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Bone marrow laminins influence hematopoietic stem and progenitor cell cycling and homing to the bone marrow

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Abstract

Hematopoietic stem and progenitor cell (HSPC) functions are regulated by a specialized microenvironment in the bone marrow - the hematopoietic stem cell niche - of which the extracellular matrix (ECM) is an integral component. We describe here the localization of ECM

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Authors contributions

K.S. performed most experiments, analyzed data and participated in writing the manuscript. E.K. performed immunofluorescence stainings, confocal microscopy and edited the manuscript. J.H. performed qPCR experiments and edited the manuscript. C.W. was involved in the bone marrow reconstitution assays. I.S. performed the cell cycle experiments. F. R. provided materials, interpreted data and edited the manuscript. C.M.T. provided materials and helped design experiments. S.K. provided materials, helped design experiments, interpreted data and edited the manuscript. L.S. designed and supervised all experiments, analyzed the data and wrote the manuscript.

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Declaration of interest

The authors declare to have no competing interests.

molecules, in particular the laminin α4, α3 and α5 containing isoforms in the bone marrow. Laminin 421 (composed of laminin α4, β2, γ1 chains) is identified as a major component of the bone marrow ECM, occurring abundantly surrounding venous sinuses and in a specialized reticular fiber network of the intersinusoidal spaces of murine bone marrow (BM) in close association with HSPC. Bone marrow from *Lama4* $^{-/-}$ mice is significantly less efficient in reconstituting the hematopoietic system of irradiated wildtype (WT) recipients in competitive bone marrow transplantation assays and shows reduced colony formation in vitro. This is partially due to retention of Lin^{-}c-kit^{+}Sca-1^{+}CD48^{-} long-term and short-term hematopoietic stem cells (LT-HSC/ST-HSC) in the G0 phase of the cell cycle in *Lama4* $^{-/-}$ bone marrow and hence a more quiescent phenotype. In addition, the extravasation of WT BM cells into *Lama4* $^{-/-}$ bone marrow is impaired, influencing the recirculation of HSPC. Our data suggest that these effects are mediated by a compensatory expression of laminin α5 containing isoforms (laminin 521/522) in *Lama4*$^{-/-}$ bone marrow. Collectively, these intrinsic and extrinsic effects lead to reduced HSPC numbers in *Lama4* $^{-/-}$ bone marrow and reduced hematopoietic potential.

Keywords

Laminin; Bone marrow; Hematopoietic stem and progenitor cells (HSPC)

Introduction

Hematopoietic stem cells (HSC) reside in the specialized bone marrow microenvironment, which consists of various cell types and extracellular matrix molecules (ECM). Molecular information provided by cell-cell and cell-matrix interactions, combined with biophysical forces, regulate the survival and self-renewal of hematopoietic stem and progenitor cells (HSPC) [1,2]. Work to date has mainly focused on the cellular components of HSC niches, with little attention to the ECM despite its abundant presence in the bone marrow. One important HSC niche is considered to be the endosteal surface, where several adhesion molecules, such as CD44 and the ECM molecules, osteopontin and osteonectin, have been shown to play a role in HSC retention and survival [3]. However, there is evidence for additional HSC niches involving other cell types including endothelial cells, adipocytes and fibroblasts and indeed several micro-niches are now proposed to exist for different progenitor cell types [4–6]. Apart from osteonectin and osteopontin, various other ECM molecules have been implicated in hematopoietic progenitor cell adhesion and differentiation, in particular fibronectin and the laminins [7,8], both of which can induce intracellular signaling pathways in progenitors and can also influence biophysical aspects of the microenvironment [9]. Recently, it was shown that megakaryocytes are surrounded by a peri-cellular matrix, consisting of fibronectin, type IV collagen and laminin, which regulates megakaryocyte development and homeostasis [10].

Laminins are large (500–1000 kDA) glycoproteins that are secreted as α-β-γ heterotrimers and constitute one of the main components of all basement membranes. Five α (α1–α5), three β (β1–β3) and three γ (γ1–γ3) chains exist that can assemble to generate at least 15 different laminin isoforms [11]. The laminin α chains are responsible for binding cell surface receptors such as integrins, dystroglycan, Lutheran glycoproteins, MCAM, or

sulfated glycolipids [11–16] and are therefore considered to be responsible for the biological function of laminins. In the bone marrow, expression of laminin α4 and laminin α5 containing laminins was reported in basement membranes of sinusoids and larger blood vessels and within the intersinusoidal space in mouse and in man [17–21]. In vitro studies using hematopoietic cell lines have shown that both laminin α4 and α5 containing isoforms can support hematopoietic cell adhesion, however, to different degrees. In general, the adhesion of hematopoietic stem and progenitor cells as well as the more committed hematopoietic precursor cells is higher to laminin α5 than α4 containing laminins [8,19]. The differential binding capacities of the laminin α4 and α5 containing isoforms raise the possibility of specific roles for different laminin isoforms in interaction with hematopoietic stem, progenitor and precursor cells as has been shown for embryonic stem cells. Integrin α6β1 mediated binding of murine embryonic stem cells to laminin 511 in vitro was shown to maintain pluripotency via induction of the PI3K/Akt pathway, while binding to laminin α4 containing laminins favored differentiation [22]. In a previous study we have shown that the bone marrow from laminin α4 deficient mice (*Lama4*$^{-/-}$) [23] has reduced capacity to reconstitute the hematopoietic system of a lethally irradiated recipient [24], specifically implicating laminin α4 containing isoforms in development and/or maintenance of HSPC.

Laminin α4 deletion in mice results in hemorrhage during the embryonic period leading to normochromic, normocytic anemia at birth. This phenotype is not observed in adult *Lama4*$^{-/-}$ mice as laminin α5 containing laminins are deposited in the endothelial basement membranes during the postnatal period and probably compensate for the defect [23]. In the present study, we describe the different types of ECM structures in the bone marrow and use the *Lama4*$^{-/-}$ mouse to investigate how laminin α4-containing laminins affect hematopoietic stem and progenitor cells in vivo. We describe a laminin α4 rich peri-sinus matrix and a reticular fiber network in the intersinusoidal space to which HSPC localize upon intravenous injection. Our data show that laminin α4 deletion leads to a more quiescent cell cycle status of HSPC without affecting their lineage output. In vivo engraftment experiments further show reduced homing of WT HSPC to *Lama4*$^{-/-}$ bone marrow, raising the possibility of an additional defect in migration of HSPC across bone marrow vessels into the bone marrow niche.

Results

ECM of the bone marrow

Immunofluorescence staining of bone marrow preparations and confocal microscopy was used to investigate the distribution and localization of laminin isoforms and other ECM molecules in the bone marrow (Table 1). Double staining with a pan-laminin antibody, which recognizes laminin α1, β1 and γ1 chains and therefore a broad range of different laminin isoforms [25], and the endothelial marker endoglin [26] revealed basement membranes of larger blood vessels and surrounding sinusoids within the bone marrow cavity as previously described [19], as well as a filigree fiber network in the intersinusoidal space (Fig. 1A; Movie 1). A similar staining pattern was observed for laminin γ1 (Fig. 1B) and β2 (Fig. 1C), and for other basement membrane molecules, including perlecan, collagen type IV and nidogen-1 (Fig. 1D; Suppl. Fig. S1). Laminin β1 or γ3 were not detected and

antibody to laminin β3 weakly stained the sinuses only, while anti-laminin γ2 showed a slightly broader localization in sinuses, the fiber network in the intersinusoidal space and arteries (Table 1).

Double staining for basement membrane markers and fibrillar collagens or fibronectin revealed colocalization around sinuses and in the intersinusoidal fiber network, which showed similarities to the reticular fiber network of secondary lymphoid organs [27–29]: all basement membrane molecules tested were found to ensheath a central core of collagen type III and fibronectin (Fig. 1D; Suppl. Fig. S1); collagen type I was not detected (Table 1). However, the diameter of each fiber (approximately 0.5 μm) was considerably smaller than that of reticular fibers and, of several common reticular fibroblast markers tested (Table 1), only ER-TR7 was detected on the outer surface of the fibers (Table 1; Suppl. Fig. S1). This result together with the very small diameter of the intersinusoidal fibers suggests that, while they share some similarities to the reticular fibers of secondary lymphoid organs, they are structurally distinct.

