |
|---------------------|----------|---------|---------------|-----------------|
|                     |          |         | 12  | 16 | Blood | Bone | Marrow | Spleen |
| LT-HSC              | 10       | H1      | 0.01% | 0.01% | 0.01% | 0.06% | 0.03% |        |
|                     |          | H2      | 0.02% | 0.05% | 0.08% | 0.03% | 0.03% |        |
|                     |          | H3      | 0.01% | 0.11% | 0.00% | 0.05% | 0.03% |        |
|                     |          | H4      | 0.05% | 0.38% | 0.00% | 0.02% | 0.03% |        |
|                     |          | H5      | 0.41% | 0.04% | 0.00% | 0.10% | 0.11% |        |
|                     |          | H6      | 0.10% | 0.35% | 0.07% | 0.05% | 0.09% |        |
|                     |          | H7      | 0.01% | 0.14% | 0.12% | 0.05% | 0.03% |        |
|                     |          | H8      | 7.42% | 10.80% | 13.26% | 2.71% | 5.95% |        |
|                     |          | H9      | 3.67% | 1.91% | 1.17% | 2.50% | 0.48% |        |
|                     |          | H10     | 0.06% | 0.35% | 0.01% | 0.04% | 0.07% |        |
|                     |          | H11     | 20.32% | 10.49% | 9.16% | 0.07% | 0.27% |        |
|                     |          | H12     | 0.03% | 0.07% | 0.00% | 0.03% | 0.08% |        |
|                     |          | B1      | 5.46% | 3.05% | 2.07% | 0.17% | 1.46% |        |
|                     |          | B2      | 0.03% | 0.00% | 0.00% | 0.06% | 0.09% |        |
|                     |          | B3      | 0.15% | 0.00% | 0.01% | 0.03% | 0.05% |        |
|                     |          | B4      | 0.68% | 0.00% | 0.00% | 0.01% | 0.24% |        |
|                     |          | B5      | 0.03% | 0.29% | 0.01% | 0.08% | 0.05% |        |
|                     |          | B6      | 0.04% | 0.00% | 0.01% | 0.16% | 0.10% |        |
|                     |          | B7      | 0.09% | 0.00% | 0.00% | 0.05% | 0.10% |        |
|                     |          | C1      | 0.22% | 0.01% | 0.02% | 0.06% | 0.17% |        |
|                     |          | C2      | 54.98% | 59.43% | 23.36% | 2.21% | 11.69% |        |
|                     |          | C3      | 49.46% | 50.53% | 40.41% | 5.36% | 25.60% |        |
|                     |          | C4      | 33.42% | 12.03% | 19.56% | 1.40% | 1.37% |        |
|                     |          | C5      | 6.50% | 0.00% | 0.56% | 0.30% | 0.97% |        |
|                     |          | C6      | 2.24% | 0.36% | 0.00% | 0.00% | 0.08% |        |
| NOT GATE            | 10       | N1      | 0.00% | 0.05% | 0.00% | 0.04% | 0.03% |        |
|                     |          | N2      | 0.01% | 0.25% | 0.00% | 0.05% | 0.04% |        |
|                     |          | N3      | 0.01% | 0.10% | 0.06% | 0.04% | 0.06% |        |
|                     |          | N4      | 0.09% | 0.06% | 0.10% | 0.08% | 0.07% |        |
|                     |          | N5      | 0.00% | 0.17% | 0.00% | 0.06% | 0.09% |        |
|                     |          | N6      | 0.01% | 0.03% | 0.00% | 0.09% | 0.06% |        |
|                     |          | N7      | 0.00% | 0.02% | 0.07% | 0.07% | 0.10% |        |
|                     |          | N8      | 0.00% | 0.05% | 0.08% | 0.06% | 0.05% |        |
|                     |          | N9      | 0.00% | 0.03% | 0.01% | 0.07% | 0.04% |        |
|                     |          | N10     | 0.01% | 0.25% | 0.00% | 0.02% | 0.04% |        |
|                     |          | D1      | 0.06% | 0.00% | 0.02% | 0.00% | 0.05% |        |
|                     |          | D3      | 0.06% | 0.00% | 0.01% | 0.06% | 0.05% |        |
|                     |          | D4      | 0.07% | 0.00% | 0.00% | 0.04% | 0.03% |        |
|                     |          | D5      | 0.14% | 0.03% | 0.05% | 0.18% | 0.18% |        |
|                     |          | E1      | 0.04% | 0.00% | 0.01% | 0.07% | 0.18% |        |
|                     |          | E2      | 0.22% | 0.00% | 0.00% | 0.06% | 0.11% |        |
|                     |          | E3      | 0.04% | 0.00% | 0.00% | 0.12% | 0.20% |        |
|                     |          | E4      | 0.12% | 0.00% | 0.07% | 0.01% | 1.01% |        |
|                     |          | E5      | 0.12% | 0.00% | 0.10% | 0.07% | 0.10% |        |
|                     |          | F1      | 0.67% | 0.02% | 0.29% | 0.15% | 0.47% |        |
|                     |          | F2      | 12.14% | 0.46% | 1.38% | 2.22% | 0.95% |        |
|                     |          | F3      | 0.77% | 0.02% | 0.04% | 0.06% | 0.27% |        |
|                     |          | F4      | 0.04% | 0.00% | 0.10% | 0.11% | 0.24% |        |
|                     |          | F5      | 0.23% | 0.00% | 0.07% | 0.22% | 0.97% |        |

