ARTICLE

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

Bianca Nowlan,[1,2,3,4,5] Elizabeth D. Williams,[2,4,5] Michael R. Doran,[1,2,3,4,5,6,*] and Jean-Pierre Levesque[3,5,*]

[1]Stem Cell Therapies Laboratory, School of Biomedical Science, Faculty of Health, Queensland University of Technology (QUT), Brisbane; [2]School of Biomedical Science, Faculty of Health, Institute of Health and Biomedical Innovation, QUT, Kelvin Grove, Queensland; [3]Mater Research Institute – The University of Queensland, Woolloongabba; [4]Australian Prostate Cancer Research Centre - Queensland, Brisbane, Queensland; [5]Translational Research Institute, Woolloongabba, Queensland and [6]Australian National Centre for the Public Awareness of Science, Australian National University, Canberra, Australian Capital Territory, Australia

** MRD and JPL contributed equally to this work.*

Ferrata Storti Foundation

Haematologica 2020
Volume 105(1):71-82

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*^{scid}/Sz (NOD-*scid*) mice and NOD.CB17-*prkdc*^{scid} *il2rg*^{tm1Wjl}/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 linage-negative C-KIT$^+$ CD27$^+$ CD201$^+$ FLT3$^-$ CD48$^-$ CD150$^+$ population in serial dilution long-term competitive transplantation assays. We also make the novel observation that CD48 expression is up-regulated in Lin$^-$ KIT$^+$ 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.

Introduction

Blood myeloid and erythroid lineages are short-lived and require continuous replacement from hematopoietic stem cells (HSC) in the bone marrow (BM).[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 LSK population of cells, i.e., cells negative for B, T, myeloid and erythroid lineages (Lin$^-$), positive for c-KIT/CD117 and positive for stem cell antigen-1 (SCA1 or LY6A/E). Multipotent long-term reconstituting HSC (LT-HSC) are LSK cells that are negative for fms-like tyrosine kinase 3 (FLT3)/CD135 and CD48 and positive for signaling lymphocytic activation molecule (SLAMF1/CD150).[4,5] When transplanted, these HSC can clonally and serially reconstitute hematopoiesis in lethally irradiated mice.[5]

Correspondence:

JEAN-PIERRE LEVESQUE
jp.levesque@mater.uq.edu.au

MICHAEL DORAN
mike@mikedoranlab.com

Received: November 22, 2018.

Accepted: May 2, 2019.

Pre-published: May 9, 2019.

doi:10.3324/haematol.2018.212910

Check the online version for the most updated information on this article, online supplements, and information on authorship & disclosures: www.haematologica.org/content/105/1/71

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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% of blood leukocytes, whereas LY6A is expressed by 50-70% of 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 on 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.

The combination of CD27 and CD201 (endothelial protein C receptor – EPCR) 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 was 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 express both CD27 and CD201.[14,15] CD27 is a member of the tumor necrosis factor receptor family expressed on T, B, and natural killer (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 the pattern of CD201 expression is conserved between human and mouse HSC, unlike that of 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*^{scid} *il2rg*^{tm1Wjl}/Sz (NSG) mice for human xenografts has increased[19-21] relative to the parental (NOD.CB17-*prkdc*^{scid}/Sz, NOD-*scid*) mice. NSG mice do not express functional interleukin-2 receptor and therefore lack NK cells in addition to their lack of B and T cells from the parental NOD-*scid* strain, resulting in more profound immunosuppression and making the animals 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 be examined for the drugs' effects on the host mouse HSC content in order to detect potential adverse effects of the drugs. 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 BM cells in NOD-*scid* and NSG mice. We demonstrate that 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 those in C57BL/6 mice.

Methods

Mice

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

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 and a C-type tube (Bergisch Gladbach, Germany). Blood was collected via cardiac puncture into 3.2% sodium citrate. Each fraction was counted using a Coulter AcT Diff Analyzer (Beckman Coulter).

To isolate BM stromal/endothelial cells, bones were harvested from NSG and C57BL/6 mice. BM was flushed and discarded and the bones were 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. n. 19771 StemCell Technologies) following the manufacturer's protocol.

BM-MSC were isolated from NSG femurs using a modification of a previously described protocol[24] (see *Online Supplementary Methods*).

Flow cytometry

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

HSC stains were applied to $5x10^6$ BM cells, while lineage stains were applied to 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. The 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 (Beckman Coulter).

Stromal and endothelial cells were stained with an "endosteal" stain (*Online Supplementary Methods* and *Online Supplementary 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, USA).

Transplantations

Male donor BM cells were enriched for c-KIT by magnetic-acti-

vated cell sorting. LK CD27$^+$CD201$^+$FLT3$^-$CD48$^-$CD150$^+$ and LK CD27$^+$CD201$^+$FLT3$^-$CD48$^+$CD150$^-$ (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 h after 2.5 Gy total-body γ irradiation (^{137}Cs, Gammacell 40 Exactor, Best Theratronics, Ontario, Canada).