Laminin isoform localization in the bone marrow

Laminin α2, α3, α4 and α5 but not laminin α1 were detected in wild type (WT) murine bone marrow; laminin α2 was restricted to basement membranes around nerve fibers (not shown); while laminin α4 and α5 chain double staining revealed colocalization in basement membranes of larger blood vessels and of the sinusoids (Fig. 2A). Only laminin α4 was clearly detectable in the intersinusoidal fiber network (Fig. 2A) with some laminin α5 occurring exclusively in the thicker fibers immediately subjacent to the sinusoidal wall (Fig. 2A). Expression of laminin β2 but not laminin β1, suggests the existence of laminin 421 in the intersinusoidal fiber network, and laminins 421 and 521 at sinusoidal and blood vessel basement membranes. The presence of laminin γ2 which has been reported to form a heterotrimer with laminin α5 and β2 chains in human bone marrow stromal cells [18], also raises the possibility of laminin 522 around larger blood vessels and venus sinuses.

Laminin α3 staining was sparce and occurred mainly in association with the central artery, and weakly in the reticular fiber network and surrounding sinuses (Fig. 2B), showing a similar pattern to that observed for laminin γ2. Weak staining for laminin β3 (data not shown) only around sinuses suggests low levels of laminin 332 at sinuses and not elsewhere. The presence of laminin γ1 and, to a lesser extent, laminin γ2 but not β3 in the intersinusoidal fiber network and around arteries suggests the existence of low levels of laminin 321, a rare isoform previously only reported in human amnion [30], and potentially also laminin 322 at these sites. The antibody employed for laminin α3 detection recognizes both the short form, typical of the skin, and the less well investigated long form of this chain [31]. Data is summarized in Table 1.

All hematopoietic stem and progenitor cells occur in the so-called lineage negative (Lin^-) population of bone marrow cells, which lack expression of cell-surface markers present on mature hematopoietic cells [32], including CD3, CD11b, CD41, B220, Ter119 and Gr-1. Therefore, to investigate the localization of hematopoietic precursor cells in relation to the bone marrow ECM, Lin^- enriched bone marrow cells isolated from green fluorescence protein (GFP)-mice were injected intravenously (i.v.) into WT recipients, and the bone

marrow was removed 16 h later and was examined by immunofluorescence staining and confocal microscopy. Hematopoietic precursor cells possess the ability to re-enter the bone marrow, a process referred to as 'homing'. The majority of the GFP^+ donor cells were found scattered throughout the bone marrow cavity as previously reported [4], in close association to the intersinusoidal fiber network (Suppl. Fig. S2).

ECM of *Lama4*$^{-/-}$ bone marrow

The distribution of most of the analyzed ECM molecules was similar in WT and *Lama4*$^{-/-}$ mice. Only laminin α3 staining was broader and more intense in *Lama4*$^{-/-}$ mice, becoming detectable in the venous sinuses and in the intersinusoidal fiber network (Fig. 2C, D), while laminin α5 showed a slightly broader expression around venous sinuses (Fig. 2C) and remained intense around larger blood vessels and undetectable in the intersinusoidal fiber network (Fig. 2C). Up-regulation of other laminin α chains or ECM molecules within the bone marrow could not be detected by immunofluorescence analyses (Suppl. Fig. S3).

Flow cytometry of hematopoietic stem and progenitor cell numbers in WT and *Lama4*$^{-/-}$ bone marrow

As previous studies suggested a slower reconstitution of the hematopoietic system by *Lama4*$^{-/-}$ bone marrow compared to WT bone marrow (Suppl. Fig. S4) [24], we assessed the relative amounts of different hematopoietic stem, progenitor and precursor cells present in the bone marrow by flow cytometry using common HSPC marker combinations.

Within the Lin^- population, $Lin^-Sca\text{-}1^+c\text{-}kit^+$ (LSK) cells contain those with stem cell activity [33,34] and consist of long-term HSC (LT-HSC), short-term HSC (ST-HSC) and multipotent progenitor cells (MPP), which can be differentiated on the basis of the cell surface markers listed in Table 2 [35,36]. LT-HSC have a repopulation capacity of >16 weeks, ST-HSC of <10 weeks [33,37], and MPP can give rise to more than one cell type but have a more limited self-renewal capacity than the pluripotent HSC populations [35,38]. Common myeloid progenitor cells give rise to more lineage-restricted cell populations. Using these cell markers, no differences were detected in proportions of the different stem and progenitor cell populations in *Lama4*$^{-/-}$ mice compared to WT mice: 0.19% of the WT and 0.17% of the *Lama4*$^{-/-}$ bone marrow cells were $Lin^-Sca\text{-}1^+ckit^+$ (LSK) (Fig. 3A), of which approximately 5.6% were LT-HSC, 41% ST-HSC and 52% MPP in both *Lama4*$^{-/-}$ and WT mice according to CD34/flt3 marker combinations (Fig. 3B). Use of the alternative CD150/CD48, so-called SLAM, marker combination, revealed the same pattern of results with no differences between *Lama4*$^{-/-}$ and WT mice, but with slightly different values for the ST-HSC and MPP populations (Suppl. Fig. S5), which is probably due to some CD48 expression by ST-HSCs [39]. The proportions of these different hematopoietic cell populations measured in WT mice with the two marker combinations are consistent with data from other groups [35,36].

To assess more committed progenitor cells, Sca-1 expression was examined within the $Lin^-c\text{-}Kit^+$ population (Table 2). During early hematopoietic development Sca-1 expression is down-regulated, so that more committed progenitor cells such as common myeloid cells (CMP) are $Sca\text{-}1^-$ [40]. The proportions of common myeloid progenitor cells were

comparable in WT (82.23%) and *Lama4*$^{-/-}$ mice (82.5%). Also, the proportions of LT-HSC/ST-HSC and MPP did not vary between WT and *Lama4*$^{-/-}$ mice within the Lin^{-}c-Kit^{+} cell population (Fig. 3C); which is in accordance to the data presented above.

Competitive bone marrow reconstitution assays for HSPC quantification

To more precisely quantify hematopoietic stem and progenitor cells from *Lama4*$^{-/-}$ and WT bone marrow competitive reconstitution assays were performed in vivo. Different ratios of WT and *Lama4* $^{-/-}$ cells carrying distinct CD45 allelic markers were transplanted into lethally irradiated WT recipients, and the blood was analyzed by flow cytometry at 4, 8, 12 and 16 weeks for CD45.1^{+} WT versus CD45.2^{+} *Lama4*$^{-/-}$ immune cells. Transfer of equal numbers of WT and *Lama4*$^{-/-}$ cells resulted in 4 times higher numbers of WT derived cells in the peripheral blood (Fig. 4A). This difference was statistically significant at 4, 8 and 12 weeks, but not at 16 weeks, suggesting reduced numbers of ST-HSC in *Lama4*$^{-/-}$ bone marrow. Although not statistically significant, even injection of ten times more *Lama4*$^{-/-}$ bone marrow cells resulted in slightly higher numbers of WT derived leukocytes in the blood, i.e. a ratio of 1.5 WT:*Lama4*$^{-/-}$ cells (Fig. 4B). Only 100 times more *Lama4*$^{-/-}$ bone marrow cells led to higher numbers of *Lama4*$^{-/-}$ derived leukocytes in the blood (Fig. 4C).

