HEMATOPOIESIS AND STEM CELLS

CD41 expression marks myeloid-biased adult hematopoietic stem cells and increases with age

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Key Points

- Integrin CD41, thought to absent on adult HSCs, marks a novel subset of myeloid-biased long-term HSCs that becomes prevalent with age.
- Loss of CD41 leads to transplantable hematopoietic defects that affect HSC survival and maintenance and are, in part, mediated by platelet loss.

The hematopoietic stem cell (HSC) compartment is heterogeneous, yet our understanding of the identities of different HSC subtypes is limited. Here we show that platelet integrin CD41 (αIIb), currently thought to only transiently mark fetal HSCs, is expressed on an adult HSC subtype that accumulates with age. CD41+ HSCs were largely quiescent and exhibited myeloerythroid and megakaryocyte gene priming, governed by Gata1, whereas CD41− HSCs were more proliferative and exhibited lymphoid gene priming. When isolated without the use of blocking antibodies, CD41+ HSCs possessed long-term repopulation capacity on serial transplantations and showed a marked myeloid bias compared with CD41− HSCs, which yielded a more lymphoid-biased progeny. CD41-knockout (KO) mice displayed multilineage hematopoietic defects coupled with decreased quiescence and survival of HSCs, suggesting that CD41 is functionally relevant for HSC maintenance and hematopoietic homeostasis. Transplantation experiments indicated that CD41-KO-associated defects are long-term transplantable, HSC-derived and, in part, mediated through the loss of platelet mass leading to decreases in HSC exposure to important platelet released cytokines, such as transforming growth factor β1. In summary, our data provide a novel marker to identify a myeloid-biased HSC subtype that becomes prevalent with age and highlights the dogma of HSC regulation by their progeny. (*Blood*. 2013;121(22):4463-4472)

Introduction

Hematopoietic stem cells (HSCs) are responsible for the production of all blood lineages for the duration of an individual's life span. During the aging of mice, HSCs undergo cell-intrinsic and cell-extrinsic alterations characterized by attenuated lymphoid-cell production and a decline in their functional potential.[1-3] These changes were originally envisioned to take place within a homogeneous HSC population that changes over time.[2] However, several recent studies indicate that even phenotypically well-defined HSC subsets are functionally heterogeneous, differing in their life span, cycling status, and lineage bias.[4-8] Intriguingly, the proportions and numbers of these subtypes change with time throughout fetal, young, and old life stages.[9] Thus, hematopoietic aging can be viewed as the outcome of gradual changes in the clonal composition of the HSC compartment with respect to distinct HSC subtypes.[9-12] However, the cell surface phenotypes of the different subtypes and the mechanisms behind these shifts remain poorly understood. Here we identify integrin CD41 as a novel marker for adult and aging HSCs and as functionally relevant for hematopoiesis.

Integrins are α/β heterodimeric transmembrane receptors mediating broad effects on cytoskeletal organization, motility, and survival in many cell types.[13] On HSCs, integrins play important roles for mobilization, localization, and engraftment.[14,15] Integrin αIIb (*itga2b*, CD41, GPIIb), the classic platelet marker, heterodimerizes with integrin β3 (*itgb3*, CD61, GPIIIa) and binds to fibronectin, fibrinogen, vitronectin, and von Willebrand factor.[16] The CD41/CD61 complex is required for platelet aggregation and clotting, and mutations of either CD41 or CD61 in humans causes Glanzmann's thrombasthenia.[17] Notably, CD41 is also the first blood-specific marker during in vitro differentiation of embryonic stem cells[18] and is expressed on all hematopoietic stem and progenitor cells in the early embryo.[19-22] However, CD41 expression is downregulated in midgestation embryos, and it is currently thought that CD41 expression on adult HSCs remains switched off throughout adult life.[23]

We show that CD41 expression increases with age to specifically mark most long-term (LT)-HSCs. CD41-knockout (KO) mice displayed pancytopenia coupled with increased HSC apoptosis and proliferation. The defects were LT transplantable and partly mediated through a reduced platelet mass, affecting transforming growth factor β1 (TGFβ1) and vascular endothelial growth factor (VEGF)-A targets in CD41-KO HSCs. Finally, we show that CD41+ wild-type (WT) HSCs exhibit robust, LT reconstitution capacity and are predominantly myeloid-biased. Our results uncover CD41 as a previously unknown marker for adult/aging HSCs, providing a novel strategy for the prospective isolation of a myeloid-biased LT-HSC subset.

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Methods

Animal work

Mice were housed in the animal facility of the Barcelona Biomedical Research Park, and procedures were approved by the Generalitat de Catalunya. CD41-KO mice were previously generated.[24] Unless otherwise stated, mice were 2 to 4 months old.

FACS analysis

Fluorescence-activated cell sorter (FACS) analysis was performed on a BD LSRII (BD Bioscience), and sorting on a BD Aria II (BD Bioscience). FACS data were analyzed with FlowJo v9.60.

Transplantation assays

Mice were inoculated retro-orbitally with 500 000 (noncompetitive setup) or 200 000 (competitive setup) BM cells. For antibody blocking, 80 ng CD41-phycoerythrin (MWReg30, BD) was used on 200 000 BM cells. One-tenth of a femur was used for serial transplantations.

Microarray analyses

RNA from 1000 HSCs (Lin-sca1+ckit+ (LSK) flt3− or LSKCD150+ CD48−) from pools of 4 to 5 mice was extracted as described.[25] Labeled cDNA was hybridized onto Agilent 8 × 60 000 chips in triplicate. Data analysis was performed with DAVID (http://david.abcc.ncifcrf.gov/) and gene set enrichment analysis (GSEA; www.broadinstitute.org/gsea/). The microarray data are deposited as GSE45561.

Single HSC methylcellulose cultures

CD41+ and CD41− LSKCD150+CD48− were single-cell sorted into 96-well plates containing 100 μl methylcellulose (M3434, StemcellTech) and cultured for 14 days. Colony size was measured using a Leica DMI6000B and LAS-AF v.2.6.0 software (Leica Microsystems).

Ex vivo HSC culture

Two hundred to 300 CD41+ and CD41− LSKCD150+CD48− HSCs were cultured in 24-well plates containing StemSpan SFEM (09600, StemcellTech) supplemented with heparin (10 μg/mL), stem cell factor (20 μg/mL), recombinant human thrombopoietin (rhTPO) (20 μg/mL), basic fibroblast growth factor (bFGF) (10 μg/mL), and insulin growth factor 2 (10 μg/mL; all Peprotech) and cultured for 7 days.

5-fluorouracil treatment of mice

Mice were injected intraperitoneally with 150 mg/kg 5-fluorouracil (Sigma) and bone marrow (BM) analyzed after 7 days.

