CD166 REGULATES HUMAN AND MURINE HEMATOPOIETIC STEM CELLS AND THE HEMATOPOIETIC NICHE

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Running title: CD166 identifies primitive murine and human HSC

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KEY POINTS

1. CD166 identifies human and murine long-term repopulating stem cells.

2. CD166 is a functional marker that regulates human and murine marrow repopulating hematopoietic stem cells.

ABSTRACT

We previously showed that immature CD166+ osteoblasts (OB) promote hematopoietic stem cell (HSC) function. Here, we demonstrate that CD166 is a functional HSC marker that identifies both murine and human long-term repopulating cells. Both murine LSKCD48-CD166+CD150+ and LSKCD48-CD166+CD150+CD9+ cells as well as human Lin-CD34+CD38-CD49f+CD166+ cells sustained significantly higher levels of chimerism in primary and secondary recipients than CD166- cells. CD166-/- (KO) LSK cells engrafted poorly in wild type (WT) recipients and KO bone marrow cells failed to radioprotect lethally irradiated WT recipients. CD166-/- hosts supported short- but not long-term WT HSC engraftment confirming that loss of CD166 is detrimental to the competence of the hematopoietic niche. CD166-/- mice were significantly more sensitive to hematopoietic stress. Marrow-homed transplanted WT hematopoietic cells lodged closer to the recipient endosteum than CD166-/- cells suggesting that HSC-OB homophilic CD166 interactions are critical for HSC engraftment. STAT3 has 3 binding sites on the CD166 promoter and STAT3 inhibition reduced CD166 expression suggesting that both CD166 and STAT3 may be functionally coupled and involved in HSC competence. These studies illustrate the significance of CD166 in the identification and engraftment of HSC and in HSC-niche interactions and suggest that CD166 expression can be modulated to enhance HSC function.
INTRODUCTION

How HSC-hematopoietic niche (HN) interactions maintain HSC function remains unknown. Several markers on HSC have a ligand on cells of the HN. However, these markers are neither obligatory for HSC function nor are they universally expressed on HSC across species, or on cells of the HN. A role for OB in maintaining HSC is well documented\textsuperscript{1-3}. We previously showed that more immature OB with high Runx2 expression maintain hematopoietic function\textsuperscript{4}. Recently, we found that anti-Activated Leukocyte Cell Adhesion Molecule (ALCAM, or CD166) expression on OB directly correlates with Runx2 expression and high hematopoiesis enhancing activity\textsuperscript{5}. CD166 expression decreased with OB maturation concomitant with a decline in Runx2 expression and OB-mediated ex vivo maintenance of HSC\textsuperscript{5}.

Expression of CD166 on niche cells has been reported\textsuperscript{6}. CD166, which can mediate CD166-CD166 homophilic interactions, is a member of the immunoglobulin superfamily and can also bind the only other known ligand, CD6\textsuperscript{7}. CD166 was originally used to identify a subset of human adult bone marrow (BM) and mobilized peripheral blood (PB) CD34+ cells enriched for progenitor activity\textsuperscript{8}. However, functional studies with CD166 were not pursued. CD166 expression on Stro-1+ stromal cells\textsuperscript{9} and binding of hematopoietic cells via CD166 to a yolk sac-derived stromal cell line were also demonstrated\textsuperscript{10}. These, and our data\textsuperscript{2,4,5,11} confirmed that CD166 is expressed on hematopoietic progenitors and on OB, suggesting the unique possibility that these cells may interact with one another through CD166-CD166 interactions.

Recently, Jeannet et al\textsuperscript{12} reported that CD166 is differentially regulated in adult hematopoiesis and that CD166\textsuperscript{−−} HSC have an engraftment defect although young CD166\textsuperscript{−−} mice displayed normal hematopoietic counts and numbers of phenotypically defined HSC.
However, these studies\textsuperscript{12} did not examine the potential of CD166 to identify bona-fide normal murine and human HSC, nor did they investigate the functional capacity of CD166 in the niche.

In this report, we demonstrate that CD166 is a universal functional marker of murine and human HSC and OB within the HN and that it is involved in modulating HSC-niche interactions and HSC fate. The conserved homology between murine and human CD166 provides an excellent translational bridge between these systems to advance future interventions for enhancing HSC engraftment and clinical benefit.
METHODS

Mice, human cord blood, and transplantation. Breeding pairs of CD166<sup>−/−</sup> mice (B6.129(FVB)-<em>Alcam<sup>−/−</sup></em>/J mice; stock number 010635) were obtained from The Jackson Laboratories. CD166<sup>−/−</sup>, B6.SJL-Pt-cqPep3b/BoyJ (BoyJ), C57BL/6, C57BL/6-BoyJ F1, and NSG mice were bred and housed at Indiana University. Mice 8-12 weeks old were used. Recipients other than NSG mice received 1100cGy (700 and 400cGy split-dose) or 950cGy single dose for homing studies; NSG mice received 275cGy. All procedures were approved by the IACUC of the Indiana University School of Medicine and followed NIH guidelines. Human umbilical cord blood (UCB) was obtained from St. Vincent’s Hospital (Indianapolis, IN) with IRB approval. This study was conducted in accordance with the Declaration of Helsinki. Test cells were injected intra-venously in 200µL PBS and where needed, 10e5 competitor cells per recipient were used. In murine and human studies, chimerism was assessed monthly in the PB and at 4 or 6mo post-transplantation (PT) in the BM of primary recipients as indicated. Secondary recipients received the equivalent of half a femur from primary recipients without competitor cells.