Colony forming assays for assessment of ST- and LT-HSCs and lineage specification

Bone marrow from WT and *Lama4*$^{-/-}$ mice was plated in semi-solid medium and the number of colonies that formed at 8 days was counted. As only cells with stem cell characteristics can survive and grow in these culture conditions, and not stromal cells that could secrete ECM molecules, this method assesses the number of hematopoietic stem cells in the donor bone marrow and does not contain secreted laminins in the cultures. The first plating allows analysis of the more mature hematopoietic precursor cells, whereas subsequent replatings reveal information about the differentiation and cell division ability of earlier hematopoietic precursor cells. Plating of total primary bone marrow cells showed a significant reduction in colony numbers derived from *Lama4*$^{-/-}$ mice compared to WT mice (Fig. 4D). The proportions of BFU-E, CFU-G, CFU-M, and CFU-GEMM, which give rise to erythrocytes, granulocytes, monocytes and macrophages, and all three groups of myeloid cells, respectively, in the first plating did not differ in cultures derived from WT or *Lama4*$^{-/-}$ bone marrow (Fig. 4E). The second plating revealed even lower numbers of colonies in cultures derived from *Lama4*$^{-/-}$ bone marrow cells compared to WT bone marrow with mean colony numbers of 200 colonies/mouse from WT BM compared to 77 colonies/mouse from *Lama4*$^{-/-}$ bone marrow (Fig. 4F). Taken together this confirms reduced hematopoietic stem and progenitor cell numbers in the *Lama4*$^{-/-}$ bone marrow but no defect in differentiation capacity.

Steady state hematopoiesis analyses of bone marrow and blood

To further investigate whether loss of laminin α4 affects lineage differentiation of hematopoietic stem and progenitor cells, flow cytometry was performed for Gr-1^{+}CD11b^{high} monocytes, Gr1^{+}CD11b^{low} granulocytes, CD3^{+} T-lymphocytes, B220^{+} B-lymphocytes, CD41 $^{+}$ megakaryocytes and TER119 $^{+}$ erythroid cells, revealing no statistically significant differences between WT and *Lama4*$^{-/-}$ mice either in the bone marrow (Suppl. Fig. S6A) or the spleen (Suppl. Fig. S6B). Accordingly, blood counts revealed similar numbers of

erythroid, lymphoid and megakaryocytic lineage cells in WT and *Lama4*$^{-/-}$ mice. The total lymphocyte, granulocyte and monocyte numbers did not reveal a quantitative difference between WT and *Lama4*$^{-/-}$ mice (Suppl. Fig. S6C). Platelet numbers were slightly elevated in the blood of *Lama4*$^{-/-}$ mice compared to WT mice, however, this was not statistically significant even though the mean platelet volume (MPV) was statistically significantly lower in *Lama4*$^{-/-}$ mice compared to WT mice (Suppl. Fig. S6D, Suppl. Table 1). Also, the red blood cell (RBC) count and relevant RBC parameters were not altered between WT and *Lama4*$^{-/-}$ mice (Suppl. Fig. S6E, Suppl. Table 1).

Lama4 and *Lama5* expression by LSK cells

As immune cells and hematopoietic cells have been reported to express ECM molecules, including laminins [10], we investigated whether bone marrow LSK cells can express laminin α4 mRNA (*Lama4*). As laminin α5 is reported to compensate for loss of laminin α4 in some tissues [23,24], we also investigate expression of laminin α5 mRNA (*Lama5)*. Compared to b.End3 endothelial cells, which express both *Lama4* and *Lama5* mRNA [41], LSK cells sorted by flow cytometry from the bone marrow of WT and *Lama4*$^{-/-}$ mice expressed little or no *Lama4* and *Lama5* as defined by real-time PCR, and importantly no differences were detected between WT and *Lama4* $^{-/-}$ mice (Suppl. Fig. S7), excluding the possibility of cell intrinsic effects.

Cell cycling analyses of HSPCs

The results from the competitive reconstitution assays suggest reduced hematopoietic stem and progenitor cell numbers in the *Lama4*$^{-/-}$ bone marrow compared to WT. As laminins have been implicated in stem cell turnover [42], we performed a flow cytometry cell cycle analysis of bone marrow for the Lin^{-}c-kit^{+}Sca-1 $^{+}$CD48 $^{-}$ hematopoietic stem cells with short-and long-term repopulating activity (LT-HSC/ST-HSC), as well as Lin^{-}c-kit $^{+}$Sca-1^{+}CD48^{+} multipotent progenitor cells and Lin^{-}c-kit^{+}Sca-1^{-}CD48^{+} common myeloid progenitor cells. Significantly more Lin^{-}c-kit^{+}Sca-1^{+}CD48^{-} LT-HSC/ST-HSCs accumulated in the G0 phase of the cell cycle in *Lama4*$^{-/-}$ mice, and correspondingly fewer in G1, compared to WT mice (Fig. 5A). Although statistically not significant, the same tendency was observed for multipotent progenitor cells (Lin^{-}c-kit^{+}Sca-1^{+}CD48^{+}) (Fig. 5B) and common myeloid progenitors (Lin^{-}c-kit^{+}Sca-1^{-}CD48^{+}) (Fig. 5C), suggesting that the absence of laminin α4 slows cycling of both stem and progenitor cells.

HSPC homing to WT and *Lama4*$^{-/-}$ bone marrow

Given that reconstitution of the bone marrow hematopoietic system after irradiation requires extravasation of hematopoietic stem and progenitor cells from the blood to the bone marrow niche, we employed homing assays to investigate whether migration to and entry of hematopoietic stem and progenitor cells into the bone marrow was altered in *Lama4*$^{-/-}$ mice. First, the same number of bone marrow cells from WT or *Lama4*$^{-/-}$ mice was injected i.v. into WT recipients and, secondly, the same number of bone marrow cells from WT mice was injected i.v. into WT or *Lama4*$^{-/-}$ recipients. Sorted CD45^{+} bone marrow cells were isolated and plated in semi-solid medium 16 h after injection and the number of colonies that formed over 8 days was counted. This permitted estimation of colony forming units (CFU) per defined number of donor bone marrow cells. Colony formation was significantly reduced in

WT recipients that received bone marrow transplantations from *Lama4*$^{-/-}$ mice (Fig. 5D), consistent with lower numbers of hematopoietic stem and progenitor cells in *Lama4*$^{-/-}$ bone marrow. However, colony formation was also significantly reduced when WT bone marrow cells were injected into *Lama4*$^{-/-}$ recipients compared to WT recipients (Fig. 5E). Here, the capacity of WT bone marrow cells to induce colony formation in WT recipients was two-fold higher that in *Lama4*$^{-/-}$ recipients. Control mice that did not receive bone marrow transplantation showed no colony formation. This suggests an additional defect in hematopoietic stem and progenitor cell entry into the bone marrow hematopoietic niche in *Lama4*$^{-/-}$ mice.

Discussion

The data presented here indicate that laminin α4 isa major constituent of bone marrow ECM and that in its absence hematopoietic stem and progenitor cell cycling is reduced, but not differentiation into different hematopoietic lineages. Additionally, reduced homing of WT hematopoietic stem and progenitor cells to the bone marrow of *Lama4*$^{-/-}$ mice suggests a defect in extravasation across bone marrow vessels and, thereby, impaired hematopoietic stem and progenitor cell circulation.

We demonstrate that only laminin α3, α4 and α5 chains occur in the bone marrow, with laminin α4 showing the most extensive distribution, occurring in basement membranes of larger vessels and underlying the venous sinusoids, as previously reported [19], but also in a reticular fiber-like network in the intersinusoidal space. Costaining for laminin β and γ chains suggests the occurrence of predominantly laminin 421 in the intersinusoidal fibers, and laminin 421 and 521 around larger vessels and venous sinuses. In the absence of laminin α4, the only notable alteration was a slightly broader expression of laminin α3, most likely in the form of laminin 321 but possibly also as laminin 322, in the intersinusoidal fiber network. While we have previously shown laminin α4 to be a major constituent of the mouse and human bone marrow [17,19], we have not previously analyzed whole mount bone marrow samples, which has revealed a laminin α4-rich intersinusoidal fiber network that interconnects arterioles and venous sinuses, two potential sites of HSC localization [43]. We show here that this intersinusoidal fiber network is a unique structure, comparable to the reticular fiber network of secondary lymphoid organs, but because of its dimensions, extracellular matrix composition and absence of common reticular fibroblast markers it is not likely to have a conduit function as in secondary lymphoid organs [29], but rather a structural role or a role in maintaining specialized adhesive niches for HSC [44]. In particular this intersinusoidal fiber network was altered in the *Lama4*$^{-/-}$ mice, suggesting that some of the effects observed on hematopoietic stem and progenitor cell cycling may be due to a role of this structure in maintaining these cell populations.