Engraftment was monitored with regular bleeds. At 18 weeks after transplantation, BM, spleen, and blood were harvested. Chimerism by donor male cells was determined by Y-chromosome polymerase chain reaction analysis based on previous protocols,[6,25] as outlined in the *Online 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$^+$ CD201$^+$ cells in C57BL/6, NOD-*scid* and NSG mice

BM cells from C57BL/6, NOD-*scid*, and NSG mice were stained with a cocktail of antibodies combining the traditional markers (Lin, 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 *Online 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$^+$ CD201$^+$ population labeled 0.019% ± 0.007% (mean ± standard deviation) of live BM nucleated cells in C57BL/6 mice, 0.100% ± 0.012% in NOD-*scid* mice and 0.041% ± 0.014% in NSG mice (Figure 1D). When calculated as cells per femur, NOD-scid mice had significantly more LK CD27$^+$ CD201$^+$ cells than had C57BL/6 and NSG mice (*Online Supplementary Table S2* and Figure 1E). When the LK CD27$^+$ CD201$^+$ were back-gated for SCA1 and c-KIT expression, SCA1 staining was lower in NOD-*scid* and NSG mice than in C57BL/6 mice, which were predominantly SCA1$^+$ (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 the proportion from C57BL/6 mice (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 investigated whether 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 in the three strains. The frequency of LK CD27$^+$ CD201$^+$ FLT3$^-$ CD48$^-$ CD150$^+$ cells among live BM nucleated cells was similar in C57BL/6 and NOD-*scid* mice but reduced in NSG mice (*Online Supplementary Table S2* and 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 (*Online Supplementary Table S2* and 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% of LK FLT3$^-$ CD48$^-$ CD150$^+$ cells were positive for both CD27 and CD201 (*Online Supplementary Figure S2*). As it has been previously reported that all HSC reconstitution activity is within the Lin$^-$ CD27$^+$ CD201$^+$ population,[13] this suggests that it is necessary to add CD27 and CD201 stains 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% of LK FLT3$^-$ CD48$^-$ CD150$^+$ cells 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 than in C57BL/6 mice 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 that in 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 (*Online Supplementary Figures S3* and *S4*) 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 and 4) to measure CD244 expression on each cell subset defined in *Online Supplementary Figures S3* and *S4*. In C57BL/6 mice, subsets of CD244$^+$ cells were observed on all BM lineages (Figure 3C, F) but predominantly on NK cells (*Online Supplementary Table S3* and Figure 3F). Within the C57BL/6 spleen (Figure 4), CD244 was highly expressed on a subset of NK cells as well as on monocytes, macrophages, and neutrophil/myeloid progenitors. In NOD-*scid* and NSG mice, the frequency of CD244$^+$ was less than 1% of all lineages examined (Figures 3 and 4).

We detected some lymphocyte-type cells that were B220$^+$ in BM and spleen in both NOD-*scid* and NSG mouse BM (*Online Supplementary Figures S3* and *S4*). In addition, NSG mice had rare NK1.1$^+$ cells whereas both NSG and NOD-*scid* had few CD3ε$^+$ cells.

Long-term hematopoietic stem cells 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 competitive repopulation unit (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 LK CD27$^+$ CD201$^+$ population from the BM of male NSG mice, namely (i) LK CD27$^+$ CD201$^+$ FLT3$^-$ CD48$^-$ CD150$^+$ cells (CD48$^-$CD150$^+$ gate) and (ii) LK