Homing and mobilization. Homing of transplanted cells to the BM of irradiated recipients was performed as previously described<sup>13</sup>. Briefly, low-density BM cells were stained with CellTrace Violet (CTV) tracking dye (Invitrogen), washed, and injected intra-venously into non-irradiated recipients to avoid radiation-induced injury to the marrow microenvironment. Recipient mice were sacrificed 16h PT and BM cells recovered and analyzed for CTV+ cells. Mobilization of hematopoietic stem and progenitor cells (HSPC) was performed as previously described<sup>14</sup>.

Cell staining, flow Cytometry, and cell sorting. Cells washed with stain wash (PBS, 1% BCS, and 1% penicillin-streptomycin) were stained for 15 minutes on ice. Low-density BM
cells were stained with fluorescein isothiocyanate (FITC)-conjugated CD3, CD4, CD45R, Ter119, and Gr1; allophycocyanin (APC)-conjugated c-Kit (CD117); phycoerythrin (PE)-Cy7-conjugated Sca-1; PE-conjugated CD166 (eBiosciences, Cat # 12-1661-82; clone -eBioALC48); AF700-conjugated CD48; and PerCpCy5.5-conjugated CD150. In some experiments, FITC-conjugated CD48 was used to simultaneously select Lin-CD48- cells. Cell sorting was performed on a BD FACS Aria and flow cytometric analysis was done on a BD LSRII.

**Chromatin Immunoprecipitation (ChIP).** ChIP was done using IL-7-stimulated TAIL7 cells with immunoprecipitation of protein-bound DNA sequences performed using antibodies for STAT3 (Cell Signaling), RNA polymerase-II (Millipore), or an irrelevant Ab (Jagged2; Santa Cruz). After purification of eluted DNA, ChIP-qPCR was performed using sequence-specific probes for promoter regions of CD166, Survivin, GAPDH or the control ORF-free intergenic region IGX1A (SABiosciences), in a 7900HT PCR system (Applied Biosystems).

**Two-photon microscopy.** Mice were injected with CFSE labeled cells as described above. Sixteen hours PT, mice were anesthetized and a jugular vein catheter was inserted to allow fluorescent tracer injections on the microscope stage. The dorsal skull surface was exposed, placed in a custom-designed atraumatic stereotaxic device, and submerged in microscope oil. Images were acquired with an Olympus FV1000 confocal system custom-modified for multiphoton imaging. Images were collected in a non-descanned mode using Olympus XLUMPLFL 20xW, NA 0.95 objective and 830nm excitation wavelength. Imaging was performed at a scan rate of 4µs/pixel. Sections through the depth of tissue (Z-stacks) were collected from 6 regions of the calvarium. Z-stacks were collected at step-size settings of 1µm.
and 512x512 pixels. Image analysis was performed using Amira 3D Visualization and Image Analysis software v.4.1 (FEI, Burlington MA).

**Statistical analysis.** Data are presented as mean ± SD and where applicable triplicate samples were measured. Two tailed Student's t-tests were performed when only two groups were compared. One-way factorial analyses of variances were used for multiple group comparisons. Significance was set at 0.05.
RESULTS

**CD166 identifies murine and human long-term BM repopulating cells.** We examined the repopulating potential of CD166+ and CD166- subsets of putative HSC rigorously identified by LSK and SLAM markers. As shown in Fig. 1A, CD166 and CD150 fractionated LSK48-cells into 4 distinct groups (FMO controls are shown in Suppl Fig. 1A). A total of 25, 50, and 100 cells from groups 1-4 were competitively transplanted. Chimerism at 4mo PT (data from 25 cells/mouse shown in Fig. 1B) demonstrated that engraftment of CD150+ cells was predominantly restricted to cells co-expressing CD166 although some low level engraftment was observed among CD166-CD150+ cells (group 4). The reconstitution kinetics of cells in groups 2, 3, & 4 were strikingly different (data from 100 cells/mouse shown in Fig. 1C). CD166+CD150+ cells (group 3) displayed long-term HSC (LT-HSC) kinetics whereas CD166+CD150- cells (group 2) provided declining levels of repopulating potential, reminiscent of short-term HSC (ST-HSC) activity. CD166-CD150+ cells (group 4) appear to be intermediate-term HSC or LT-HSC with a limited, but consistent, repopulating potential. Limiting dilution analysis (LDA, Fig. 1D) revealed that the frequency of LT-HSC was highest among CD166+CD150+ cells (1/17). Serial transplantation in which primary recipients received 10 cells from groups 1, 2, 3, or 4 (Fig. 1E and 1F) demonstrated that CD166+CD150+ cells (group 3) provide approximately 20-fold higher levels of chimerism than other groups examined. The number of repopulating units (RU) per 10 cells (Fig. 1G) illustrated that; LDA and RU calculations generated very similar results, and ascertained that repopulating HSC among LSK48-CD166+CD150+ cells are approximately six-fold higher than among LSK48-CD166-CD150+ cells.