Results

CD41 is expressed on adult murine HSCs and increases with age

We first analyzed the expression of CD41 on HSCs in young and older mice. Subfractionation of the BM LSK compartment with CD34 and flt3 ('34F' code) or CD48 and CD150 ('SLAM' code) revealed a composition shift during aging (supplemental Figure 1A, available on the *Blood* Web site). Specifically, the LT-HSC population (LSKCD34−Flt3− or LSKCD48−CD150+) accumulated with age, whereas the LSKCD34+Flt3+ population (containing lymphoid-primed multipotent progenitors [LMPPs]) decreased (supplemental Figure 1A), as previously described.[1] In young mice (aged 2 months), CD41 expression on LSKs was low (~3%-5%; Figure 1A), which is consistent with the notion of CD41 downregulation during midgestation.[21,26] However, CD41+ LSKs were significantly increased with age (6 months: ~10%; 16 months: ~30%; Figure 1A). CD41 expression was predominantly confined within the LT-HSC subset, being largely absent on short-term (ST)-HSCs (LSKCD34+flt3− or LSKCD48+CD150−), LSKCD34+Flt3+ progenitors, and mature leukocytes (Figure 1B, supplemental Figure 1B, and not shown). Notably, 35% to 60% of LT-HSCs were CD41+ in mice older than 6 months (Figure 1B-C). CD41 expression correlated largely with that of CD150, as CD150-high-expressing LSK cells displayed higher levels of CD41 than CD150− low cells (supplemental Figure 1C). Finally, CD41+ HSCs possessed high dye-efflux capacity (ie, lower side population) and high levels of ESAM (supplemental Figure 1D), 2 additional immunophenotypical definitions of LT-HSCs.[4,27] The absence of another platelet marker (glycoprotein IbA [GpIbA, CD42b]) on LSKs excluded the possibility that CD41 expression was a result of adhering platelets (supplemental Figure 1E).

Together, these data reveal an age-related heterogeneous expression of CD41 on phenotypical LT-HSCs.

Loss of CD41 leads to perturbations of the hematopoietic system

To determine whether CD41 was functionally relevant for adult hematopoiesis, we turned to our $CD41^{YFP/YFP}$ knock-in mouse model (CD41-KO).[24] Compared with age-matched C57BL6 WT controls, 2- to 3-month-old and 9- to 10-month-old CD41-KO mice displayed reduced platelet count (Figure 2A). As mean platelet volume was unaltered, total platelet mass was reduced (supplemental Figure 2A). Moreover, CD41-KO mice exhibited reductions in erythrocyte and all leukocyte lineages (Figure 2A and supplemental Figure 2B-C). Whereas with age all blood compartments increased in WT mice, CD41-KO mice displayed unchanged or decreased blood cell counts, resulting in the exacerbation of the pancytopenic phenotype in older mice (Figure 2A and supplemental Figure 2B). Similarly, the CD41-KO BM was hypocellular in both myeloid and B-cell lineages and more pronounced in older animals (Figure 2B-D). In contrast, the extramedullar hematopoietic sites spleen and thymus displayed normal cellularity and progenitor cell numbers (supplemental Figure 2D and data not shown), suggesting that the BM cellularity decrease was not a result of increased HSC mobilization.

Together, these results show that CD41 loss leads to the perturbation of adult hematopoiesis.

Survival and maintenance of HSCs is affected in CD41-KO mice

We next asked whether CD41-KO HSC and progenitor cell compartments were likewise perturbed. The LSK compartment composition was mildly altered (Figure 2E and supplemental Figure 2E), resulting in a reduction in ST-HSC numbers and myeloerythroid progenitors (Figure 2F and supplemental Figure 2E-F).

To understand the cause behind HSC loss, we assessed apoptosis in CD41-KO mice. Notably, CD41-KO LSKCD34− LT-HSCs and LSKCD34+ cells (ST-HSC+LMPP) were significantly more apoptotic than WT, as determined by either AnnexinV or TUNEL staining (Figure 2G and supplemental Figure 2G). Moreover, in vivo 5-bromo-2′-deoxyuridine (BrdU) incorporation assays revealed that CD41-KO LT-HSCs, but not LSKCD34+ cells, showed an increased proliferation rate (Figure 2H).

Taken together, these data indicate that loss of CD41 expression leads to decreased survival and quiescence of HSCs.

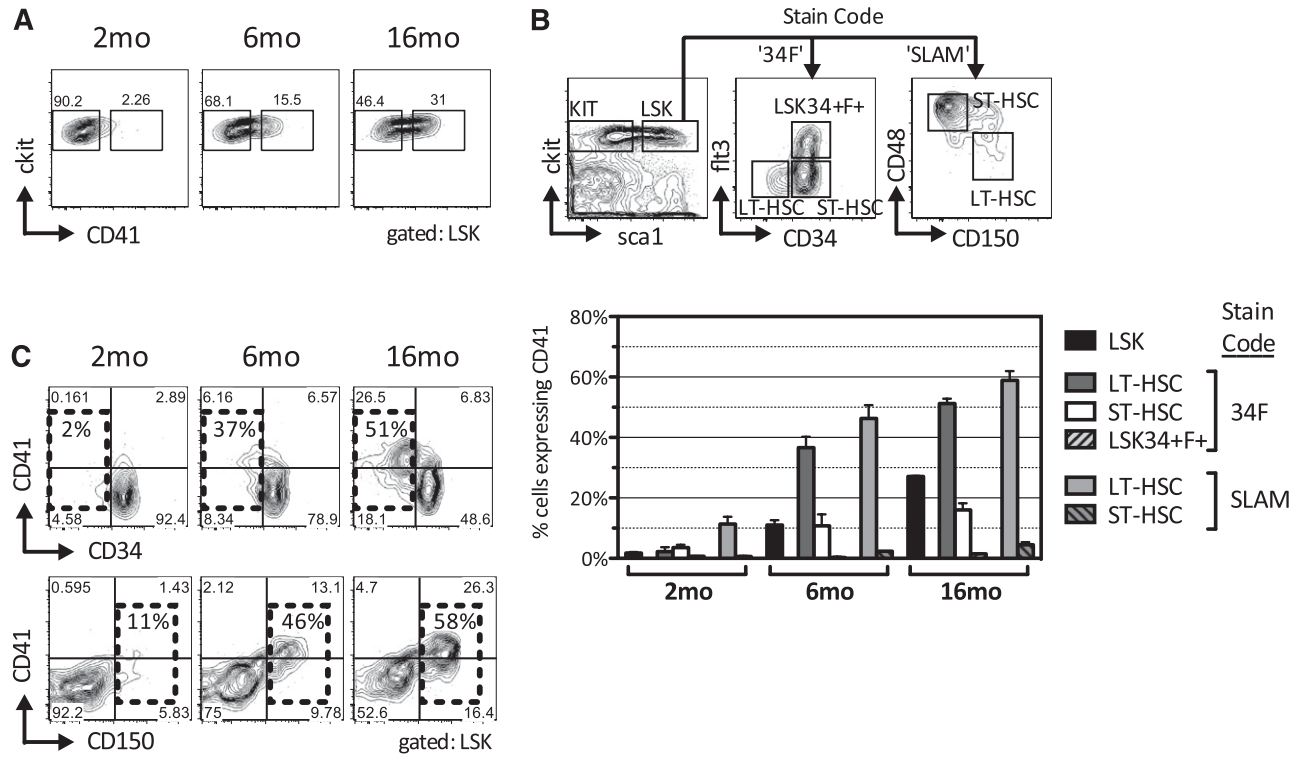

Figure 1. CD41 expression increases with age to mark the majority of LT-HSCs. (A) Representative FACS plots of CD41 expression within LSK cells in 2-month-old, adult 6-month-old, and 16-month-old mice. (B) Top panel shows gating strategies for analysis of the LSK compartment according to the 34F (CD34/flt3) or SLAM (CD150/CD48) stain codes. Bottom panel shows a bar diagram depicting the proportion of cells expressing CD41 within each population shown gated in the top panel. The LSKCD34+Flt3+ (LSK34+F+) gate contains LMPPs (n = 3). Error bars, SEM. (C) Representative FACS plots showing the expression of CD41 plotted against either CD34 (top) or CD150 (bottom) in mice of different ages. Quadrant statistics are frequency of parent, whereas statistics in bold text (%) correspond to the proportion of CD41+ cells within the LT-HSC subset defined as CD34− (top) or CD150+ (bottom). Note that in addition to the increase in the proportion of phenotypical LT-HSCs with age, the proportion of LT-HSCs expressing CD41+ increases as well. HSC, hematopoietic stem cell; LT, long-term; ST, short-term.