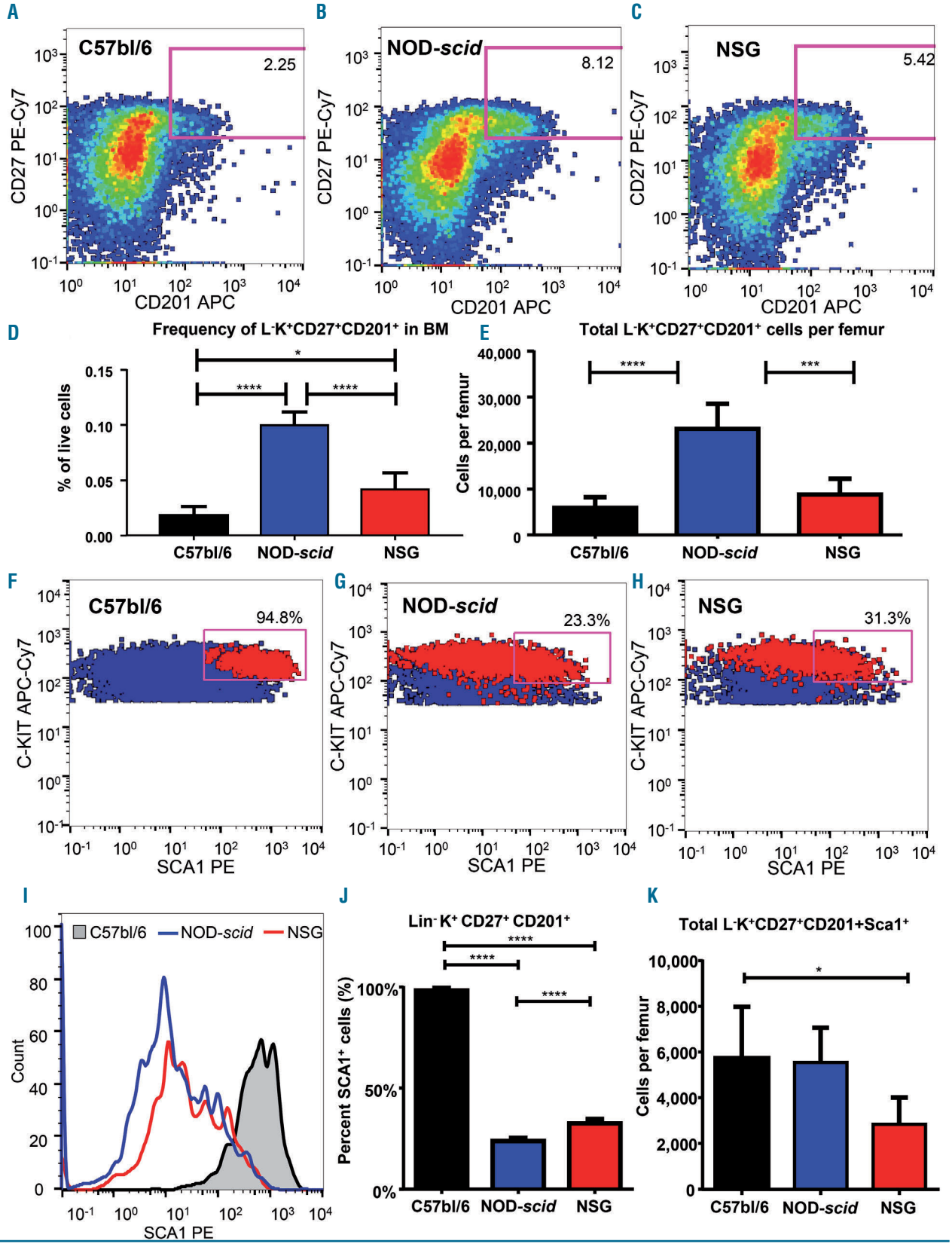


Figure 1. CD27 and CD201 expression in Lin⁻ KIT⁺ bone marrow hematopoietic stem and progenitor cells from different NOD-*scid*-derived mouse strains. (A-C) Expression of CD27 and CD201 in viable Lin⁻ KIT⁺ bone marrow (BM) hematopoietic stem and progenitor cells from C57BL/6 (A), NOD-*scid* (B) and NSG (C) mice. (D) Frequency of Lin⁻ KIT⁺ CD27⁺ CD201⁺ cells within BM leukocytes of the three mouse strains. (E) Absolute number of Lin⁻ KIT⁺ CD27⁺ CD201⁺ cells per femur in the three strains. (F-H) 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 (red) mice. (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 the mean ± standard deviation of five mice per group. *P* values were calculated by analysis of variance with Tukey corrections, **P*≤0.05, ***P*≤0.01, ****P*≤0.001, *****P*≤0.0001.

CD27$^+$ CD201$^+$ FLT3$^-$ cells that were not in the CD48$^-$ CD150$^+$ gate (NOT GATE) (Figure 5H, isotype controls in *Online Supplementary Figure S5*). We transplanted serial dilutions of these two populations (Figure 5H) 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 polymerase chain reaction 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 *vs.* female cells to demonstrate that the assay readout reflected the linear dilution series (*Online 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 (*Online Supplementary Table S4*).

In transplanted recipients, we measured chimerism between 8 and 18 weeks. There was robust long-term male donor chimerism in recipients that received 50 or 150 CD48$^-$ CD150$^+$ gated cells whereas there was a very "low" frequency of long-term chimerism in recipients of NOT GATE cells (Figure 5I, J). Poisson distribution analysis