Previous data from our laboratory showed slower reconstitution of the hematopoietic system by *Lama4*$^{-/-}$ than WT bone marrow [24], which is probably explained by the combined effects of slower stem and progenitor cell cycling and the reduced extravasation of stem cells into the bone marrow cavity, resulting in reduced hematopoietic and progenitor cell numbers. This was suggested by the results of the in vivo competitive reconstitution assays, which, in particular, showed reduced short-term HSPC numbers in the *Lama4*$^{-/-}$ bone

marrow. Flow cytometry could not confirm reduced short-term HSPC numbers in *Lama4*$^{-/-}$ bone marrow, probably due to the lower sensitivity of this method compared to in vivo competitive reconstitution assays and the low numbers of HSPC in the bone marrow. The statistically significant accumulation of Lin^{-}c-kit^{+}Sca-1^{+}CD48^{-} HSC with long-term and short-term repopulation activity in the G0 phase in *Lama4*$^{-/-}$ mice suggests that the reduced HSPC numbers in these mice is due to their slower cycling. Although not significant, Lin^{-}c-kit^{+}Sca-1^{-}CD48^{+} common myeloid progenitor cells and Lin^{-}c-kit^{+}Sca-1^{+}CD48^{+} multipotent progenitor cells showed the same tendency towards reduced cycling in the *Lama4*$^{-/-}$ mice, suggesting that the effects are not specific for stem cells and early progenitor cells but also for more differentiate populations.

The colony forming assays, which showed fewer colonies in both first and second platings of *Lama4*$^{-/-}$ bone marrow versus WT bone marrow, further support reduced progenitor cell numbers in *Lama4*$^{-/-}$ bone marrow. However, in such experiments it is not HSC but rather myeloid-restricted progenitors and precursors that are assessed and their clonal expansion, i.e. a single progenitor cell that clonally expands into a colony. The first plating reflects mature progenitor numbers, whereas the second plating addresses earlier progenitors. In both platings, the number of colonies was significantly reduced in the case of *Lama4*$^{-/-}$ bone marrow, suggesting lower numbers of both early and mature myeloid progenitors. However, as the proportions of different CFU types did not differ between WT and *Lama4*$^{-/-}$ bone marrow, i.e. the proportion of cells differentiating towards granulocyte, monocyte/macrophage or erythrocyte lineage, this indicates no defects in differentiation capacity. This was supported by unaltered peripheral blood cell counts and numbers of mature immune cell populations including granulocytes, lymphocytes, megakaryocytes and erythrocytes in the bone marrow and spleens of *Lama4*$^{-/-}$ and WT mice. Taken together, these results suggest that although stem and progenitor cells are reduced in the bone marrow of *Lama4*$^{-/-}$ mice they have normal differentiation capacity. In addition, the CFU and in vivo limiting dilution data suggest that laminin α4/α5 probably exert their effects upstream of the myeloid progenitors, at the level of the multipotent progenitors or the short-term HSC. The slower cycling of these stem cells may lead to reduced numbers of progenitors cells committed to myeloid, and possibly also lymphoid lineages, although the latter remains to be investigated.

Since *Lama4*$^{-/-}$ mice still express laminin α5, at least surrounding sinuses and larger blood vessels, and show a broader expression of laminin α3, whether reduced numbers and cycling of stem and progenitor cell populations is due to the absence of laminin α4 or to the presence of laminin α5 and/or α3 cannot be distinguished from the experiments performed here. However, previous studies have reported that only laminin α5 and not laminin α4 or α3 containing isoforms inhibit embryonic stem cell cycling and maintain pluripotency, consistent with our data [22,42,45]. Additionally, laminin α5 containing isoforms support high levels of high affinity binding of bone marrow cells and stem cell lines, while laminin α4 containing isoforms are considerably weaker substrates and laminin α3 containing isoforms have not been shown to support binding [8,17,19]. Taken together, this suggests that the reduced HSPC numbers and cycling observed here is most likely due to inhibitory effects induced by strong adhesion of stem cell and progenitor cell to laminin 521 and

potentially also laminin 522 [18]. Whether weak interactions with laminin 421 stimulate differentiation, however, remains an open question.

Differential effects of laminin α4 and α5 containing isoforms on HSPC formation and function are likely, given differences in their abilities to self-polymerize into networks and to engage different cell surface receptors. Laminin 411 is an isoform with severe N-terminal truncation of one of the three short arms needed to polymerize, it binds to heparin and sulfatides, and is reported to exhibit weak integrin and dystroglycan binding [46,47]. Laminin 421, of which little is known, can be predicted to have the same polymerization and dystroglycan-binding attributes but might bind differently to integrins [12,48]. By contrast, α5-laminins bind strongly to a variety of integrins, including α6β1 [46], α5β1 and αvβ3 [49], all of which are expressed by HSPC [50] and, of which, α6β1 has been shown to mediate binding of primary and hematopoietic progenitor cell lines to laminin 511 in a 2D system [17,19]. In support of integrin-laminin induced signaling in HSPC numbers and cycling, kindlin-3-mediated integrin activation was recently reported to be essential for the retention of short-term repopulating HSC (ST-HSC) but not the long-term repopulating HSC [51] in the bone marrow, resulting in reduced ST-HSCs in the circulation and exhaustion of the hematopoietic system. Additionally, short-term homing of LSK into the bone marrow was impaired in these kindlin 3-mutant mice [51]. While the phenotypes described in this study are more extreme than those described here for the *Lama4*$^{-/-}$ mice they show the same pattern, supporting the possibility that the bone marrow laminins (probably laminin 521/522) represent at least one of the integrin substrates controlling HSPC cycling and extravasation.

In addition to effects on HSPC numbers and cycling, experiments addressing HSPC homing to the bone marrow suggested defects in the hematopoietic niche and/or in HSPC extravasation into the bone marrow niche. The analysis of WT mice transferred with *Lama4*$^{-/-}$ donor cells resulted in 67% fewer colonies than in the case of WT donor cells, consistent with reduced HSPC cells in the *Lama4*$^{-/-}$ bone marrow. However, the converse experiment, where WT cells were transferred to *Lama4*$^{-/-}$ recipients, showed a similar reduced number of colonies in the *Lama4*$^{-/-}$ versus WT recipients. Given that bone marrow was excised at 16 h after transfer this suggests either a defect in HSC survival in the *Lama4*$^{-/-}$ bone marrow or, as has been shown for immune cell infiltration into the brain of *Lama4*$^{-/-}$ mice [24], impaired extravasation from the blood into the bone marrow. As in inflammatory situations, in bone marrow transplantations the HSC must exit from the blood at the level of venules in order to enter into the bone marrow niche. Even though the venous sinuses and their underlying basement membrane are considered to have a fenestrated phenotype, these fenestrations are roughly 200 nm in diameter [52], hence, extravasating cells must still interact both with the endothelium and the basement membrane. As laminin α5, which is still present in the blood vessel basement membranes of *Lama4*$^{-/-}$ mice, inhibits this transmigration process in the case of T lymphocytes and neutrophils during inflammation [24,53], it may have a similar effect on HSC extravasation. As a small population of HSC constantly circulates in the peripheral blood [54] such an effect would reduce both entry into and exit from the bone marrow niche, reducing HSC peripheral circulation. A further factor crucial for stem cell homing and survival in the bone marrow is the chemokine CXCL12/SDF-1, the localization of which within the bone marrow has recently been shown to affect HSC function [4,44]. Whether laminin α4 and/or laminin α5

containing isoforms can differentially bind and/or present this chemokine at specific sites within the bone marrow is a possibility that requires future investigation.

In conclusion, while laminin α4 and α5 containing isoforms have been previously identified in the bone marrow and show to interact with HSCs in vitro, an in vivo function for such interactions has not been shown to date. We provide evidence here that the balance of laminin α4 and α5 containing isoforms in the bone marrow influence self-replication of hematopoietic stem and progenitor cells without affecting lineage output, and influences their recirculation between blood and bone marrow. Our data support an inhibitory role for laminin α5 containing isoforms (laminin 521/522) in both these functions. Such knowledge about the bone marrow extracellular matrix is important for tissue engineering strategies that focus on the generation of extracellular matrix substrates that promote in vitro expansion of HSPC. Future studies will have to address the in vivo signals originating from laminin α4 and α5 containing isoforms, and their effects on stem and progenitor cell expansion and survival in 3D systems reflecting the in vivo architecture.