The CD41-KO hematopoietic defects are LT transplantable and negatively affect WT hematopoiesis

To assess CD41-KO HSC function, we transplanted WT or CD41-KO BM cells noncompetitively into lethally irradiated congenic WT recipients (Figure 3A). Donor chimerism levels vs endogenous cells reached ~95%, as expected, confirming recipient myeloablation (Figure 3B). Notably, analysis of peripheral blood after 4 months showed that CD41-KO HSCs yielded reduced blood leukocyte counts (Figure 3C) with a normal lineage distribution (supplemental Figure 3A), recapitulating the steady-state phenotype of CD41-KO mice. Inverse transplantations of WT BM into irradiated CD41-KO recipients revealed no discernible hematopoietic defects in blood, BM, spleen, or thymus (supplemental Figure 3B). Together, these data indicate that the CD41-KO hematopoietic defects are LT transplantable and HSC-derived.

We next performed competitive transplantations of WT or CD41-KO BM mixed 1:1 with congenic WT competitor cells and injected into irradiated hosts (Figure 3D and supplemental Figure 3C). Donor chimerism levels vs competitors were similar between WT and CD41-KO HSCs at 4 months (Figure 3E), and donor lineage distribution was normal (supplemental Figure 3D). However, closer inspection of peripheral blood at 4 to 6 months revealed that mice receiving CD41-KO + WT cells displayed reduced leukocyte and platelet counts compared with mice receiving WT + WT BM cells (Figure 3Fi-ii), suggesting that the hematopoietic defect of CD41-KO mice is dominant over and affects WT hematopoiesis. Consistent with this interpretation, the WT competitor lineage distribution was altered when it was cotransplanted with CD41-KO cells (supplemental Figure 3E).

Taken together, these data suggest that the HSC-derived hematopoietic defect in CD41-KO mice influences WT hematopoiesis, hinting at a downstream-of-HSC feedback mechanism affecting HSC homeostasis.

Gene expression analysis of CD41-KO HSCs shows downregulation of TGFβ targets

To elucidate the nature of the CD41-KO HSC defects, we performed microarray gene expression analyses of HSCs from young (2 months) and older (1 year) WT and CD41-KO mice. Because of the rarity of these cells, we used a whole-transcriptome amplification approach[25] to amplify RNA from 1000 sorted LSKCD48−CD150+ LT-HSCs obtained from a pool of 4 mice (*Materials and Methods*). Analysis of differentially expressed genes shared in both ages by GSEA revealed changes in several signaling pathways (supplemental Figure 4A). Notably, the top enriched pathway downregulated in CD41-KO HSCs was TGFB_UP.V1_UP ($P < .01$, NES; 1.48), a gene set composed of targets upregulated by TGFβ1 signaling, including transcription factors *Jun*, *JunB*, and Klf family members as well as signaling molecules such as thrombopoietin (*thpo*; Figure 4A and supplemental Figure 4B). In addition, a VEGF-A target gene set was significantly downregulated in CD41-KO LT-HSCs (Figure 4A).

Thus, the microarrays suggest that HSCs in CD41-KO mice are exposed to decreased levels of important signaling molecules such as TGFβ and VEGF-A, both of which are known to be abundant in platelets.[28]