Percent male (sry+) engraftment of female mice at each bleed or harvest. Mice >1% in bold to indicate successful engraftment. Tx= transplanted.
Supplementary Table S5. Competitive reconstitution units per sort gate based on LT-HSC frequencies determined by serial dilution transplants

| Mouse | LK CD27+ CD201+ FLT3- | CD48- CD150+ | CRU Frequency | CRU/Gate | NOT GATE | CRU Frequency | CRU/Gate | Proportion CRU in CD48-CD150+ gate versus parent LK CD201+ CD27+ FLT3- gate |
|-------|------------------------|--------------|---------------|-----------|----------|---------------|-----------|----------------------------------------------------------------------------------|
| 1     | 10,407                 | 560          | 1/179         | 3.1       | 9,847    | 1/5,786       | 1.7       | 64.6%                                                                              |
| 2     | 11,912                 | 784          | 4.4           | 11,128    | 1.9      | 69.8%         | 0.8       | 72.4%                                                                              |
| 3     | 5,014                  | 374          | 2.1           | 4,640     | 1.5      | 76.9%         | Mean      | 70.9%                                                                              |
| 4     | 9,492                  | 892          | 5             | 8,600     |          | SD            | 5.2%      |                                                                                   |

Abbreviations: LK= lineage C-KIT+; CRU= competitive reconstitution units; SD= standard deviation.
Supplementary Figure S1. Gating Strategy to identify phenotypic HSC in C57BL/6 mice. 5x10^6 BM cells were stained with HSC antibody cocktail and analyzed by flow cytometry. (A-C) Viable single cells were gated as (D) Lineage-negative, c-KIT+ and (E) CD201 and CD27 positive. F) Isotype control for CD27 and CD201. Lin- KIT+ CD27+CD201+ cells were further gated on Flt-3 (G). Lin- KIT+ CD27+CD201+ cells gated to define the primitive FLT3-HSPC and subsequently CD48-CD150+ phenotypic HSC (H). (I) Lin- KIT+ CD27+ CD201+ cells (red) were examined for SCA1 and KIT expression and (J) overlaid with Lin- KIT+ CD27+CD201+ cells (blue). The position of the SCA1+ KIT+ gate is highlighted in red.
Supplementary Figure S2. Combination of CD48+ CD150+ gating and CD27+ CD201+ gating is necessary to identify phenotypic HSCs. 5x10^6 BM cells from each mouse strain were stained with HSC antibody cocktail and analyzed on flow cytometry. (A-C) Viable single cells were gated as (D) Lineage negative, c-KIT+ and (E) FLT3 negative, and then examined for CD48 and CD150 for the mice stains, C57BL/6 in black box (F), NOD-scid in blue box (I) and NSG in red box (L). L-K+FLT3-CD48-CD150+ cells were gated to CD27 and CD201 expression in C57bl/6 (G), NOD-scid (J) and NSG (M). Only a minority of Lin− KIT+ FLT3− CD48− CD150+ cells are also CD27+ CD201+. SCA1 expression heterogeneity was demonstrated at this level by taking the CD27+ CD201+ gate (red) versus the NOT GATE (blue) for the three mouse strains (H, K, and N).
**C57bl/6**

- **A**: Single Live cells
  - T cell gate
    - CD11b
    - B220
  - NK1.1
  - B220