Analysis of multilineage differentiation 4mo PT suggested that differentiation of CD150+ cells (regardless of CD166 expression status) is skewed towards the myeloid lineage (Fig. 1H),
while that of CD150- cells, especially the CD166+CD150- cells is skewed towards the lymphoid lineage as previously reported for both subgroups\textsuperscript{19}. Skewed lineage differentiation of CD150+ and CD150- cells was also observed in mice receiving different size grafts (Suppl. Fig. 2). Recently, Karlsson et al\textsuperscript{20} reported a new phenotypic characterization of murine HSC using the tetraspanin CD9. We examined whether CD9+ HSC\textsuperscript{20} express CD166. Approximately 1/3 of LSKCD48-CD150+ cells expressed CD9 (Fig. 1I, FMO controls shown in Suppl Fig. 1B). Only CD166+CD9+ cells sustained significant in vivo engraftment (Fig. 1J) suggesting that CD166 expression is obligatory for the complete identification of functional HSC. That CD166 marks HSC was also gleaned from co-expression of CD166 and side population (SP) cells\textsuperscript{21} (Fig. 1K).

The majority of Lin-CD34+CD38- cells in human umbilical cord blood (CB) expressed CD166 (Fig. 2A). The repopulating potential of Lin-CD34+CD38-CD166+ cells in NSG mice (Fig 2B) was >4-fold higher than that of equal numbers of Lin-CD34+CD38-CD166- cells and far exceeded chimerism sustained by Lin-CD34+CD38- cells. Recently, CD49f (along with Lineage markers, CD34, CD38, and CD90) was used to identify robust human HSC capable of engrafting in a xenotransplantation model at the single cell level\textsuperscript{22}. A modified staining protocol (Fig. 2C; FMO controls shown in Suppl Fig. 1C) demonstrated that CD166 and CD49f fractionate CB Lin-CD34+CD38- cells into four distinct subgroups (Fig 2C). Repopulating cells in NSG mice were predominantly CD166+ (Fig. 2D; groups 2 & 3) regardless of the status of CD49f suggesting that CD166+, but not CD166- cells within the CD34+Lin-CD38- fraction contains the majority of long-term engrafting cells. Long-term engraftment of CD49f+ cells was principally restricted to cells co-expressing CD166 demonstrating the importance of CD166 in identifying human long-term HSC. Serial transplantation studies (cells isolated as described in Figure 2C) confirmed that CD166+, but not CD166- cells sustained durable long-term engraftment (groups 2 and 3; Fig. 2E and 2F). CD166 identified similar subgroups in adult
human BM (Suppl. Fig. 3) illustrating that CD166 identifies putative HSC from different hematopoietic tissues at different stages of ontogeny.

**CD166-/- HSC do not engraft in WT recipients.** Since CD166 is expressed by HSC (Fig. 1) and OB critical for their function, we reasoned it may play an important role in the niche via CD166-mediated HSC-OB interactions. To investigate the role of CD166 in hematopoiesis, we examined the functional properties of HSC from CD166 KO mice. As expected, BM cells from the central (C) region, considered here the vascular niche, and from flushed then digested bones, considered here the endosteal (E) region of KO mice did not express CD166 (Suppl. Fig. 4A) nor did OB from these mice (Suppl. Fig. 4B). Absolute numbers of LSK48- and LSK48-CD150+ cells in the BM and Lin-CD48- cells in the PB of CD166-/- mice were significantly reduced (Fig. 3A). Lineage composition in the C region revealed that KO mouse BM contained a slightly, but significantly, higher percentage of lymphoid cells and a slightly, but significantly, lower percentage of myeloid cells (Suppl. Fig. 5A). However, cell composition in the E fraction did not show significant differences between WT and KO mice (Suppl. Fig. 5B). The frequency of HSPC including LSK, LT-HSC (LSKCD34-CD135-), and CD150+LT-HSC was significantly lower in the BM of KO mice (Fig. 3B) although the frequencies of MPP (LSKCD34+CD135+) and ST-HSC (LSKCD34+CD135-) were not significantly reduced in KO mice (Fig. 3B). The clonogenic potential of steady state BM- and PB-derived progenitors was significantly reduced in the BM and PB of KO mice (Fig. 3C). However, CD166-/- mice mobilized normally in response to G-CSF although the overall number of circulating clonogenic progenitors was significantly lower than in WT mice (Fig. 3D) suggesting that CD166 may play a role in G-CSF-induced mobilization of HSPC. The size and cellularity of the spleens of CD166-/- mice were not different than those of WT mice.
LSK cells from KO mice collected from the C (Fig. 3E and 3F) or E regions (Fig. 3G) almost failed to engraft in lethally irradiated recipients. Given the low chimerism levels in primary recipients, secondary transplants were not performed. Multilineage differentiation analysis demonstrated a significant increase in lymphoid and a significant decrease in myeloid reconstitution in recipients of KO cells (Suppl. Fig. 6) reflecting the same skewness in KO mice (Suppl. Fig. 6). When used as recipients of LSK cells from GFP C57BL/6 mice, only short-term repopulating cells engrafted efficiently in KO mice (Fig. 3H). Chimerism in WT recipients at 4mo PT was more than 12-fold higher than in KO hosts (Fig. 3I) demonstrating that the CD166^/- HN cannot support LT-HSC engraftment. In addition, BM cells from KO mice also had a compromised radioprotective function (Fig. 3J).

Homophilic interactions between CD166 expressed on OB and LSK cells was evident from adherence studies in which significantly fewer CD166^/- clonogenic cells within the LSK fraction adhered to OB when either or both LSK and OB were derived from KO mice (Fig. 3K). The negative impact of loss of homophilic CD166 interaction between OB and LSK was evident in the number of clonogenic cells produced in mixed co-cultures (Fig. 3L). The highest level of loss of hematopoietic function was evident between co-cultures of WT OB plus CD166^+ LSK cells and KO OB plus CD166^- LSK cells (bars 3 and 6 in panel L). These data demonstrate that selection of CD166^+ LSK cells enriches for hematopoietic progenitor cell activity and suggest that other HSC-OB compensatory adhesion interactions may be operable. However, we did not detect any significant difference in the expression patterns of several adhesion molecules on WT or KO marrow cells (Fig. 3M).