Material and methods

Animals

C57BL/6 mice (Charles River Laboratories) and *Lama4* $^{-/-}$ mice [23] of 8–16 weeks were employed. C57BL/6 mice carrying CD45.1 were crossed with C57BL/6 mice carrying CD45.2 to create CD45.1/CD45.2 mice. Experiments were conducted according to German animal welfare guidelines and approved by ‘Landesamt fuer Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen’.

Antibodies

Antibodies against the following extracellular matrix molecules were employed in immunofluorescence staining: perlecan (clones C11L1, A7L6) [55], collagen type I (Chemicon, catalog number AB765P), collagen type III (Southern Biotech, catalog number 1330–01), collagen type IV (Chemicon, catalog number AB765P), nidogen-1 [56], laminin 111 (455/pan-laminin), laminin α1 (clone 200) [57], laminin α2 (clone 4H8–2) [58], laminin β1 (clone 3A4) [59], laminin β2 (antibody name 409) [60], laminin γ1 (clone 3E10) [59], laminin α5 (clone 4G6) [41], fibronectin (Sigma, catalog number F3648), monoclonal antibody ER-TR7 [61] (Dianova/BMA catalog number T-2109), laminin α4 domain III (antibody name 377) [41], laminin α3A (a3 LEc1110) [31], laminin β3 VI/V, laminin γ2, laminin γ3 (kind gift from T. Sasaki) [49,62], fibrillin-1 [63], desmin (Progene, catalog number 10570), α-SMA (alpha smooth muscle actin) (Sigma, Catalog number C6198), gp38 (clone 8.1.1, Developmental Studies Hybridoma Bank, University of Iowa). Rat anti-mouse endoglin (clone MJ7/18) [26] was employed as an endothelial marker in immunofluorescence stainings.

Antibodies against the following surface markers were used in flow cytometry: CD3 (clone 145–2C11), PE-cy5.5-anti-CD3 (clone 7D6) [64], CD4 (clone GK1.5), PE-Cy5.5-anti-CD4 (clone RM4–5, Invitrogen), CD8 (clone 5H10), CD8a (clone 53–6.7, Invitrogen), CD11b (clone M1/70) [65], CD19 (clone 6D5), B220 (clone RA3–6B2), Gr-1 (clone RB6–8C5)

[66], CD41 (clone MWreg30) [67], TER-119 (BD Bioscience, catalog number 553673) [68], c-kit (clone 2B8) [69], Sca-1 (clone E13–161.7) [70], CD34 (clone RAM34) [71], flt3 (clone A2F10.1), CD45.1 (clone A20) [72], CD45.2 (clone 30G12) [73], CD48 (BioLegend, clone HM48–1) [74] (all purchased from BD Biosciences, unless otherwise stated).

Additional reagents used in flow cytometry were: PE-Cy7 streptavidin (catalog number 557598; BD Bioscience), anti-Ki67 (clone B56; BD Pharmingen) and Hoechst33342 (catalog number 14533, Sigma).

Tissue collection

Mice between 8 and 16 weeks of age were employed. Murine bone marrow cells were collected from femur and tibia. Lysis of enucleated red blood cells was achieved by adding RBC lysis buffer (BD Pharma Lyse™, 555899) according to the manufacturer's protocol.

Immunohistochemistry

Femurs were embedded in 8% gelatin/PBS and immediately frozen at −80 °C; 20 μm or 40 μm cryosections were used for immunofluorescence staining. Sections were air-dried at room temperature and fixed in ice cold methanol for 5 min at −20 °C. Subsequent blocking with 1% bovine serum albumin (BSA) in PBS was performed in a humidified chamber at room temperature for 1 h. Incubation with primary antibody followed overnight at 4 °C or for 2 h at room temperature, after washing, secondary antibodies were incubated for 1 h at room temperature. Secondary antibodies were diluted in PBS containing 1 μg/ml DAPI to visualize nuclei.

In the case of pan-laminin and endoglin costaining, bone marrow was removed from the bone and stained as a whole mount as described above, with the exception that 1% Triton-×100 was included in all solutions.

Sections were washed with PBS and mounted in Elvanol. Slides were analyzed using a Zeiss AxioImager microscope equipped with epifluorescence (Zeiss) or a LSM700 confocal laser-scanning microscope (Zeiss). Images from Zeiss AxioImager microscope were captured using an ORCA-1 camera (Hamamatsu) and Volocity 6.0.1. software (Improvision).

Blood analyses

Blood was taken from the tip of the tail and collected into a heparinized tube. Blood parameters were measured using the veterinary blood cell counter Vet abc (Horiba).

Flow cytometry (FACS) and cell cycle analyses

Single cell suspensions of bone marrow or spleen cells were obtained by sieving through a 70-μm filter. The cell surface marker combination, $Lin^{-}Sca^{-}1^{+}c\text{-}kit^{+}$ (LSK), was used to identify a population of bone marrow cells enriched for hematopoietic stem and progenitor cells (Table 2). This subpopulation was further subdivided into long-term HSC (LT-HSC), short-term HSC (ST-HSC), multipotent progenitor cells (MPP), lineage-restricted hematopoietic precursor cells (HPC) or myeloid cells based on their differential expression of CD34, flt3, CD48 or CD150 (Table 2). Cell cycle analysis was performed as described

previously [75]. Briefly, all single nucleated cells were identified by flow cytometry using the fluorescent vital dye Hoechst 33342 and subsequent gating on lineage^{-}c-kit^{+} cells, which represent a population enriched for HSPC and more committed progenitors. Sca-1 and CD48 expression was used to subdivide cells into LT-HSC/ST-HSC, MPP and common myeloid progenitors (CMP). Classification into G0, G1 and S/G2/M phase was performed based on Hoechst33342 fluorescence intensity and Ki67 analyses. Flow cytometry was performed using a Calibur™ flow cytometer with CellQuest (BD Bioscience) software or Becton Dickinson FACS Canto™ flow cytometer with Becton FACS Diva (BD Bioscience) software or FlowJow software. Cell sorting was performed with Becton Dickinson FACS Aria™ flow cytometer (BD Bioscience).

Colony forming unit assay (CFU assay)

7×10^4 bone marrow cells were added to 3.5 ml sterile methylcellulose medium in triplicate and colony forming unit assays were performed according to the manufacturer's instructions (MethoCult M3434, Stem Cell Technologies, Vancouver, BC, Canada). Colonies were counted on day 8 after plating. Colony types were classified as burst forming units erythrocytes (BFU-E), colony-forming units granulocytes (CFU-G), colony-forming units monocytes (CFU-M), colony-forming units granulocytes/monocytes (CFU-GM) and colony-forming units granulocytes/erythrocytes/monocytes/macrophages (CFU-GEMM) according to their morphology. After counting, cells were replated: 7×10^4 cells were used for second platings, and analyzed as for the first plating.

Competitive reconstitution assay

CD45.1^{+}/CD45.2^{+} mice between 12 and 16 weeks of age were employed as recipient mice and irradiated with 11 Gy 4 h prior to bone marrow transplantation. Donor bone marrow cells from CD45.1^{+} WT mice and CD45.2^{+} *Lama4*$^{-/-}$ mice were harvested and three different cell suspensions were prepared. Suspension one contained 100000 CD45.1^{+}, 100000 CD45.2^{+}; suspension two contained 10000 CD45.1^{+}, 100000 CD45.2^{+} and suspension three contained 10000 CD45.1^{+}, 1000000 CD45.2^{+} cells. WT CD45.1^{+}/CD45.2^{+} donor bone marrow cells were co-transferred with suspension 1 and 2 to make up the total number of cells transferred to 1×10^6 in order to guarantee the survival of irradiated recipients. Cells were transferred in 200 μl PBS injected i. v. into the tail veins of recipient mice. The reconstitution of the hematopoietic system was analyzed by flow cytometry quantification of the single positive populations only, i.e. CD45.1 WT versus CD45.2 *Lama4*$^{-/-}$ cells, at 4, 8, 12 and 16 weeks after transfer. Data were expressed as the CD45.1 WT/CD45.2 *Lama4*$^{-/-}$ ratio.