- **D**: 7-AAD
  - CD11b
  - CD169
  - F4/80

- **G**: Myeloid
  - BM monocytes
  - BM macrophages

- **K**: BM T cell
  - NK1.1
  - B220

**NOD-scid**

- **B**: Single Live cells
  - T cell gate
    - CD11b
    - B220
  - NK1.1
  - B220

- **E**: 7-AAD
  - CD11b
  - CD169
  - F4/80

- **H**: Myeloid
  - BM monocytes
  - BM macrophages

- **J**: BM T cell
  - NK1.1
  - B220

**NSG**

- **C**: Single Live cells
  - T cell gate
    - CD11b
    - B220
  - NK1.1
  - B220

- **F**: 7-AAD
  - CD11b
  - CD169
  - F4/80

- **I**: Myeloid
  - BM monocytes
  - BM macrophages

- **L**: BM T cell
  - NK1.1
  - B220

**Cells per femur**

- **M**: BM monocytes
  - C57bl/6: 1x10^6
  - NOD-scid: 1.5x10^6
  - NSG: 2x10^6

- **N**: BM macrophages
  - NS: 3x10^6

- **O**: BM Neutrophil+ myeloid progenitors
  - C57bl/6: 6x10^6
  - NOD-scid: 2x10^6
  - NSG: 1x10^6

- **P**: BM B cell
  - C57bl/6: 6x10^6

- **Q**: BM NK
  - C57bl/6: 5x10^6

- **R**: BM T cell
  - C57bl/6: 2x10^6
Supplementary Figure S3. Quantification of lineage-positive leukocytes in the bone marrow of different mouse strains. Each mouse strain appears in a different coloured column box: Viable single cells were gated into lymphoid (CD11b- low side scatter) and myeloid (CD11b+) gates for each mouse strain (A-C). Myeloid cells were further separated using CD169 and F4/80 (D-F) as CD11b+ F4/80+ CD169+ monocytes, CD11b- F4/80+ CD169+ macrophages and CD11b+ F4/80- CD169+ gate which includes granulocytes and immature myeloid cells. Lymphoid cells (G-I) were plotted with B220 against NK1.1 to identify B (CD11b- B220+ NK1.1-) and NK (CD11b- NK1.1) cells. The B220- NK1.1- gate was plotted for CD3ε expression to identify CD3ε+ T cells (J-L). Total cells per femur for each cell type in histograms (M-R). Data are average ± SD of n=5 mice per group. P-values were calculated by ANOVA with Tukey corrections with multiple comparisons, * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001, **** p ≤ 0.0001.
Supplementary Figure S4. Quantification of lineage positive leukocytes in the spleen of different mouse strains. Each mouse strain appears in a different coloured column box: Viable single cells were gated into lymphoid (CD11b- low side scatter) and myeloid (CD11b+) gates for each mouse strain (A-C). Myeloid cells were further separated using CD169 and F4/80 (D-F) as CD11b+ F4/80+ CD169+ monocytes, CD11b+ F4/80+ CD169+ macrophages and CD11b+ F4/80- CD169- gate which includes granulocytes and immature myeloid cells. Lymphoid cells (G-I) were plotted with B220 against NK1.1 to identify B (CD11b- B220+ NK1.1-) and NK (CD11b- NK1.1) cells. The B220- NK1.1- gate was plotted for CD3ε expression to identify CD3ε+ T cells (J-L). Total cells per spleen for each cell type in histograms (M-R). Data are average ± SD of n=5 mice per group. P-values were calculated by ANOVA with Tukey corrections with multiple comparisons, * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001, **** p ≤ 0.0001.
Supplementary Figure S5. CD27, CD201, FLT3, CD48, and CD150 sort controls. A-C) single cell sorting. (D) Lineage negative, KIT+ gate to determine the full minus one controls for setting up sort parameters and gates. (E) is the minus CD201-APC control, (F) the minus CD27-PECy7 control. (G) shows the FLT3-PECF594 minus control and (H) shows the CD48-Pacblue and CD150-PE minus controls.
Supplementary Figure S6. qPCR titration of male donor genomic DNA versus female DNA for chimerism quantification. Amplification curves of sry (A) and il6 gene (B) in different mixtures with different proportions of male and female leukocytes. (C) The linear relationship between the proportion of male cells and intensity of sry genomic DNA relative to il6 genomic DNA.