**Stress response and homing of hematopoietic cells from CD166 KO mice.** The behavior of CD166^/- LSK cells and the compromised competence of the HN of KO mice, prompted us to examine the response of KO HSPC to stress. Following a single 150mg/Kg BW
injection of 5-Fluorouracil (5-FU), survival of CD166<sup>-/-</sup> mice was significantly reduced compared to WT mice (Fig. 4A). The BM cellularity of surviving KO mice 5, 10, 17, and 24d after 5-FU injection was unchanged from that of WT mice (Fig. 4B). At these time points, some classes of HSPC<sup>23,24</sup>, specifically LSK, LT-HSC (LSKCD34-CD135-), and GMP (Lin-Scal-CD117+CD34+CD16/32w+), were significantly reduced in KO relative to WT mice (Fig. 4C), while recovery of ST-HSC, MPP, CLP, CMP, and MEP was not different between the two genotypes (Fig. 4C). A significantly larger fraction of d0, d5, d10, d17, and d24 KO LSK cells were in G1 and S/G2+M phases of cell cycle (Fig. 4D and E) demonstrating the cell cycle-dependent sensitivity of KO HSPC to 5-FU.

We also assessed CD166 expression on LSK cells in response to radiation. BM cells from irradiated mice exhibit a persistent engraftment defect post-irradiation<sup>25,26</sup>, while those from mice treated with 5-FU engraft successfully<sup>27</sup>. Expression of CD166 on LSK from sublethally irradiated mice was significantly reduced (Fig. 4F). However, 5-FU treatment did not significantly reduce CD166 expression suggesting that the engraftment defect observed in cells exposed to radiation may be associated with decreased CD166 expression.

Homing of KO cells to the E or C regions of the marrow of KO recipients was inefficient (Fig. 5A). Using intra-vital imaging, we examined the behavior of BM-homed transplanted cells to assess the impact of CD166 on interactions between transplanted cells and elements of the HN. Figure 5B illustrates that both WT and KO cells home to the marrow of both genotypes. However, distances between WT cells and the endosteum of WT recipients (Suppl. Fig 7), were significantly shorter than those measured between KO cells homed to the marrow of KO mouse and the endosteum of these recipients (Fig. 5C). Interestingly, this defect was not as pronounced (Fig. 5C) when CD166 was absent on either transplanted cells or the endosteum of
recipient mice only (KO→WT or WT→KO) suggesting that other compensatory adhesion interactions between transplanted HSC and the endosteum are also critical for HSC function. Most importantly, differences in the settings WT→WT versus KO→KO were consistent whether grafts contained low-density BM cells (data not shown), lineage depleted BM cells, or LSK cells (Fig 5C). A smaller percentage of BM-homed KO cells from the C region were in G0/G1 compared to WT cells (Fig. 5D) but these differences were not significant, suggesting that increased cell cycle kinetics\textsuperscript{28,29} are most likely not responsible for the gradual decline in the engraftment of these cells. Interestingly, expression of CXCR4 on KO HSPC cells was not altered (Fig. 5E) suggesting that CD166 is not involved in directed homing of HSPC to the BM.

**Bone phenotype and function in CD166 KO mice.** To assess the impact of loss of CD166 on OB phenotype and function, we analyzed multiple properties of OB from KO mice. A significantly smaller fraction of 2-day calvarial OB from KO mice were in G0/G1 during days 1 and 2 of culture (Suppl Fig. 8A). Except for alkaline phosphatase, expression of mRNA levels of collagen type 1, osteocalcin, osteopontin, and Runx2, were similar between WT and KO osteoblasts (Suppl Fig. 8B). Expression of HoxB4 and N-cadherin which have been implicated in maintaining HSC function\textsuperscript{30-32}, but not SDF-1 were significantly lower among KO OB (Suppl Fig. 8C). Although these changes were significant, they were not substantial suggesting that reduced signaling through HoxB4 and N-cadherin may play a role in the altered function of HSPC from KO mice and the diminished competence of the CD166\textsuperscript{−/−} HN. Expression levels of fibulins, which negatively influence the adhesive properties of the endosteal niche\textsuperscript{33}, were significantly increased in KO OB (Suppl Fig. 8D) suggesting that loss of CD166 upregulates the expression of fibulins and indirectly reduces the adhesive properties of OB possibly through increased expression of MMP2, a pathway known to be triggered by CD166\textsuperscript{34}. 
**CD166 regulation.** We analyzed the promoter region of CD166 and recognized the presence of three consensus motifs for STAT3 binding. Interestingly, using a hematopoietic cell-targeted deletion of STAT3^{35}, we recently established that the engraftment potential of STAT3^{-/-} BM cells was significantly reduced^{36}. Chromatin immunoprecipitation with Polymerase-II using the leukemia cell line TAIL7^{37}, which exhibits STAT3 activation and strong expression of CD166 (Suppl. Fig. 9) showed significant enrichment in GAPDH, but not of the control ORF-free intergenic region IGX1A (Fig. 6A). However, immunoprecipitation with STAT3 antibody showed marked enrichment for the CD166 promoter sequence, as well as for Survivin, a well-known transcriptional target of STAT3. To determine whether STAT3 transcriptional activity regulates CD166 expression on HSC, we examined BM cells from WT and STAT3^{-/-} mice. Figure 6B illustrates a substantial decrease in the percentage of CD166+ cells within the LSK and LSK48-CD150+ fractions in STAT3^{-/-} mice relative to controls. Expression of CD166 was significantly reduced on murine BM-derived Lin- cells treated for 24hr with the STAT3-selective inhibitor STATTIC (Fig. 6C). Finally, suppression of STAT3 expression with shRNA concomitantly reduced CD166 expression on MOLT4 cells (Fig. 6D). Collectively, these data suggest that expression of CD166 can be modulated by STAT3 and present the possibility that loss of engraftment potential of STAT3^{-/-} cells^{36} is most likely linked to decreased CD166 expression on these cells.
DISCUSSION