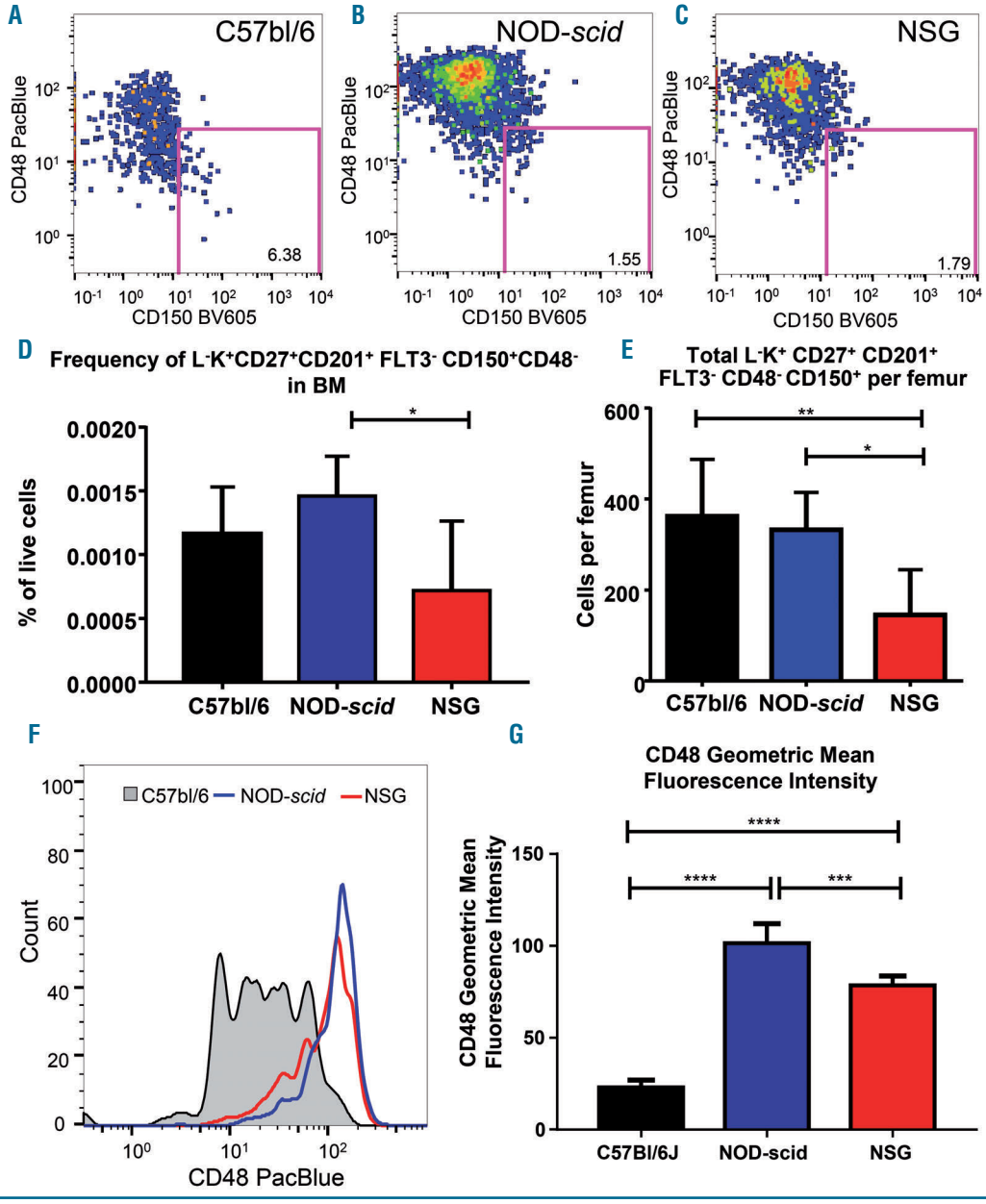


Figure 2. Complementarity of CD27 and CD201 with FLT3, CD48 and CD150 staining in different mouse strains. The gating strategy is shown in *Online Supplementary Figure S1*. (A-C) Lin$^-$ KIT$^+$ CD27$^+$ CD201$^+$ FLT3$^-$ bone marrow (BM) cells were gated and analyzed for CD150 and CD48 expression in C57BL/6 (A), NOD-*scid* (B) and NSG (C) mice. (D, E) 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 in the three mouse strains. Data are the mean ± standard deviation of five mice per group. *P* values were calculated by analysis of variance with Tukey corrections for multiple comparisons, **P*≤0.05, ***P*≤0.01, ****P*≤0.001, *****P*≤0.0001.