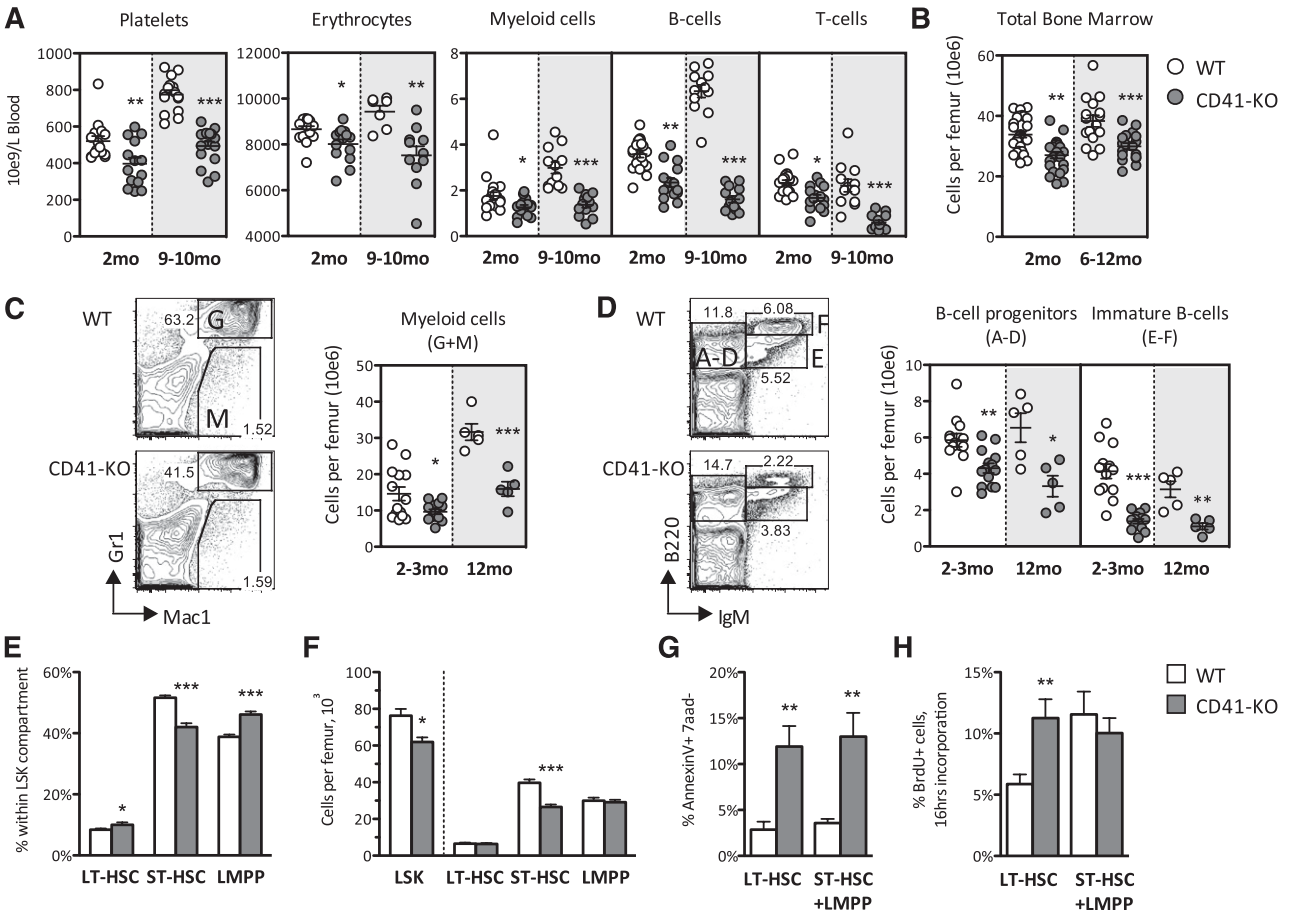

Figure 2. CD41-KO mice are pancytopenic, and CD41-KO HSCs display increased apoptosis and proliferation. (A) Complete blood cytometry quantification of the platelet, erythrocyte, and leukocyte compartments in 2- to 3-month-old and 9- to 10-month-old mice. Leukocytes were lineage analyzed by FACS for markers of myeloid (Mac1), B-cells (B220), and T-cells (CD4/CD8a) (n = 12-19). (B) Whole-BM cellularity in 2- to 3-month-old and 6- to 12-month-old mice (n = 16-29). (C-D) FACS plots of BM myeloid (C) and B-lymphoid (D) markers. Scatter plots show total cellularity per femur of gated populations in 2- to 3-month-old and 12-month-old mice. (A-D) Pre-proB and pre-B (B220+IgM−) cells, Frs. (E-F) Immature B-cells (B220+IgM$^{lo\text{-}high}$) (n = 5-14). Bar diagrams showing the composition (E) and total numbers per femur (F) of the LSK compartment in WT and CD41-KO mice (n = 8-10). (G) Levels of Annexin-V+7aad- apoptotic HSC subsets (n = 8-10). (H) In vivo BrdU incorporation assays of HSC subsets (n = 8-10). Error bars, SEM. *$P < .05$, **$P < .01$, ***$P < .001$ by Student *t* test. Frs, fractions; G, granulocytes (Mac1+Gr1+); M, monocytes/macrophages (Mac1+Gr1−).

CD41+ and CD41− HSCs display gene expression differences with respect to lineage priming and cell cycle activity

To shed light into the molecular identity of CD41+ HSCs, we performed microarray analyses of WT CD41+ and CD41− LSKflt3− HSCs (*Materials and Methods*).

Gene ontology terms enriched in CD41+ HSCs included response to wounding, cell adhesion, and chemotaxis, and comprised several megakaryocyte/platelet genes (eg, *pf4, gp9, selp*, and *vwf*; Figure 4B and supplemental Figure 5A). Notably, key myeloerythroid and megakaryocyte transcription factors, including *gata1, zfpm1* (Fog-1), *gfi1b,* and *klf1*,[29-31] were selectively expressed by CD41+ HSCs (Figure 4B). Extending these results, the TGFβ type 3 receptor, a direct target of growth factor independent 1B (Gfi-1B),[30] was specifically expressed on CD41+ LT-HSCs (Figure 4C). Conversely, gene ontology terms associated with CD41− HSCs included regulation of apoptosis, cellular response to stress, cell cycle process, and M phase, suggesting a higher proliferative propensity compared with CD41+ HSCs (supplemental Figure 5A). Moreover, CD41− HSCs selectively expressed lymphoid-restricted genes; for example, transcription factors *ikzf1*, *ikzf2*, and *notch1-2* and cytokine receptors *flt3* and *il7ra* (Figure 4B).

Transcription factor motif analysis by GSEA on genes enriched in CD41+ HSCs revealed the enrichment of the GATA1 motif, indicating that Gata1 is selectively active in CD41+ HSCs (Figure 4D and supplemental Figure 5B). In contrast, motifs of E2F family members (eg, E2F1) and Myc/Max, which are known cell cycle regulators and also involved in apoptosis,[32,33] were enriched in CD41− HSCs (Figure 4D, supplemental Figure 5B, and data not shown). The expression of some of the aforementioned genes was validated by reverse transcription–polymerase chain reaction (supplemental Figure 5C).

In summary, these data suggest that CD41+ HSCs exhibit priming of myeloerythroid and megakaryocyte genes, whereas CD41− HSCs selectively express lymphoid-associated genes and appear to be mitotically more active.

CD41 expression correlates with a quiescent state of HSCs

To functionally validate the microarray data, we assessed the in vivo proliferation rates of the 2 HSC subsets. The CD41+ LSK compartment incorporated significantly less BrdU than CD41− LSKs in mice of different ages (Figure 5A). Likewise, a more detailed analysis in 2-month-old mice showed that CD41+ LT-HSCs and ST-HSC/LMPPs incorporated significantly less BrdU compared with the corresponding CD41− fractions (Figure 5B).

Next, we single-cell sorted and cultured CD41+ and CD41− LSKCD150+CD48− LT-HSCs in methylcellulose for 14 days.