Interactions between HSC and cells of the HN, which are vital for stem cell pool maintenance, depend on ligand-receptor molecules co-expressed independently on interacting cells. Amongst the most characterized interactions in the HN are those between HSC and OB which sustain HSC functions, especially self-renewal\(^1,3,6\). To date, not a single, functional surface marker has been identified as a common marker on murine and human HSC and on cells of the HN, nor has there been any description of a marker capable of homophilic interactions that is co-expressed on all these cell types.

Using strict phenotypic definitions of murine\(^1^6\) and human\(^2^2\) engrafting cells, we demonstrated that CD166 is a critical and functional common marker that positively identifies repopulating HSC beyond what is currently possible with previous approaches. To our knowledge, this is the first report to describe a common functional marker on both human and murine HSC and to demonstrate that loss of this marker not only impacts stem cell function, but also compromises the competence of the HN. In addition, we also demonstrate a possible novel regulatory pathway impacting HSC function that involves STAT3-mediated regulation of CD166 expression. STAT3, which plays a significant role in the hematopoietic system, is activated by several factors involved in homeostatic and stress hematopoiesis including IL6, G-CSF, EPO, TPO, and LIF. Although STAT3 deletion markedly decreases the hematopoietic repopulating ability\(^3^6\), little is known about the downstream effectors of this defect. Our studies demonstrate a direct link between loss of STAT3 in HSC and decreased CD166 expression (Fig. 6B) and suggest this pathway as a possible explanation for the decreased repopulating potential of STAT3\(^{-}\) BM cells and a likely tentative mechanism for the role of STAT3 in hematopoiesis.
In our transplantation studies, the significant majority of both murine and human long-term repopulating cells were CD166+ demonstrating the importance of CD166 in identifying LT-HSC in both species. The degree to which CD166 facilitated the enrichment of long-term repopulating cells was evident from the frequency of these cells among LSK48-CD166+CD150+ cells; 1/17. While the frequency of 1/17 long-term HSC among this phenotype is lower than the 1/3 frequency reported by Kiel et al\textsuperscript{16} for CD150+ cells, calculation of the frequency of repopulating units using a different approach\textsuperscript{18} yielded results (<2RU per 10 cells, Fig. 1G) almost identical to those from Kiel et al\textsuperscript{16}. These analyses suggest that exact HSC enumeration or quantification may be limited by the sensitivity of the currently available experimental protocols and subtle differences between practices in different laboratories. Similar observations can be made about CD49f in the human system. While our studies do not contradict the findings of Notta et al\textsuperscript{22}, they suggest that only a subfraction of CD49f+ cells that also expresses CD166 contains bona fide marrow reconstituting cells.

Our current and previous investigations\textsuperscript{2,4,5,11} illustrate that CD166 is a functional marker on both HSC and OB. Functionality of CD166 was demonstrated by the almost complete inability of CD166\textsuperscript{-/-} LSK cells to engraft in WT recipients and by the failure of the CD166\textsuperscript{-/-} microenvironment to support the engraftment of WT LT-HSC. Furthermore, recent data (BRC and EFS, unpublished observations) suggest that human CB cells treated with CD166 shRNA have a reduced engraftment potential in NSG mice suggesting again that CD166 is a functional marker of engrafting stem cells. Imaging data suggest that simultaneous expression of CD166 on HSC as well as cells of the niche is critical for engraftment and repopulation. Our results are consistent with those reported by Jeannet et al.\textsuperscript{12} where the engraftment potential of purified CD166\textsuperscript{-/-} LSK cells in secondary transplantation recipients was significantly reduced.
While loss of CD166 did not repress the expression of other stem cell markers on putative HSC, homing of CD166\(^{+/−}\) cells to the marrow of CD166\(^{−/−}\) recipients was significantly compromised (Fig. 5A). Jeannet et al\(^{12}\) only examined the homing of KO cells to the BM of WT recipients and hence no defect in homing was observed. Our data (Fig 5A) demonstrate that overall, homing of KO cells to the BM is inefficient, and that homing of KO cells to the microenvironment of KO recipients is significantly reduced. In the studies of Jeannet et al\(^{12}\), the HN of KO mice was not analyzed. That CXCR4 expression on CD166\(^{+/−}\) HSPC is normal (Fig. 5E), suggests that CD166 may not play a critical role in homing and retention of HSPC in the marrow but may be critical for HSC-HN interactions that ensure survival and function of these cells. Imaging studies (Fig. 5) confirm this premise and lend support to the suggestion that CD166 may be critical for HSC-OB interactions that sustain stem cell survival and engraftment. More definitive studies are required to ascertain the role of CD166 in HSC-niche interactions. Our studies examined lodgment of marrow-homed CD166\(^{+/−}\) cells under permutations not examined by Jeannet et al\(^{13}\) leading perhaps to different conclusions regarding homing of KO cells to the BM of recipient mice.