Bone marrow homing assays

4 h after irradiation of recipient mice with 11 Gy 5×10^6 donor bone marrow cells were injected into the tail vein of recipient mice. Control mice did not receive bone marrow transplantations. Recipient mice were sacrificed 16 h after injection and 1×10^6 bone marrow cells were gained to perform the CFU assay as described above.

Statistical analyses

Statistical analyses were performed using GraphPad Prism. All values are expressed as means ± standard deviation (SD). Data were tested for normality (Kolmogorov–Smirnov test in GraphPad Prism). If there was no normal distribution, then we used the non-parametric Mann–Whitney test, otherwise we employed the two-sided Student's t-test. Pearson's R value and mean fluorescent intensity were analyzed by ImageJ.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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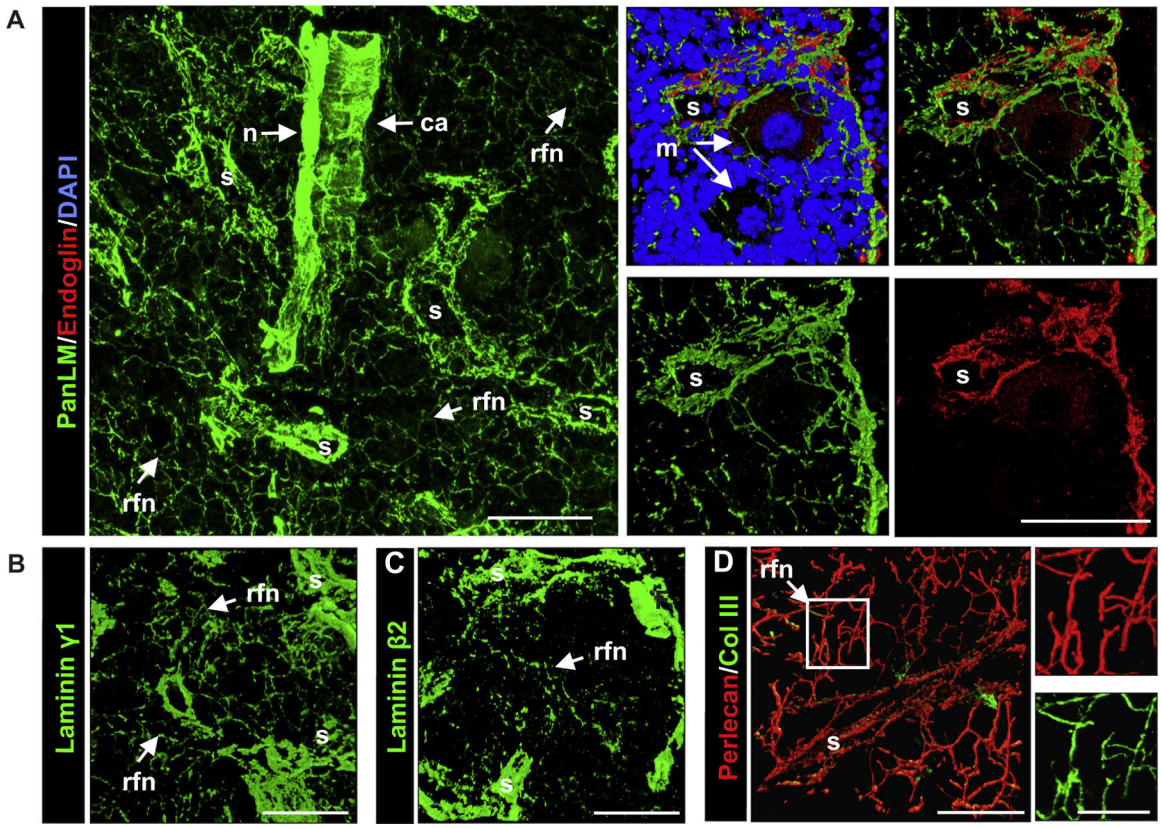

Fig. 1.

ECM localization in WT bone marrow. (A) Immunofluorescence staining of whole mount bone marrow preparations for pan-laminin (PanLM) provides an overview of the central region of the bone marrow, which contains the central artery (ca), nerves (n), the sinusoids (s) and the reticular fiber-like network (rfn) between sinusoids. Costaining of PanLM with endoglin, an endothelial cell marker (shown in right panels) reveals laminin in basement membranes underlying the endothelial layer of sinusoids. The reticular fibers form a net-like structure around the megakaryocytes (m) that reside in close vicinity to the outer surface of sinusoidal wall. (B) Laminin γ1 and (C) laminin β2 occur in the reticular fibers of the intersinusoidal network and in the sinusoidal basement membranes. (D) Double staining for perlecan and collagen type III shows colocalization in the reticular fiber-like structures, where collagen type III forms the cores of the fibers and is ensheathed by perlecan. Boxed area in D is shown at higher magnification to the right. Scale bars are 50 μm in A–C, 25 μm in D, and 10 μm in the insert of D. Data shown are representative of at least 6 mice analyzed.

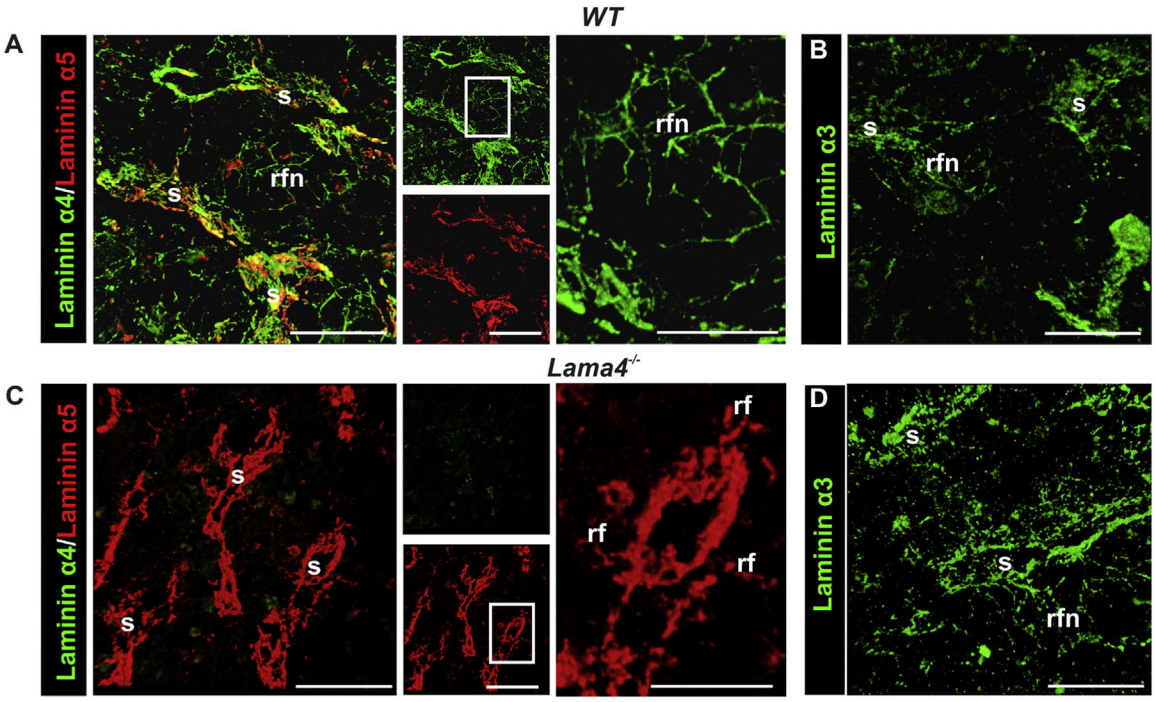

Fig. 2.