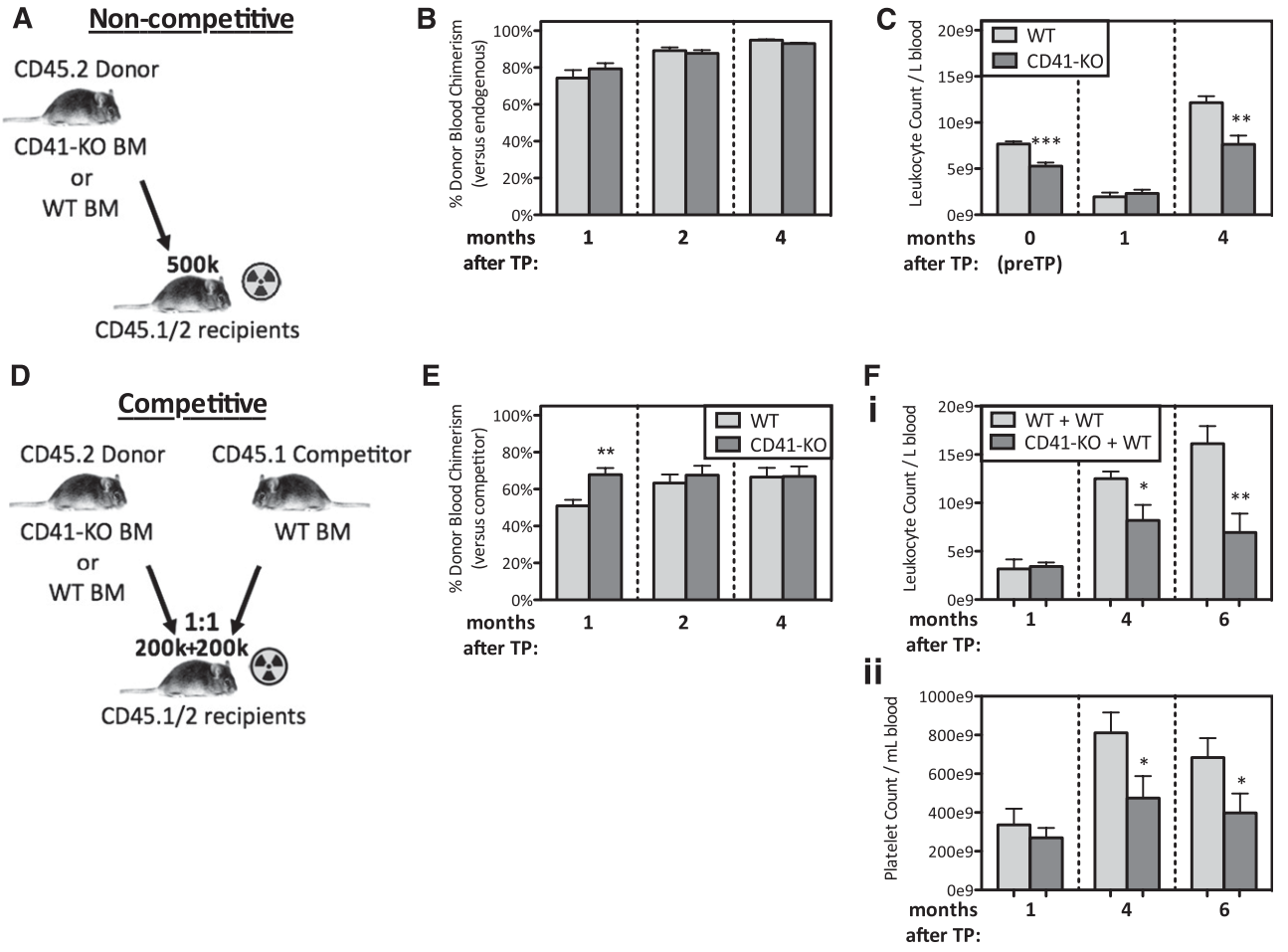

Figure 3. The hematopoietic defects of CD41-KO mice are LT transplantable and negatively affect WT hematopoiesis. (A) Noncompetitive transplantation setup: 500 000 WT or CD41-KO unfractionated BM were injected per recipient. (B) Donor chimerism vs endogenous cells shows near-total myeloablation of recipients (n = 5). (C) Complete blood cytometry analysis showing total leukocyte count in peripheral blood under steady-state conditions (0 months) and in recipient mice 1 and 4 months after BM transplantation with WT or CD41KO cells (n = 11-13). (D) Competitive transplantation setup: WT or CD41-KO BM cells were injected into recipients mixed at equal ratios (1:1; 200 000 + 200 000) with congenic WT BM competitor cells. (E) Donor chimerism level in peripheral blood of recipient mice at 1, 2, and 4 months after transplantation (n = 18-23). (F) Complete blood cytometry analysis of total peripheral blood showing (i) leukocyte and (ii) platelet numbers in recipients receiving either WT+WT or WT+KO cells (n = 4). Error bars, SEM. $*P < .05$, $**P < .01$, $***P < .001$ by Student t test.

The clonogenicity of both HSC fractions was high (~85%; data not shown). However, CD41− LT-HSCs consistently yielded a larger proportion of high proliferative colonies (>2 mm), whereas CD41+ HSC-derived colonies were smaller and many wells contained fewer than 40 cells (Figure 5C). Similar results were obtained with liquid cultures (data not shown).

Finally, we investigated the effect of proliferative stress on the expression of CD41 on WT HSCs. Of note, 7 days after 5-FU-induced myeloablation, CD41 expression was significantly down-regulated compared with nontreated controls (Figure 5D).

Together, these results suggest that CD41 expression correlates with a relative quiescent state of HSCs in the adult BM.

CD41+ HSCs are hierarchically related to CD41− HSCs

We next investigated the hierarchical relationship between CD41+ and CD41− HSCs. Although currently not technically possible to fully maintain HSCs ex vivo, we opted for culture conditions that maximize HSC viability and maintenance for up to 7 days.[34] CD41+ and CD41− LSKCD48−CD150+ cells were isolated and cells analyzed by FACS for immunophenotypical changes at different times. As expected, many cells lost their Lin−sca1+ckit+CD48−CD150+ ('LT-HSC') phenotype during the culture period, as HSCs started to differentiate (data not shown); however, cells retaining the LT-HSC phenotype were present for 7 days and analyzed for CD41. Notably, we found that CD41+ LT-HSCs gave rise to both CD41+ and CD41− LT-HSC cells in all times analyzed (Figure 5E). However, CD41− LT-HSCs appeared to yield predominantly CD41− LT-HSC cells at 5 to 7 days, although a small, transient population of CD41+ LT-HSC cells was detectable at 2 to 3 days (Figure 5E).

These data suggest that, at least under these culture conditions, CD41+ HSCs reside at a hierarchical position equal or above that of CD41− HSCs.

MWReg30 antibodies impair the LT repopulation ability of WT HSCs

To functionally assess CD41+ HSCs in vivo, we performed control experiments using the anti-CD41 antibody clone MWReg30, which is known to block CD41 function on platelets.[35] The specificity of MWReg30 for HSC-expressed CD41 was confirmed, as WT but not CD41-KO LSKCD150+ LT-HSCs were stained (Figure 6A). Next, we incubated BM cells from 2- to 3-month-old WT mice for

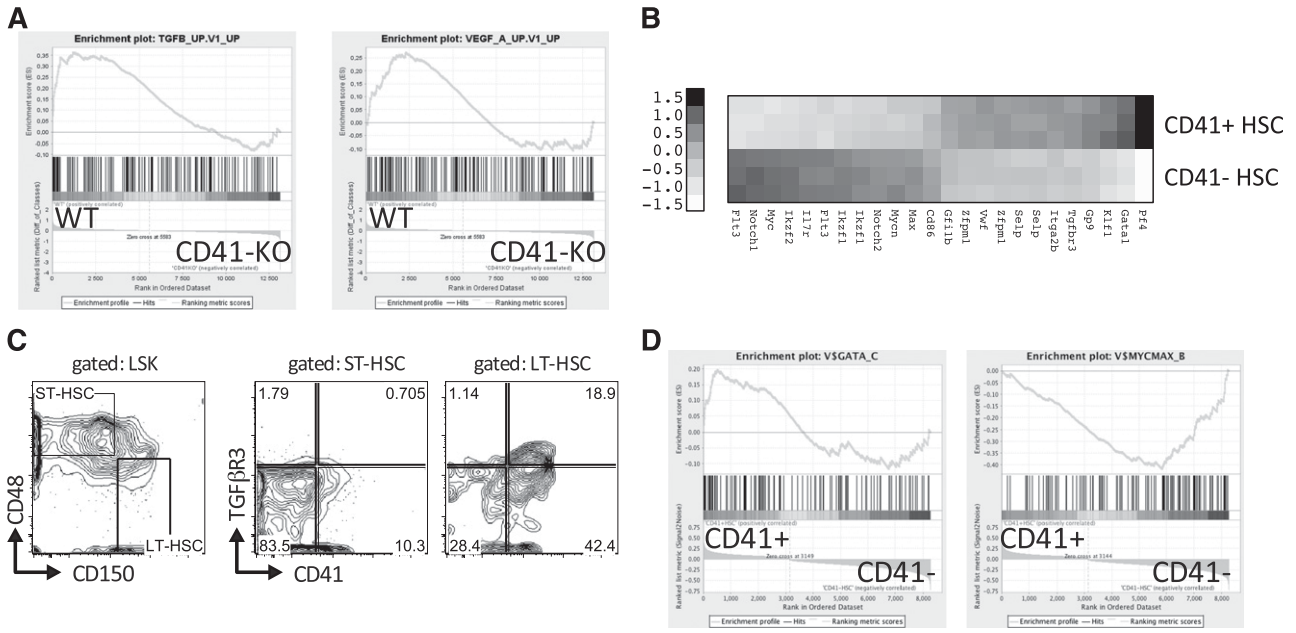

Figure 4. Gene expression analyses show alterations in CD41-KO HSCs and suggest differential lineage priming of CD41+ WT HSCs compared with CD41− WT HSCs. (A) GSEA plots showing top gene sets downregulated in CD41-KO LSKCD150+CD48− LT-HSCs compared with WT. (B) Heat map showing the expression of selected genes differentially expressed in CD41+ and CD41− WT LSKflt3− HSCs and mentioned in the text. Black/white indicates enrichment/depletion. (C) FACS plots showing the coexpression of TGFβR3 within a subset of CD41+, but not CD41−, LT-HSC (LSKCD48−CD150+), or ST-HSC+LMPP (LSKCD48+CD150−) fractions. (D) GSEA enrichment plots for transcription factor motifs enriched in CD41+ (left) and CD41− (right) HSCs.