The extent to which loss of CD166 impacts HSC function and the fact that KO mice are viable with no apparent major defects is interesting. While the engraftment defect of CD166\(^{−/−}\) HSC is severe and almost total, the defect observed in the HN of CD166\(^{−/−}\) mice is partial. Collectively, data in Figures 3H and 3J suggest that ST-HSC survive momentarily in KO mice but are quickly depleted early after transplantation. These defects predict an acute hematopoietic injury in CD166\(^{−/−}\) mice that would impact survival. The susceptibility of CD166\(^{−/−}\) mice to metabolic stress underscores the abnormalities of their hematopoietic system that are not detected under steady-state hematopoiesis. Our data suggest that loss of CD166 is more critical for HSC function than for the competence of the HN and highlight osteoblast
differentiation and maturation irregularities in CD166−/− mice that may partially contribute to the overall defect of the HN. A more detailed analysis of the bones of KO mice (presented elsewhere) did not reveal major defects that can explain loss of competence of the niche observed in these studies. Nevertheless, our analysis documents significantly higher levels of alkaline phosphatase mRNA in CD166−/− OB (Suppl Fig. 8B) suggesting that these OB are more mature than their WT counterparts. Since we have already demonstrated that immature CD166+ OB are responsible for the hematopoiesis enhancing activity2,4,5,11, these data suggest that maturation of OB may be a contributing factor to the inability of the HN of KO mice to support the engraftment of LT-HSC (Fig. 3H). Our studies also illustrated that HoxB4 and N-Cadherin expression is moderately, but significantly reduced in OB from KO mice. While the importance of HoxB4 in sustaining HSC function is well documented38, the role of N cadherin in hematopoiesis is controversial32,39. Whether the observed differences in the expression levels of HoxB4 and N-cadherin contribute to the inability of the KO microenvironment to support LT-HSC engraftment remains to be determined.
ACKNOWLEDGEMENTS

The authors thank the operators of the Indiana University Melvin and Bren Simon Cancer Center Flow Cytometry Resource Facility for their outstanding technical help and support. This work was supported in part by grant NHLBI HL55716 (E.F.S.) and the Indiana Center for Excellence in Molecular Hematology (NIDDK P30 DK090948). The Flow Cytometry Research Facility is partially funded by NCI P30 CA082709.

We acknowledge the In Vivo Therapeutics Core of the Indiana University Simon Cancer Center (partially funded by NCI P30 CA082709 and NIDDK P01 DK090948) as well as the nursing staff and Dr. Arthur Baluyut at the St. Vincent Hospital (Indianapolis, IN) for providing umbilical cord blood samples for this study.

None of the authors of this manuscript has any financial conflict of interest that might be construed to influence the results or interpretation of the results reported in this communication.
AUTHORSHIP CONTRIBUTIONS

Brahmananda R. Chitteti performed the majority of the experimental work and helped writing the paper. Michihiro Kobayashi performed many of the animal studies. Ying-Hua Cheng performed all the experimental work involving preparation and culturing of osteoblasts. Huajia Zhang performed all the chromatin immunoprecipitation studies. Bradley Poteat contributed to ex vivo culture studies and animal work. Hal E. Broxmeyer contributed to data analysis and interpretation. Louis M. Pelus contributed to data analysis and interpretation. Helmut Hanenberg contributed to experimental design and data analysis. Amy Zollman assisted in animal preparation for the imaging studies and performed imaging. Malgorzata M. Kamocka performed a substantial volume of the imaging work and analyzed imaging data. Nadia Carlesso assisted in experimental design and data interpretation. Angelo A. Cardoso assisted in experimental design, data interpolation, and animal modeling. Melissa A. Kacena designed all the work related to osteoblast preparation and culturing, helped in the design of other experiments, and assisted in writing the manuscript. Edward F. Srour designed the research, interpreted data, assisted in some experimental work, and wrote the manuscript.
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**FIGURE LEGENDS**

**Figure 1.** Characterization of the functional properties of murine HSC fractionated with CD166.  
(A) C57BL/6 low-density BM cells were analyzed for the expression of Lineage markers and CD48 (left dotplot). Lineage-CD48- (Lin-48-) cells were analyzed for the expression of Sca-1 and cKit (middle dotplot) to identify Lin-Sca1+cKit+CD48- (LSK48-) cells (group 1 in middle dotplot). LSK48- cells were analyzed with CD150 & CD166 (right dotplot) to define groups 2, 3, and 4. Percentages next to each group represent the relative size of each population within the dotplot. A total of 100, 50, & 25 cells from groups 1 through 4 were transplanted into F1 mice with 10e5 BoyJ competitor cells (5 mice/group).  
(B) Chimerism 4mo PT for the 25-cells/mouse group. *p<0.05 vs. group 3.  
(C) Engraftment kinetics for groups 1, 2, 3, & 4 over 4mo measured in the PB of recipients of 100-cells/mouse group. *p<0.05 vs corresponding values from LSK48-CD166+CD150+ cells. These data are from one of three independent experiments with similar results.  
(D) LDA analysis from BM engraftment at 4mo PT in mice receiving 25, 50, and 100 cells (5 mice/group). p=0.01 for the overall test for differences in HSC frequencies between any of the groups. Table shows the estimated frequencies and range of repopulating cells within each group tested.  
(E) Chimerism at 4mo PT in primary recipients following the competitive transplantation of 10 cells/mouse from groups 1-4 identified in panel (A) along with 10e5 BoyJ competitor cells.  
(F) Chimerism at 4mo PT in secondary recipients of the equivalent of ½ femur from primary recipients (BM cells were collected 4mo post-primary transplant) shown in (E) without competitor cells (4-5 mice/group in (E) and (F)).  
(G) Repopulating Units (RU) per 10 cells calculated from data shown in (E) as described in Harrison & Astle. *p<0.05 vs. group 3.  
(H) Multilineage differentiation 4mo PT of groups 1 through 4 in mice receiving 100 cells/animal. *p<0.05 between LSK48-CD166+CD150+ cells and other phenotypes within each group.  
(I) Expression of CD166 on LSK48-CD150+CD9+ cells (in the 3 dotplots from L to R).  
(J) Chimerism at 4mo PT in mice receiving 10 cells per mouse from groups I, II, and III transplanted
competitively into F1 mice with 10e5 BoyJ competitor cells (5 mice/group). *p<0.05 vs. group III.