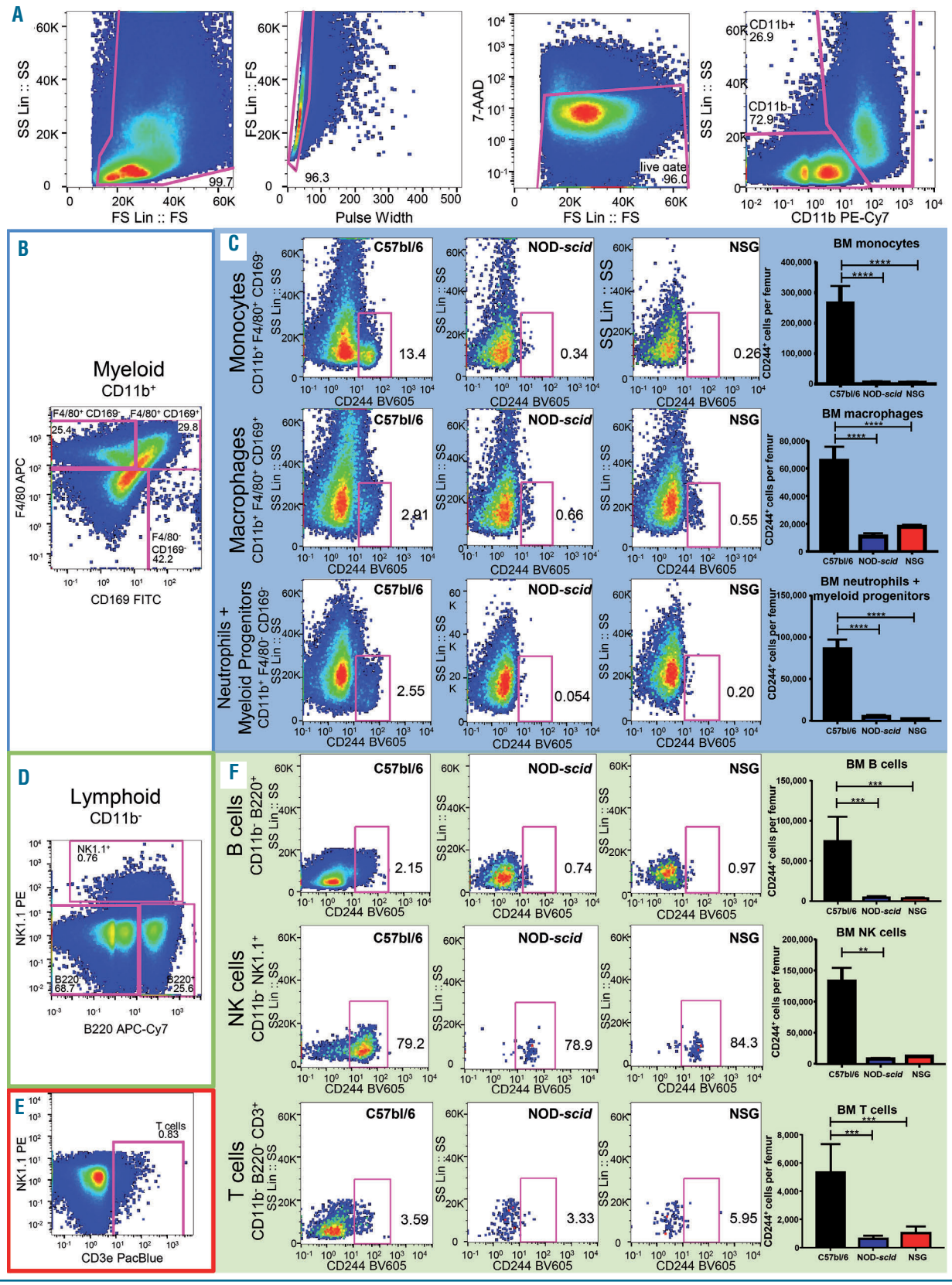


Figure 3. CD48 ligand CD244 is poorly expressed in the bone marrow of NOD-*scid* and NSG mice. (A) Viable single cells were gated into lymphoid (CD11b$^-$ low side scatter) and myeloid (CD11b$^+$) cells. (B) Myeloid cells were further separated using CD169 and F4/80 antigens. Monocytes were CD11b$^+$ 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 of CD244$^+$ cells per femur. (D) Lymphoid cells were separated using B220 and NK1.1 antigens to identify B cells (CD11b$^-$ B220$^+$ NK1.1$^-$) and natural killer (NK) cells (CD11b$^-$ NK1.1$^+$). (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 of CD244$^+$ cells in each subset per femur. Data are mean ± standard deviation of five mice per group. *P* values were calculated by analysis of variance with Tukey corrections for multiple comparisons: ***P*≤0.01, ****P*≤0.001, ****P≤0.0001.