30 minutes with MWReg30 or control IgG antibodies, mixed 1:1 with untreated WT competitor and injected into lethally irradiated congenic WT mice (Figure 6B). After 1 month, no differences in peripheral blood chimerism were observed (Figure 6C). However, at 4 months (LT), MWReg30-treated cells showed significantly reduced donor chimerism in blood (Figure 6C), BM, spleen, and thymus (data not shown), whereas lineage distribution was unaltered (supplemental Figure 6A).

These results show that MWReg30 blocking antibodies impair the LT repopulation ability of WT HSCs.

CD41+ HSCs exhibit robust, serial repopulation capacity and are more myeloid-biased than CD41− HSCs

Finally, we sought to determine whether the observed differential lineage priming of CD41+ HSCs is functionally relevant in vivo. To avoid the use of blocking antibodies, we used cells from $CD41^{YFP/+}$ heterozygote (CD41-HET) animals, which were functionally WT, as shown by normal hematopoietic parameters including leukocyte, platelet, and BM counts (supplemental Figure 7A and data not shown). Although the YFP+ HSC fraction was uniformly CD41+ (supplemental Figure 7Bi), the YFP− fraction contained false-negative CD41+ cells (supplemental Figure 7Bii), as previously observed in platelets from these mice.[24] With this caveat in mind, we isolated 50 to 100 YFP+ and YFP− LT-HSCs from 4- to 6-month-old CD41-HET mice, mixed with a standard dose of competitor BM cells and injected into lethally irradiated congenic recipients (Figure 7A). YFP+ LT-HSCs exhibited robust LT competitive repopulation ability at levels comparable to YFP− HSCs at 4 months (Figure 7B, top). Importantly, YFP+ HSCs showed a significantly higher myeloid bias compared with YFP− HSCs (Figure 7B, bottom, and supplemental Figure 7C) or with unfractionated BM (supplemental Figure 3A,D-E).

At 4 months, we performed secondary transplantations of unfractionated BM from representative mice of each cohort. Most secondary recipients in the YFP+ cohort showed further increased donor chimerism after 4 months, whereas many secondary mice in the YFP− cohort showed unchanged or decreasing chimerism levels (supplemental Figure 7D). Donor lineage distribution in the YFP+ cohort was still more myeloid-biased than in the YFP− cohort (Figure 7B-C, bottom, and supplemental Figure 7C), although less pronounced than in the primary recipients.

In summary, these data show that CD41+ adult HSCs, isolated without blocking antibodies, possess robust competitive LT repopulation capacity and are myeloid-biased.

Discussion

Although CD41 was identified as a marker of embryonic HSCs a decade ago,[18,19,22] it remains controversial whether CD41 marks adult HSCs, and a possible function of CD41 for either embryonic or adult HSCs is not known. Two previous studies suggested its involvement in adult hematopoiesis and maintaining multipotent progenitor cell numbers.[36,37] In contrast, another study showed that only CD41−, and not CD41+, HSCs could reconstitute transplanted recipients.[23] However, the HSCs used in that study were mobilized by Cy/G-CSF (cyclophosphamide/granulocyte colony stimulating factor) and isolated from the spleen using the CD41 antibody clone MWReg30,[23] which we here show impairs LT reconstitution of WT HSCs. In addition, Cy/G-CSF-mobilized HSCs are known to undergo rapid changes in integrin expression patterns, affecting their homing properties.[14] Here we show that when isolated from the BM without the use of blocking antibodies, adult CD41+ LT-HSCs exhibit robust, multilineage competitive repopulation of both primary and secondary hosts, which is the definition of HSCs. These findings conclusively show that CD41 marks a subset of functional adult HSCs and warrant caution against the use of MWReg30 antibodies for isolating adult HSCs.

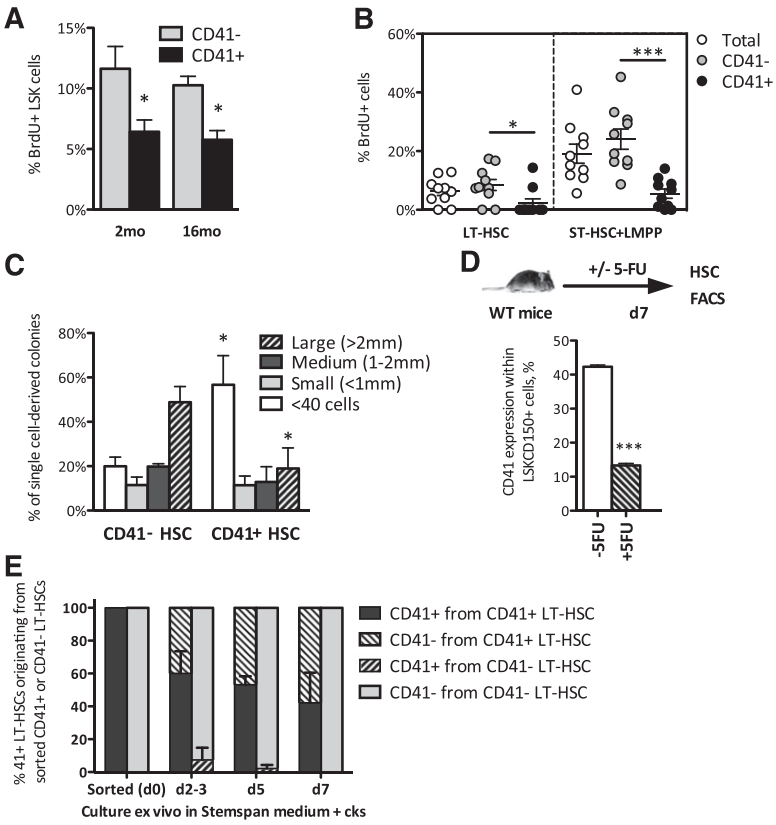

Figure 5. CD41 expression correlates with a quiescent state of HSCs. (A) Bar diagrams showing in vivo BrdU incorporation in CD41+ and CD41− LSK fractions in 2-month-old (n = 10) and 16-month-old (n = 3) mice. (B) Scatter plots showing in vivo BrdU incorporation of CD41+ (dark gray) and CD41− (light gray) LT-HSC (LSKCD34−) and ST-HSC+LMPP (LSKCD34+) cell fractions in individual 2-month-old mice. White symbols indicate total cell fraction (n = 10). (C) Size distribution of 224 single CD41− and CD41+ LT-HSC (LSKCD48−CD150+) derived colonies after 14 days in methylcellulose culture (n = 3). Error bars, SEM. (D) Bar diagram showing expression of CD41 within the LSKCD150+ (LT-HSC) fraction in control (-5FU) or mice 7 days after 5-FU treatment (n = 3). (E) Bar diagram showing the proportion of cells with the LT-HSC phenotype (LSKCD150+CD48−) expressing CD41 after 2 to 3, 5, and 7 days of ex vivo culture of sorted CD41+ or CD41− LSKCD150+ CD48− LT-HSCs. *$P < .05$, ***$P < .001$ by Student *t* test.