Localization of laminin α chains in the bone marrow of WT (A, B) and *Lama4*$^{-/-}$ mice (C, D). (A) Double immunofluorescence staining of WT bone marrow sections for laminin α4 and α5, showing colocalization in basement membranes of sinusoids (s), and filigree staining for laminin α4 throughout the bone marrow between sinusoids in a reticular fiber-like network (rfn); laminin α5 staining is restricted to the reticular fibers (rf) in close proximity to the sinusoids. (B) Laminin α3 staining is weak in the reticular fibers and basement membranes of sinusoids of WT mice. (C) In *Lama4*$^{-/-}$ bone marrow, laminin α5 distribution is slightly broader in the reticular fibers around the sinusoids compared to the WT bone marrow, while (D) laminin α3 staining is more pronounced in the reticular fibers and sinusoids. Boxed areas are shown at higher magnifications to the right. Scale bars are 50 μm, except in high magnifications in A and C where it is 25 μm. Data shown are representative of at least 6 WT mice and 6 *Lama4*$^{-/-}$ mice.

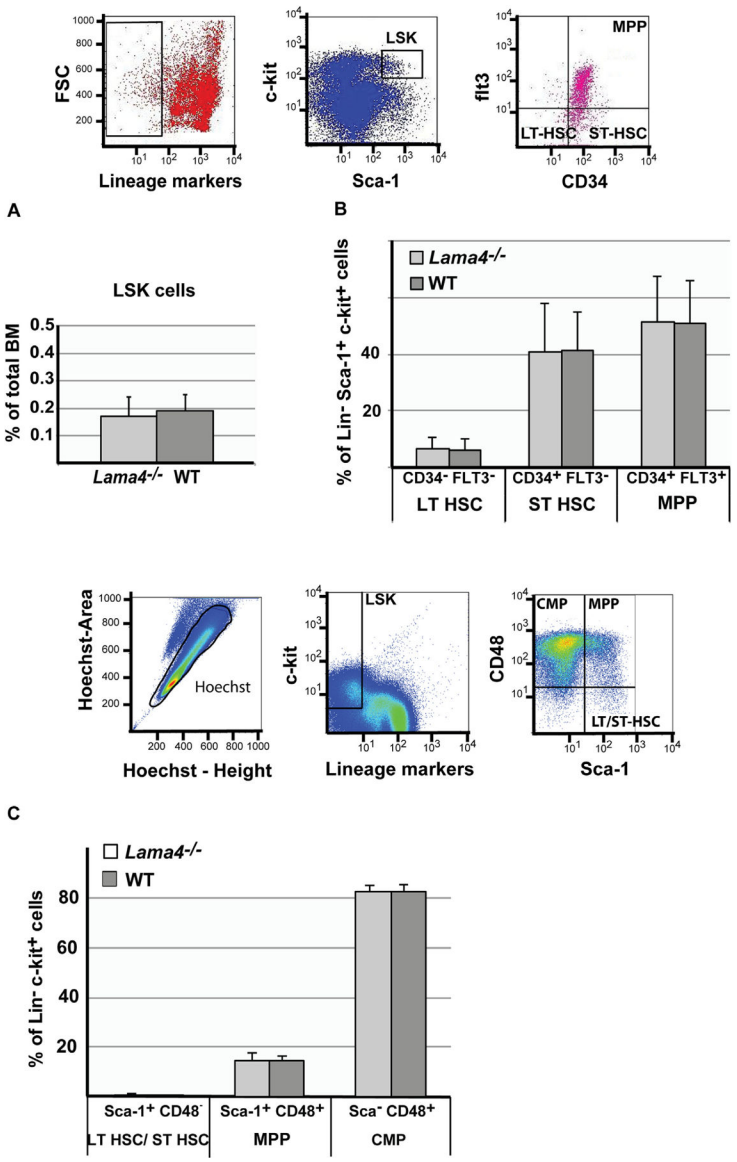

Fig. 3.

Hematopoietic stem and progenitor cell numbers in *Lama4*$^{-/-}$ bone marrow. Proportions of hematopoietic stem and progenitor cells in murine bone marrow of WT and *Lama4*$^{-/-}$ mice were quantified by flow cytometry. The upper panel shows flow cytometry gatings for graphs A and B. (A) LSK cells are lineage^{-}Sca-1^{+}c-kit^{+}; (B) LSK cells from (A) were further subdivided by expression of CD34 and Flt3 expression: Lin^{-}c-kit^{+}Sca-1^{+}CD34^{+}Flt3^{+} are multipotent progenitor cells (MPP), Lin^{-}c-kit^{+}Sca-1^{+}CD34^{+}Flt3^{-} are short-term repopulating HSC (ST-HSC), Lin^{-}c-kit^{+}Sca-1^{+}CD34^{-}Flt3^{-} are long-term repopulating HSC (LT-HSC). (C) Flow cytometry gatings employed are shown. These cell populations were further used for cell cycle analysis (Fig. 5). All nucleated live single cells are found within the gate Hoechst peak height versus Hoechst peak area. Further classification into different cell populations is based on expression of lineage markers, c-kit, Sca-1 and CD48. Lin^{-}c-kit^{+}Sca-1^{+}CD48^{-} are LT-HSC/ST-HSC, Lin^{-}c-kit^{+}Sca-1^{+}CD48^{+} are MPP and Lin^{-}c-kit

$^{+}$Sca-1^{-}CD48^{+} are common myeloid progenitors (CMP) quantification of which is show in the bar graph. Data are mean values ± SD obtained from at least 3 experiments with 2–4 WT and *Lama4*$^{-/-}$ mice/experiment.

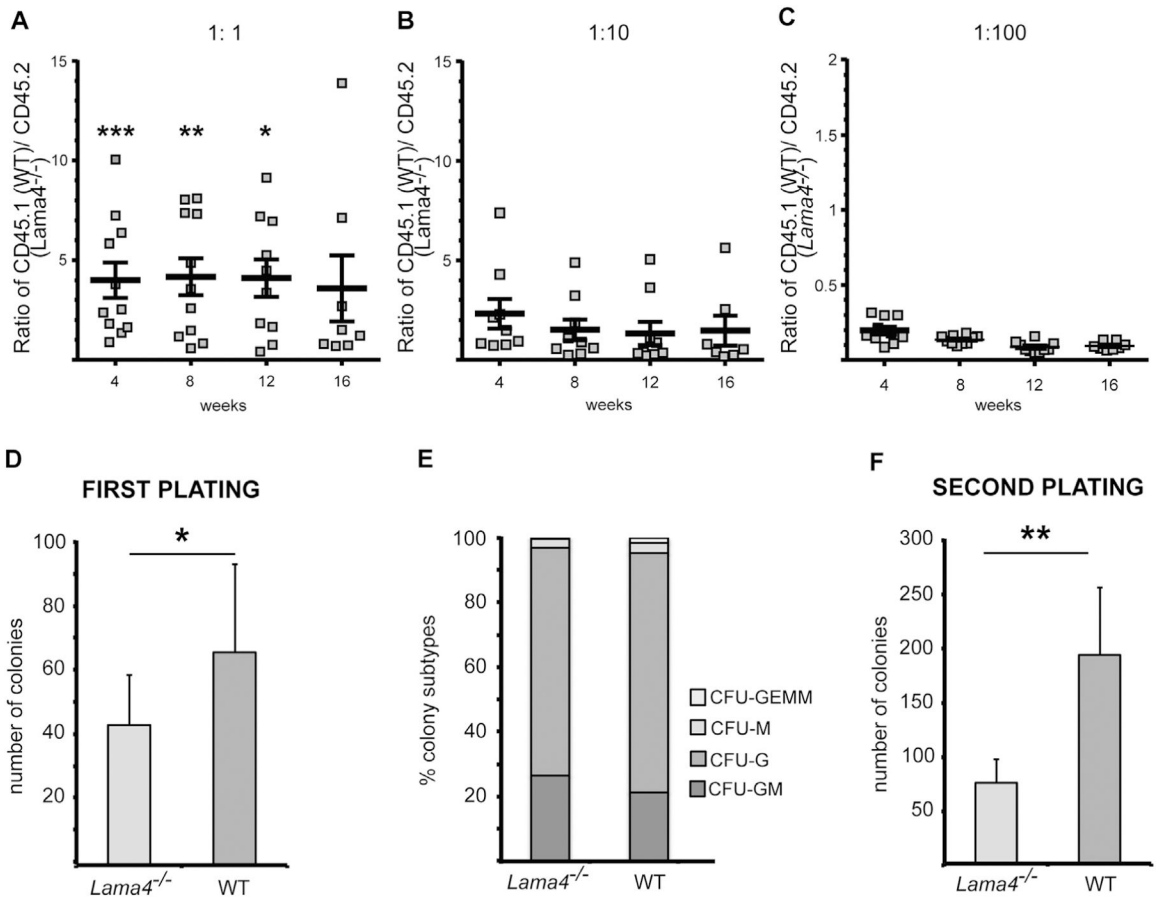

Fig. 4.