(K) Expression of CD166 on the low (L), medium (M), and high (H) fractions of murine BM side population (SP) cells. Data shown in (K) are from 1 representative experiment from 4 independent analyses with similar results.

Figure 2. Characterization of the functional properties of human CB-derived HSC fractionated with CD166. (A) Human cord blood cells were analyzed with a combination of Lineage markers, CD34 and CD38 and Lin-CD34+CD38- cells (group 1) were analyzed for the expression of CD166 to identify CD34+CD38-CD166- (group 2) and CD34+CD38-CD166+ (group 3) cells. Percentages next to each group represent the relative size of each population within the dotplot. (B) A total of 5,000 cells from groups 1, 2, and 3 were transplanted into NSG mice and chimerism was assessed 4mo later. n=3-4/group. *p<0.05, CD34+CD38-CD166+ vs. other groups. (C) Fractionation of human CB cells with CD34 and a combination of Lineage markers and CD38 to select Lin-CD34+CD38- cells (group 1 in left dotplot). Lin-CD34+CD38- cells were analyzed with CD166 and CD49f (right dotplot) to define groups 2, 3, 4, and 5. Percentages next to each group represent the relative size of each population within the dotplot. A total of 1,000 cells from groups 1 through 5 were transplanted into NSG mice. (D) BM chimerism 4mo PT. n=3-4/group. *p<0.05, groups 2 & 3 vs. other groups. Similar results were obtained in another independent experiment (4,000 cells/group; n=4/group). The 2 data sets were not pooled. (E&F) Chimerism at 4mo post-transplantation (E) sustained by 1000 cells per mouse from groups 1-4 as shown in panel (C) and in secondary recipients (F) transplanted with BM cells collected from primary recipients (1/2 a femur equivalent from pooled BM cells per recipient) shown in (E). n=4-5 per group in (E) and (F). *p<0.05, groups 2 & 3 vs. other groups. Flow cytometric analysis of representative mice from panel (F) are shown in Suppl Figure 10.
Figure 3. Hematopoietic parameters of CD166<sup>−/−</sup> mice. (A) Absolute numbers of LSK48- and LSK48-150+ cells in the BM and Lin-48- cells in the PB of WT and KO mice (6 mice /group). *p<0.05 compared to WT. (B) Percentages of HSPC classes in the marrow of WT and KO mice (6 mice /group). Classes of HSPC were identified as such: LSK (Lin-Sca1+Kit+); MPP (LSKCD34+CD135+); ST-HSC (LSKCD34+CD135-); LT-HSC (LSKCD34-CD135-); 150+LT (CD150+LSKCD34-CD135-). *p<0.05 compared to WT. (C) Numbers of CFU-GM in 1mL of PB and in BM contained in one femur from WT and KO mice. n= 5-6 mice per group. *p<0.05 compared to WT. (D) Numbers of CFU-GM in 1mL of PB and in BM contained in one femur from WT and KO mice mobilized with G-CSF (2 daily injections of 1µg/mouse for 4d). Cells were collected on day 5. n=4-5 mice/group. *p<0.05 between KO and WT vehicle (Veh) control. #p<0.05 compared to Veh in each set. †p<0.05 between G-CSF KO and WT. Values in parentheses are fold-increase between Veh and G-CSF in each tissue. (E) Lethally irradiated F1 mice (n=13/group from 3 independent experiments) received 1,000 LSK cells from the C region of CD166<sup>−/−</sup> mice plus 10e5 CD45.1 competitor cells. *p<0.05 compared to WT at corresponding time point. (F) BM chimerism at 4mo PT of LSK cells from one of the 3 experiments in (E). *p<0.05 compared to WT. (G) Lethally irradiated F1 mice (n=5/group) received 1,000 LSK cells from the E region of CD166<sup>−/−</sup> mice plus 10e5 CD45.1 competitor cells. *p<0.05 compared to WT at corresponding time point. (H) Engraftment of 1,000 GFP+ LSK cells from GFP+ WT mice transplanted with 10e5 CD45.1 competitor cells into lethally irradiated WT or KO mice (n=5/group). *p<0.05 compared to WT at corresponding data points. Chimerism is reported as percentage of GFP+ cells in PB. (I) BM chimerism at 4mo PT of GFP+ LSK cells in mice in (H). *p<0.05 compared to WT. (J) Survival of lethally irradiated recipients (700 and 400 cGy split dose) over a 30d period following transplantation with 10e5 low-density BM cells from WT or KO donors. *p<0.05 compared to WT group. (K) Numbers of colony-forming units per 10e3 sorted LSK cells from the BM of WT or KO donors retained on WT or KO OB following 16hr of co-culture of both cell types. *p<0.05 compared to WT OB-LSK co-culture. (L) Fold
increase in the number of CFU produced after 7d of co-culture combinations as shown in the table. Each culture received 1,000 LSK cells on d0. Data are reported as fold-increase relative to the CFU number obtained from freshly isolated 1000 LSK cells on day0. *p<0.05 compared to WT OB and CD166+ LSK co-culture (third bar from the left). (M) Expression of different adhesion markers on BM cells from WT and CD166−/− donors.