CD41+ and CD41− HSCs exhibited differential lineage priming, with CD41+ HSCs enriched in megakaryocyte/platelet (Meg) genes such as *pf4*, *gp9*, and *vwf*, as well as transcription factors *gata1*, *zfpm1* (Fog-1), and *klf1*. Interestingly, *itga2b* (CD41), *pf4*, and *gp9* promoters carry GATA/ets consensus binding sites,[38] known to be bound by Gata-1 and Fog-1 in megakaryocytes,[39] and we show here that the Gata1 motif is enriched in CD41+ HSCs. These observations, together with the fact that expression of *vwf* is enriched in LT-HSCs,[8] indicates the existence of a broader Meg gene module expressed in a subset of HSCs, of which CD41 is part. Moreover, we found that Gfi-1b, a transcription factor important for both dormant HSCs and megakaryocyte/erythroid development,[40] and its target TGFβR3[30] are selectively expressed by CD41+ HSCs. These data are in agreement with and extend the observations that myeloid-biased HSCs are differentially responsive to TGFβ signaling.[4]

In contrast, CD41− HSCs were found to be enriched for lymphoid-associated factors such as *ikzf1*, *ikzf2*, *notch1*, *notch2*, *flt3*, and *il7ra*, as well as CD86, which has been recently found to denote lymphopoietic potential of HSCs.[41] Analysis of HSC lineage bias in vivo confirmed the observations that CD41 marks myeloid-biased HSCs. The fact that the lineage distribution differences between CD41:YFP+ and CD41:YFP− fractions were less evident in secondary recipients could have several interpretations. First, the limitation of our YFP model, in which the YFP− fraction contained false-negative CD41+ cells, could be underestimating the actual lineage bias differences over time. Second, we show by ex vivo HSC cultures that CD41+ HSCs and, to a lesser extent CD41− HSCs, may be able to yield the other HSC phenotype in vivo, suggesting a nonrigid lineage bias state. Whether or not CD41+ HSCs reside at the top of the HSC hierarchy remains to be studied by more detailed analyses in vivo (eg, through the use of currently not commercially available nonblocking CD41 antibodies for HSC isolation). However, our data showing that lineage distribution in CD41-KO mice is not significantly altered would support the interpretation that CD41+ HSCs also can give rise to CD41− HSCs in vivo, at least on conditions of hematopoietic stress such as observed in pancytopenic CD41-KO mice.

Whereas virtually nothing is known in terms of a function of CD41 for HSCs, the role of CD41 in platelets has been studied in great detail. Activation of the CD41/CD61 complex allows platelet adhesion to fibrinogen and other ligands and results in the activation of the small GTPases Rho, Rac, and cdc42 which mediate broad downstream signaling events.[42,43] Interestingly, cdc42 was recently implicated in the aging of HSCs,[44] raising the possibility that CD41-mediated adhesion to niche components acts upstream of cdc42 also in HSCs.

Importantly, it was shown that CD41-deficient multipotent progenitors displayed reduced adhesion to fibronectin and BM-derived stroma,[36] suggesting a direct requirement of CD41 for HSC adhesion. Consistent with the role of integrins for cell survival through adhesion,[45] we found that HSCs in our CD41-KO mouse model showed increased levels of apoptosis. Moreover, CD41-KO HSCs displayed increased proliferation, which could be interpreted as either a compensation mechanism resulting from increased HSC apoptosis or a direct role of CD41-mediated adhesion to, for example, fibronectin for HSC quiescence. Supporting the latter, it was shown that human CD34+ HSC/progenitor cells show enhanced survival and quiescence when cultured on fibronectin.[46] It remains to be studied whether CD41 is also expressed on human HSCs.

Our experiments with the MWReg30 antibodies suggest that CD41 may act as an organizing molecule for other integrins and cell-adhesion molecules on the surface of HSCs. For example, CD41/CD61 on platelets is known to form noncovalent complexes with CD47 (integrin associated protein; IAP), CD9 and glycoproteins (GP)

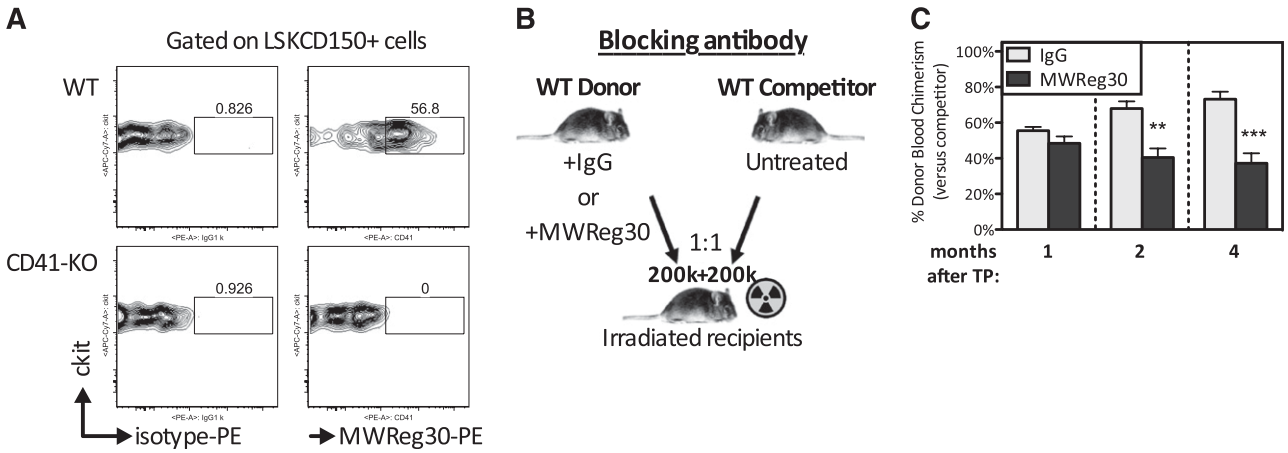

Figure 6. Antibodies against CD41 (clone MWReg30) impair LT repopulation of WT HSCs. (A) Representative FACS plots showing the staining pattern of the MWReg30-PE (anti-CD41) antibody used for blocking experiments in panels B and C, on WT (top right) or CD41-KO (bottom right) LSKCD150+ LT-HSCs. IgG1, κ isotype control staining shown in left column. Note the absence of MWReg30 staining on CD41-KO HSCs confirming its specificity for HSC-bound CD41 antigen. (B) Blocking antibody competitive transplantation setup: WT cells were incubated with isotype or MWReg30 antibodies and injected into recipients mixed at equal ratios (1:1; 200 000 + 200 000) with congenic WT BM competitor cells. (C) Bar diagrams showing donor chimerism level in peripheral blood of recipient mice at 1, 2, and 4 months after transplantation (n = 10-11). Error bars, SEM, $^{**}P < .01$, $^{***}P < .001$ by Student *t* test.