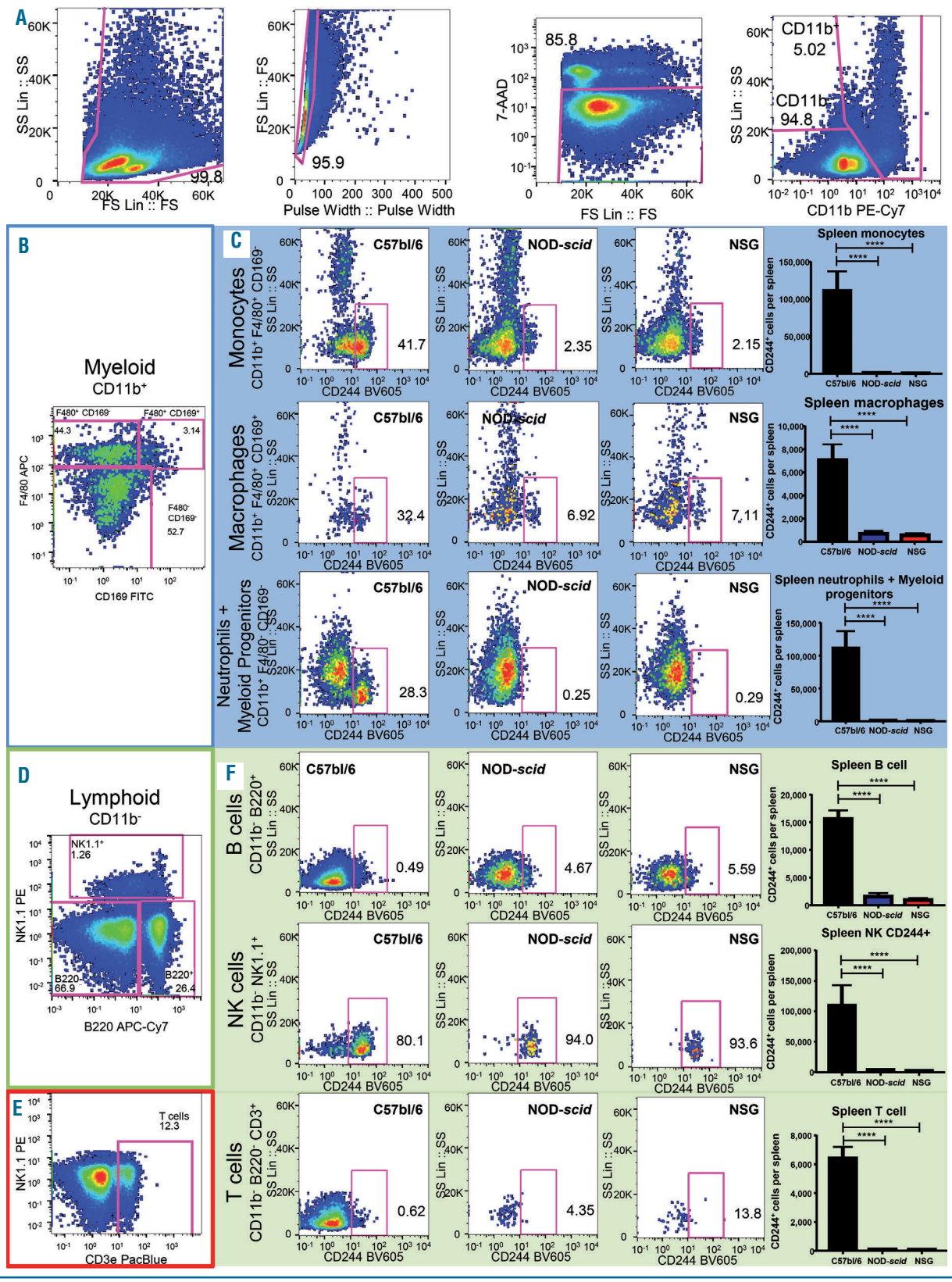
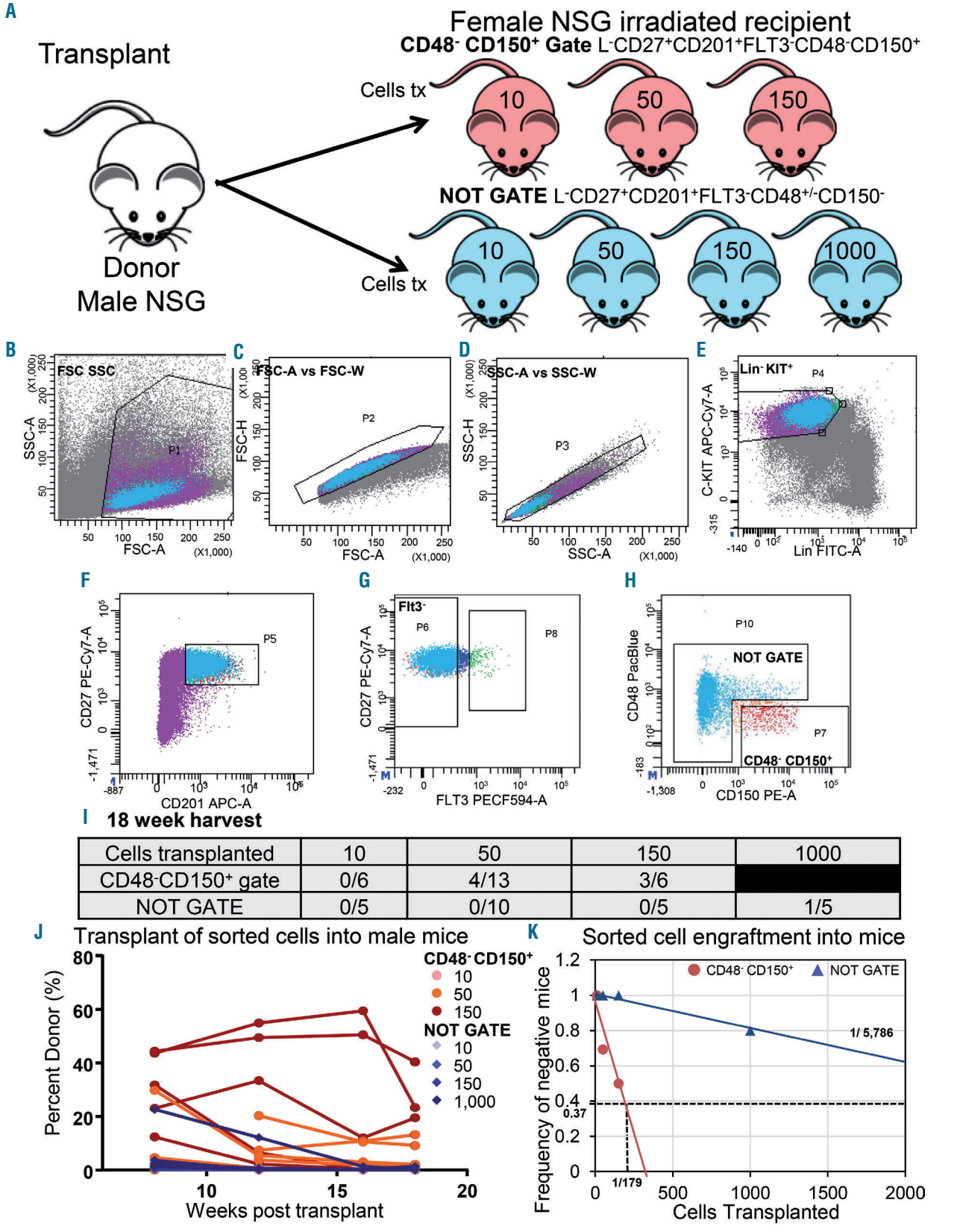


Figure 4. CD48 ligand CD244 is poorly expressed in the spleen of NOD-*scid* and NSG mice. (A) Viable single cells were gated with CD11b into lymphoid cells (CD11b$^-$ low side scatter) and myeloid cells (CD11b$^+$). (B) Myeloid cells were further separated using CD169 and F4/80 antigens. Monocytes were 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 of CD244$^+$ cells per femur. (D) Lymphoid cells were separated using B220 and NK1.1 antigens to identify B cells (CD11b$^-$ B220$^+$ NK1.1$^-$) and natural killer (NK) cells (CD11b$^-$ NK1.1$^+$). (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 of CD244$^+$ cells in each subset per femur. Data are average ± standard deviation of five mice per group. *P*-values were calculated by analysis of variance with Tukey corrections for multiple comparisons, *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.0001.