Competitive bone marrow repopulation assays (A–C) and colony forming assays (D–F) suggest reduced HSPC cells in *Lama4*$^{-/-}$ bone marrow but no defects in differentiation. Ratios of (A) 1:1 (B) 1:10 and (C) 1:100 WT (CD45.1): *Lama4*$^{-/-}$ (CD45.2) bone marrow cells were injected i.v. into irradiated CD45.1^{+}/CD45.2^{+} recipient mice and CD45.1 versus CD45.2 cells in the peripheral blood were determined by flow cytometry at 4, 8, 12 and 16 weeks. At an equal transplantation ratio, fewer *Lama4*$^{-/-}$ bone marrow derived peripheral blood cells were measured at 4, 8 and 12 weeks, suggesting a competitive repopulation deficit. Data are mean values ± s.e.m., each dot represents one recipient mouse; at least 4 experiments were performed with 3–4 mice/experiment; *P < .05; **P < .01; ***P < .001. (D) WT and *Lama4*$^{-/-}$ bone marrow cells were seeded in semi-solid medium and the resulting colonies were counted at 8 days, revealing reduced colonies derived from *Lama4*$^{-/-}$ bone marrow at first platings (D), but no differences in the proportions of different myeloid cell types that develop from WT or *Lama4*$^{-/-}$ bone marrow. (F) Second platings of colonies derived from the first plating and analyzed at day 8 show fewer *Lama4*$^{-/-}$-derived colonies. Data in D–E are mean values ± SD obtained from 3 experiments with 3 WT and 3 *Lama4*$^{-/-}$ mice/experiment. **P < .01, ***P < .001.

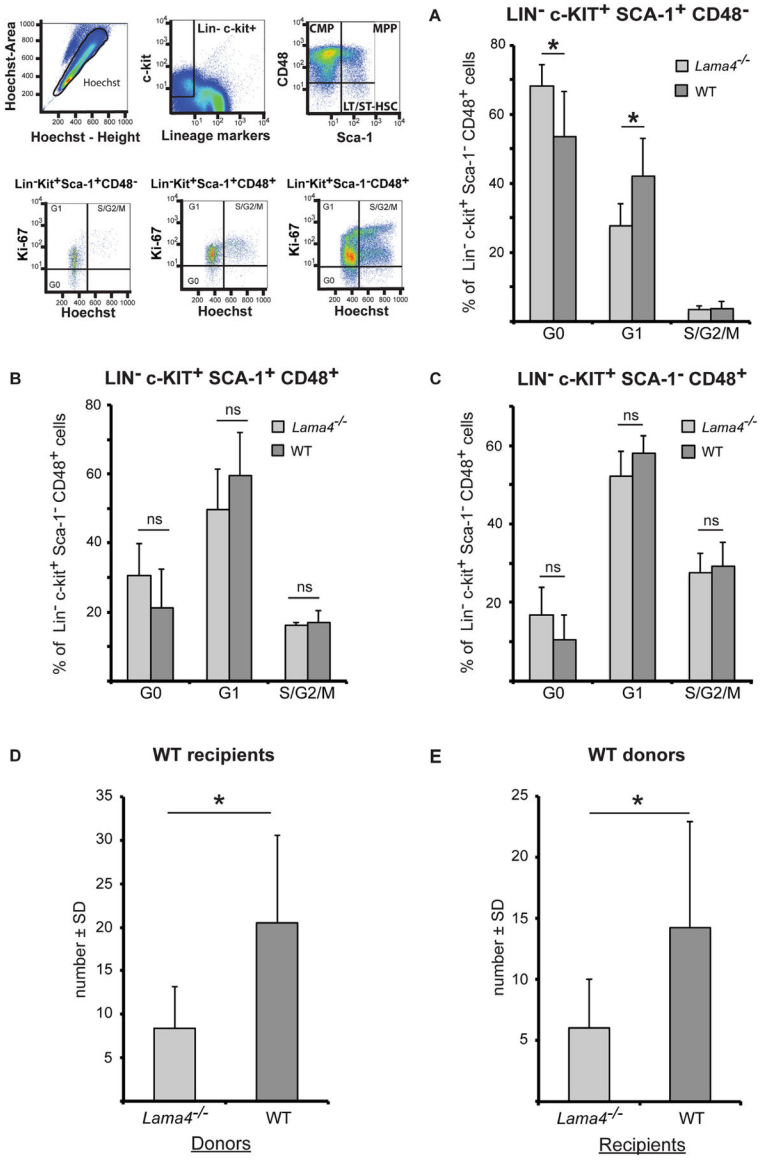

Fig. 5.

Cycling (A–C) of HSPC from *Lama4*$^{-/-}$ mice is slower and their homing to the bone marrow (D–E) is reduced. The cell cycle status of (A) Lin^{-}c-kit^{+}Sca-1^{+}CD48^{-} multipotent progenitors and HSC, (B) Lin^{-}c-kit^{+}Sca-1^{-}CD48^{+} myeloid progenitors and (C) Lin^{-}c-kit^{+}Sca-1^{+}CD48^{+} lineage restricted hematopoietic progenitors was analyzed by flow cytometry. Data shown are mean values ± s.e.m. obtained from 3 experiments with 3 WT and 3 *Lama4*$^{-/-}$ mice/experiment. *P < .05). (D, E) Bone marrow cells were injected i.v. into irradiated recipient mice and the bone marrow of recipient mice analyzed at 16 h in colony forming assays. D) Transplantation of *Lama4*$^{-/-}$ bone marrow to WT recipients reveals lower numbers of colonies compared to WT bone marrow; E) transplantation of WT bone marrow to *Lama4*$^{-/-}$ recipients also reveals fewer colonies than when WT bone marrow cells are transferred. Data shown are mean values ± SD from 3 experiments with 2–3 recipient mice/experiment. *P < .05.

Table 1.

Localization of ECM molecules and reticular fibroblast markers in murine bone marrow.

	Large blood vessels	Sinusoidal BMs	Fiber network
Collagen I	−	−	−
Collagen III	++	++	++
Collagen IV	++	++	++
PanLM	++	++	++
Laminin α1	−	−	−
Laminin α2[a]	−	−	−
Laminin α3	+	+	+
Laminin α4	++	++	+
Laminin α5	++	++	−
Laminin β1	−	−	−
Laminin β2	++	++	++
Laminin β3	−	+	−
Laminin γ1	++	++	++
Laminin γ2	+	+	+
Laminin γ3	−	−	−
Perlecan	++	++	++
Nidogen-1	++	++	++
Fibronectin	++	++	++
ER-TR7	−	−	+
Fibrillin-1	−	−	−
gp38	−	−	−
Desmin	−	−	−
SMA	−	−	−

++ strongly detectable

+ weakly detectable

− absent;

SMA is smooth muscle actin.

[a] Detected in nerves associated with arteries.

Table 2.

Cell surface markers employed to identify hematopoietic cell populations.

	Progenitor Type	Cell Type	Markers[§]
Self–Renewal ↓ ↑ Differentiation	**$Lin^-Sca{-}1^+c{-}kit^+$ (LSK)**	LT–HSC	$CD34^-$ $flt3^-$ $CD150^+$ $CD48^-$
		ST–HSC	$CD34^+$ $flt3^-$ $CD150^-$ $CD48^{-/low}$
		MPP	$CD34^+$ $flt3^+$ $CD150^-$ $CD48^+$
	$Lin^-Sca{-}1^-c{-}kit^+$	CMP	$CD150^-$ $CD48^+$

[§]according to [35,36]; LT-HSC is long-term HSC; ST-HSC is short-term HSC; MMP is multipotent progenitors; CMP is common myeloid progenitor.