Ib/V/IX.[47] Moreover, CD41 on multipotent progenitors modulates the binding affinity of VLA-4 and VLA-5,[36] two integrins with key roles for HSC homing and lodging.[15] Together, these observations suggest that the MWReg30 blocking antibody disrupts the functional interaction between CD41 and other integrins. This could help explain why MWReg30-blocked LT-HSCs display a LT repopulation defect, whereas CD41-KO HSCs, on which no CD41 is present and could be functionally replaced, appear to engraft properly.

The idea that HSC quiescence and differentiation are processes governed by the modulation of integrins and other cell-adhesion molecules was previously described.[48] Specifically, c-myc was shown to repress integrins and N-cadherin, thus favoring HSC differentiation over quiescence.[48] Our observation that c-myc and its target genes are enriched in CD41− HSCs supports this idea. These data raise the exciting possibility that CD41-mediated adhesion to, for example, fibronectin could improve strategies for maintenance and expansion of HSCs ex vivo or for the de novo generation of HSCs.

In addition to the direct effects of CD41 for HSC maintenance, our transplantation data of CD41-KO cells would suggest the presence of additional layers of feedback mechanisms important for hematopoietic homeostasis. Noncompetitive transplantations revealed that the CD41-KO phenotype was transplantable for the LT, showing that the defects

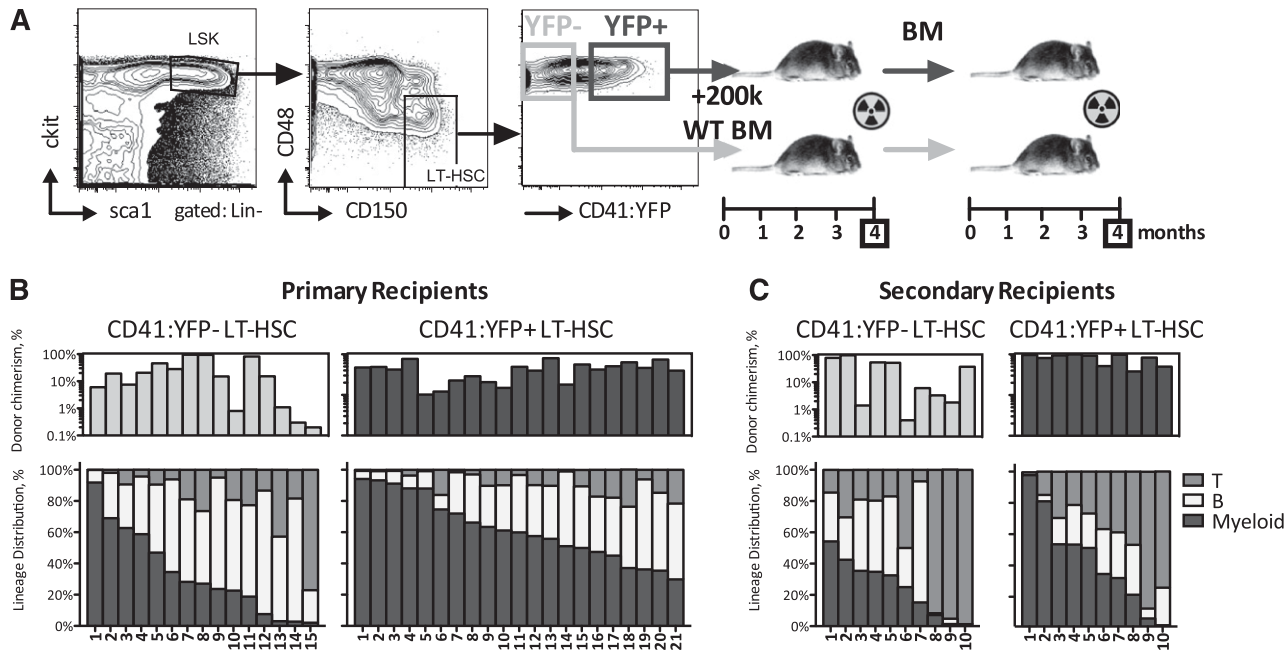

Figure 7. CD41:YFP+ LT-HSCs show robust, LT repopulation ability in secondary recipients and are more myeloid-biased than CD41:YFP− LT-HSCs. (A) Strategy of YFP+ and YFP− LT-HSC isolation and serial transplantation setup. Fifty to 100 LT-HSCs from 4- to 6-month-old CD41$^{YFP/+}$ (CD41-HET) mice were transplanted with 200 000 competitor BM into lethally irradiated primary recipients. After 4 months, unfractionated BM was transplanted into lethally irradiated secondary recipients without addition of new competitor. (B-C) Top panels show donor chimerism in peripheral blood after 4 months in individual primary (B) and secondary (C) recipients. Bar colors: light gray, CD41:YFP−; dark gray, CD41:YFP+ donor HSCs. Bottom panels show donor lineage distribution in individual mice, corresponding vertically to the top panel bars.

derive from and are continuously recapitulated by LT-HSCs. Interestingly, competitive transplantations, in which the resulting hematopoietic system is a chimera between WT and CD41-KO cells, showed that WT HSCs were negatively affected in terms of leukocyte and platelet output and altered lineage distribution.

Although the mechanism behind this result remains unclear and would be of interest for a more detailed future study, several observations prompted us to speculate that part of the CD41-KO phenotype could be mediated by loss of platelets: CD41 expression is completely restricted on HSCs, megakaryocytes and platelets; platelet mass is decreased in CD41-KO mice; platelets are known to signal back to the HSC pool through the regulation of availability of potent cytokines such as thrombopoietin,[49] known to play critical roles in HSCs as well as megakaryocytes/platelets[50]; and gene expression analysis of CD41-KO LT-HSCs revealed the downregulation of direct targets of TGFβ1 as well as VEGF-A, both of which are abundant in and released by platelets.[28]

It is interesting to note that TGFβ1 was shown to regulate myeloid-biased HSCs differently than lymphoid-biased HSCs,[4] suggesting the presence of an in vivo positive feedback loop of platelets to myeloid-biased HSCs (through, eg, TGFβ1). It is conceivable, therefore, that over time, this feedback loop would lead to an increase of myeloid-biased HSCs as well as platelet numbers, both of which are in fact observed during aging of mice (Figures 1C and 2A). Our data thus support the increasing literature that platelets, through their release of cytokines as well as adhesion characteristics, play important roles for normal hematopoiesis as well as cancer, and that expression of CD41 is required to maintain this feedback mechanism to HSCs.

In summary, and contrary to the current dogma, we identify CD41 as a novel marker for adult HSCs, expressed on an HSC subset that accumulates with age. The prospective isolation of CD41+ HSCs should facilitate future studies aimed at resolving the mechanisms of HSC heterogeneity, lineage bias, and hematopoietic aging. In addition, the data highlight the paradigm of HSC regulation by their mature progeny.

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Authorship

Contribution: C.G. designed the research, performed experiments, analyzed the data, and wrote the paper; and T.G. wrote the paper.

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