Cells transplanted	10	50	150	1000
CD48-CD150+ gate	0/6	4/13	3/6	
NOT GATE	0/5	0/10	0/5	1/5

Figure 5. Competitive repopulation unit cells 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 sublethally irradiated (2.5 Gy) female NSG mice to test competitive repopulation unit (CRU) activity. (B-H) Sorting profile from donor male c-KIT enriched bone marrow 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 weeks. (J) Time-course of male donor cell engraftment in each transplanted mouse. Warm colors are for dilutions of sorted Lin- KIT+ CD27+ CD201+ FLT3- CD48- CD150+ cells, cold colors are dilutions of Lin- KIT+ CD27+ CD201+ FLT3- NOT GATE sorted cells. (J) Frequency of donor engraftment at 18 weeks post-transplantation and Poisson distribution of donor hematopoietic stem cells. tx: transplant.

(Figure 5K) showed a 32-fold enrichment (P=1.86 x 10^{-5}) in 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 for further enrichment 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 recipients of CD48$^-$ CD150$^+$ cells (*Online 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 were within the CD48$^-$ CD150$^+$ subset (*Online Supplementary Table S5*).

SCA1 expression in unaltered in bone marrow endothelial and mesenchymal cells in NSG mice

As NSG mice and NOD-*scid* mice blood cells have low SCA1 expression in hematopoietic stem and progenitor cells (HSPC) (Figure 1I), we compared SCA1 expression in BM endothelial cells and MSC from C57BL/6 mice and NSG mice (Figure 6). Endosteal cells were collected from collagenase-treated femurs, magnetically enriched in non-hematopoietic cells, and stained against CD45, Lin, CD31, CD51, SCA1, and PDGFRα antibodies (gating strategy in Figure 6A-D). CD45$^-$ Ter119$^-$ CD31$^+$ BM endothelial cells (Figure 6E, F) 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, H), expressed similar levels of SCA1 in the PDGFRα$^+$ subset which defines the PαS cells[30] (Figure 6J).

Finally, we found that plastic-adherent BM MSC 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 demonstrated that CRU were 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 lipopolysaccharide administration.[13,27] We also noted a lower CRU frequency compared to the reported 50% CRU frequency in LK CD48$^-$CD150$^+$ cells sorted from C57BL/6 mice.[6] Competitive assays in lethally irradiated recipient mice with congenic whole BM cells as a source of competing HSC were used in these studies[6] whereas in the present study, we sublethally irradiated our recipient mice (2.5 Gy) without exogenous competing HSC. This irradiation dose depresses circulating granulocytes and monocytes for only 8 days without transplantation *(data 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 regarding 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 mice. 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. It is, therefore, 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 with CD244 gene deficiency.

Interestingly, NOD-*scid* mice still contain a NK-cell population[33] but we did not detect higher numbers of NK1.1$^+$ cells compared to the numbers in NSG mice. As we did not perform functional assays, we cannot conclude from our experiments that NSG mice had less functional NK cells compared to the NOD-*scid* mice. The literature indicates 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 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 markers of more mature B cells, 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 MSC 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 mice[39,40] and CBA mice[40] 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