Figure 4. Behavior of CD166−/− HSPC under hematopoietic stress. (A) Survival of WT and KO mice after a single IP injection of 5-FU (150mg/Kg, arrow). n= 14/genotype. Survival was monitored daily. *p<0.05; Kaplan-Meyer curves were analyzed with a log-rank (Mantel-Cox) nonparametric test. (B) Total WBC count per femur before (d0) and on days 5, 10, 17, and 24 following treatment with 5-FU. n= 4/genotype/time point. *p<0.05 compared to WT. (C) Phenotypic analysis of HSPC from BM of WT and KO mice 5, 10, 17, and 24d post 5-FU. n= 4/genotype/time point. *p<0.05 compared to WT. (D) Representative analysis of LSK cells from one mouse/genotype/time point with Hoechst 33342 and Pyronin Y. n= 4/genotype/time point. (E) Percentage of LSK cells in the G0 phase of cell cycle on days 5, 10, 17, and 24 post 5-FU treatment analyzed as shown in (D). n= 4/genotype/time point. *p<0.05 compared to WT. (F) Expression of CD166 on BM-derived LSK cells from one representative control mouse or from each of 3 separate mice irradiated with 400cGy or treated with 150mg/Kg 5-FU 10d earlier.

Figure 5. (A) Homing of CD166−/− hematopoietic cells to the BM of lethally irradiated recipients. Recovery of BM-homed cells 16hr PT (corrected for total body BM). Cells were stained with CTV, injected into lethally irradiated recipients then recovered from flushed central (C) or from digested endosteal (E) regions. n=3-4 mice/group. *p<0.05 compared to WT. (B) Intravital images of CFSE stained Lin- cells in the calvariae of non-irradiated recipients 16hr PT. Panels on the left depict WT cells in WT recipients and those on the right show KO cells in KO recipients. Top two panels show CFSE stained cells (green) in the BM microenvironment.
Images of vessels (collected at 605nm) that were stained with TRITC-conjugated dextran appear in red. Images in the middle row depict two different areas of the calvarium with the bone rendered silver to facilitate visualization of marrow-homed cells. Lower row show same areas depicted in the middle row after removal of the images of the vasculature. Distances from each cell to the surface of the endosteum was measured using Amira 3D Visualization and Image Analysis software v.4.1 using a 3D caliper tool. Additional information can be found in Suppl Figure 7. (C) Average distances between WT or KO Lin- and LSK cells and the endosteum. Distances between cells and the bone surface (reconstructed with a constant threshold value, visible in gold) were measured using a 3D caliper tool. *p<0.05 compared to WT. Data shown are from one representative experiment of a total of 3. At least one recipient mouse per condition was used in each experiment. In the analysis shown between 8 and 25 cells per condition were measured for the Lin- group and 5 to 7 for the LSK group. Similar data were obtained in the other experiments. (D) Cell cycle analysis of BM-homed cells 16hr PT. BM homed CTV+ cells were isolated by cell sorting, stained with propidium iodide, and analyzed flow cytometrically for cell cycle status to determine cells in G0, G1, and G2+M. n=3-4/group. (E) Frequencies of WT and KO marrow-derived LSK and LSK48-150+ cells expressing CXCR4.

Figure 6. (A) ChiP analysis of the binding of STAT3 to the CD166 promoter. Data are from one representative experiment from a total of 3 that showed very similar results in each. (B) CD166 expression on LSK and LSK48-150+ cells from WT and STAT3−/− mice. n=3 mice/group. Data shown are from 1 mouse/genotype. Identical results were obtained from the other 2 mice. LSK cells were gated from column “A” and LSK48-150+ were gated from column “B”. (C) CD166 expression on BM-derived Lin- cells treated with STATTIC for 24hr. Percentage of CD166+ cells declined from 53% (blue plot) to 17% (red plot) after treatment. (D) Expression of CD166 in human MOLT4 cells transduced with scrambled (Src), CD166, or STAT3 shRNA. ShRNA were cloned into Age1 and EcoR1 sites of the MISSION® vector (Sigma) then selected by puromycin.
FIGURE_1

A. Live, Lin-48-, and LSK48- CD46, cKit, Sca1, and CD150 expression.

B. % Chimerism by time post transplantation.

C. % Chimerism by number of cells transplanted.

D. Log fraction of negative mice.

E. % Chimerism for 1<sup>st</sup> Recipients.

F. % Chimerism for 2<sup>nd</sup> Recipients.

G. # RU / 10<sup>6</sup> cells.

H. % of BM cells for CD3, CD45R, and Gr1.

I. Lin-48-, LSK48-, cKit, Sca1, CD150, CD9, and CD166 expression.

J. % Chimerism for (I), (II), and (III).

K. Hoachest blue and Hoescht red.
FIGURE_3

A

B

C

D

E

F

G

H

I

J

K

L

M

CD44

CD49d

CD49e

CD61

CD62L

CD144

CD162

CXCR4

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CD166 regulates human and murine hematopoietic stem cells and the hematopoietic niche

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