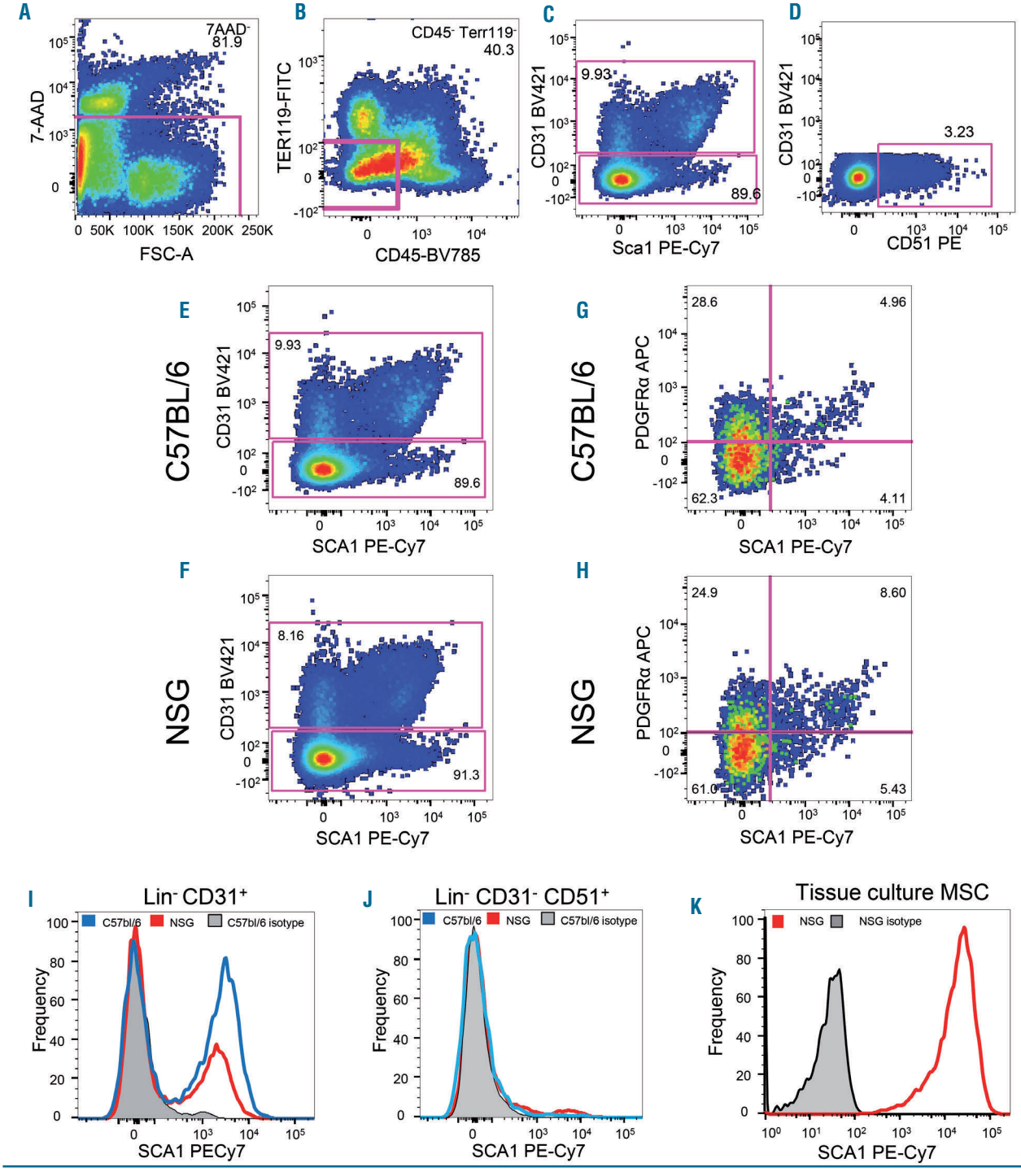


Figure 6. Comparative SCA1 expression in bone marrow endothelial and mesenchymal cells from C57BL/6 and NSG mice. (A-D) Live single cells (A) were gated for non-hematopoietic CD45$^-$ Ter119$^-$ cells (B). From these CD45$^-$ Ter119$^-$ cells, endothelial cells were gated as CD31$^+$ (C). The remaining CD31$^-$ non-endothelial cells were gated for CD51$^+$ mesenchymal cells (D). (E, F, I) SCA1 expression in bone marrow endothelial cells was similar in C57BL/6 (E) and NSG (F) mice as seen in the overlay histogram (I). (G, H, J) CD45$^-$ Ter119$^-$ CD31$^-$ CD51$^+$ mesenchymal cells were further gated for PDGFRα and SCA1 expression. SCA1 expression in mesenchymal cells was comparable in C57BL/6 (G) and NSG (H) mice in dot-plots and the overlay histograms (J). (K) SCA1 expression in mesenchymal stromal cells (MSC) cultured from NSG mice. These plots represent data from four separate mice for each strain.

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 found 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 in circumstances that SCA1 expression is weak. However, when the Lin$^-$ CD27$^+$ and CD201$^+$ phenotype is combined with the 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$^-$, CD48$^-$ and CD150$^+$ is enriched in LT-HSC activity in NSG mice in a rigorous serial dilution long-term competitive transplantation assay. This alleviates the need to stain for SCA1, which is expressed at very low levels in these mice. In addition, we identified a non-reported upregulation 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 had been established, were injected with human BM or cord blood CD34$^+$ cells. The relative quantification of the seeding of humanised ectopic bone scaffolds by human *vs.* murine 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 a human HSPC mobilization defect in NSG mice but we were unable to show a similar effect on murine HSC due to their low SCA1 expression.[50] Therefore, this new staining strategy identifying Lin$^-$ KIT$^+$ CD27$^+$ CD201$^+$ FLT3$^-$ CD48$^-$ CD150$^+$ cells as mouse HSC in NOD-*scid*-derived strains will enable a more accurate measurement of the relative colonization or distribution of mouse bones or ectopic bone organoids by endogenous mouse HSC *vs.* xenotransplanted human HSC.

Acknowledgments

The authors acknowledge the Translational Research Institute (TRI) for providing an excellent research environment and core facilities that enabled this research. We particularly thank the Flow Cytometry and the Biological Resources Core Facilities. BN was supported by an Australian Government Research Training Program Scholarship during her PhD studies. JPL is funded by Research Fellowship APP1136130 from the National Health and Medical Research Council of Australia (NHMRC). MRD is funded by a Career Development Fellowship APP1130013 and Project Grant APP1108043 from the NHMRC. EDW is supported by funding from the Movember Foundation and the Prostate Cancer Foundation of Australia through a Movember Revolutionary Team Award. The APCRC-Q is supported by funding from the Australian Government Department of Health. The TRI is supported by Therapeutic Innovation Australia (TIA). TIA is supported by the Australian Government through the National Collaborative Research Infrastructure Strategy (NCRIS) program.

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